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Liquid chromatographic mass spectrometric (LC/MS/MS) determination of plasma hydroxocobalamin and cyanocobalamin concentrations after hydroxocobalamin antidote treatment for cyanide poisoning☆

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ABSTRACT

Cyanide poisoning occurs in individuals after fire smoke inhalation and after oral ingestion of cyanide. Hydroxocobalamin (HOCbl), a hydroxylated form of vitamin B_{12}, is often used as an antidote to treat cyanide toxicity. It has a high affinity for cyanide and rapidly removes cyanide from tissue by forming cyanocobalamin (CNCbl). Little information is available on the pharmacokinetics of HOCbl and CNCbl largely because of the lack of analytical methods for analyzing HOCbl and CNCbl. In this study, we developed a new liquid chromatographic mass spectrometric (LC/MS/MS) method for the quantitative analysis of plasma HOCbl and CNCbl in the porcine (Sus scrofa) model. The method uses on-column extraction, reversed phase gradient chromatography, and multiple reaction monitoring (MRM) for quantitation. MRM transitions monitored were 664.7 → 147.3 and 664.7 → 359.2 for HOCbl and 678.8 → 147.3, 678.8 → 359.1 678.8 → 457.1 for CNCbl. The limit of detection (LOD) and the lower limit of quantitation (LLOQ) were 1.0 and 1.0 μmole/L, respectively, for plasma HOCbl and 0.1 and 0.5 μmole/L for plasma CNCbl. The within-day and between-day CVs were 4.3 and 6.4% for plasma HOCbl at 500.0 μmole/L and 5.5 and 5.7% for CNCbl at 100.0 μmole/L ($n = 6$). The plasma HOCbl and CNCbl calibrations curves were linear from 100.0 to 2000.0 and 50.0 to 500.0 μmole/L, respectively. Based on 6 separate calibration curves the average linear regression coefficient (R^2) for both HOCbl and CNCbl was 0.992. The LC/M/MS method was found to be accurate and precise and has been validated by determining the plasma HOCbl and CNCbl concentrations in 11 pigs that were treated with HOCbl for cyanide poisoning.

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1. Introduction

Cyanide poisoning frequently occurs after smoke inhalation from house and industrial fires [1–4] as well as from suicide attempts after oral ingestion of cyanide [5]. A number of antidotes are available for treating cyanide poisoning and include a combination of amyl nitrite, sodium nitrite, and sodium thiosulfate (cyanide antidote kit) [6–9]. Sodium thiosulfate forms methemoglobin from hemoglobin which complexes with cyanide to form a non-toxic cyanide derivative. HOCbl, a natural analog of vitamin B12 (Fig. 1), is also being evaluated as an antidote for cyanide poisoning [6–14]. The cobalt ion of hydroxocobalamin binds to circulating and cellular cyanide molecules to form cyanocobalamin (CNCbl) (Fig. 1) which is then excreted in the urine.

Little information exists on methods for the analysis of plasma HOCbl and CNCbl concentrations after HOCbl treatment. The lack of methodology has impeded studies on the pharmacokinetics of HOCbl as well as the formation of CNCbl in animals and in humans exposed to cyanide poisoning. Several methods, based on derivative spectrophotometric methods [15,16] or radioimmunoassay [17] have been reported for the analysis of cobalamins in biological samples. A major limitation of these methods is that they cannot distinguish between the various cobalamins. SP-Sephadex ion-exchange chromatography also has been used for the analysis of HOCbl; however, its ability to separate HOCbl and CNCbl were not examined [14].

Several high performance liquid chromatographic methods with atomic absorption spectrometry [18], or ultraviolet detection [19–22] have been developed for the analysis of plasma HOCbl and CNCbl concentrations. Two of the methods were used for the analysis of both hydroxocobalamin and cyanocobalamin [21,22]. Of these methods, one was used for the analysis of hydroxocobalamin and cyanocobalamin in human plasma [22] and the

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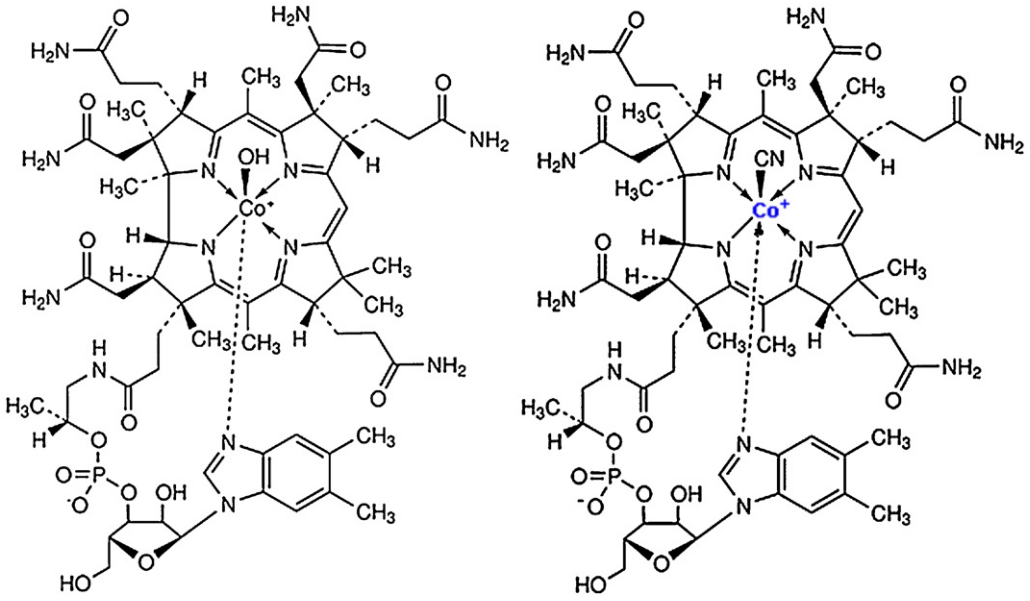


Fig. 1. Chemical structure of hydroxocobabalamin and cyanocobalamin.

other was used to analyze hydroxocobalamin, cyanocobalamin and other cobalamins in seawater [21]. The other HPLC methods were developed for the analysis of either hydroxocobalamin [18,19] or cyanocobalamin [20], but not for both compounds. Likewise, a liquid chromatographic mass spectrometric (LC/MS/MS) method has been described for the analysis of HOCbl in pharmaceutical preparations, however, the focus of the study was on the analysis of metallothionein rather than on the analysis of cobalamins [23]. While the LC/MS/MS method has been used for the analysis of HOCbl in pharmaceutical preparations, it has not been used for the analysis of plasma HOCbl and CNCbl concentrations.

The aim of this study was to develop and validate a new LC/MS/MS method for the analysis of HOCbl and CNCbl in plasma samples obtained from pigs given hydroxocobalamin as an antidote for cyanide poisoning. For the study, we evaluated several chromatographic columns and mobile phases for the on-column extraction and LC/MS/MS analysis of plasma HOCbl and CNCbl. In this report, we provide information on the validation of the LC/MS/MS method and on the pharmacokinetics of HOCbl and CNCbl after the administration of cyanide and HOCbl in the porcine animal model.

2. Experimental

2.1. Chemicals and supplies

Hydroxocobalamin hydrochloride (Cat. no. H 7126), cyanocobalamin (Cat. no. C3607), and potassium cyanide (Cat. no. 60178) were obtained from Sigma–Aldrich, acetonitrile (Cat. no. 9017-03) from JT Baker; ethanol (Cat. no. E7023) from Sigma–Aldrich, and methanol (Cat. no. A452-4) from Fisher Scientific. Microcentrifuge tubes, 1.5 mL polypropylene with indented caps, were obtained from Fisher Scientific (Cat. no. 02-681-2391). The Waters Oasis HLB on-line extraction column, 2.1 mm i.d. × 20.0 mm, 25 μm, was obtained from Waters Corporation (Cat. no. 186000706) and the Luna C18 analytical column, 2.0 mm i.d. × 150 mm, 5 μm, was obtained from Phenomenex Corporation (Cat. no. 00P-4252-00).

2.2. Animal studies

This study was part of a larger study evaluating hydroxocobalamin and sodium thiosulfate and sodium nitrite and sodium thiosulfate as antidotes for acute cyanide poisoning in the swine (Sus scrofa) model [24]. For the study, HOCbl (150 mg/kg) was injected via the venous port approximately 30 min after injection of potassium cyanide (0.4 mg/kg). Blood samples were collected with an indwelling catheter into 10 mL lithium–heparin vacutainer tubes at 0, 10, 20, 30, and 40 min. After centrifugation, the plasma samples were stored at −70 ± 4 °C until analyzed. The protocol for the animal study was approved by our Institutional Animal Care and Use Committee (IACUC).

2.3. Preparation of HOCbl and CNCbl standards and plasma calibrators

Calibrator and control stock solutions were prepared in blank (cobalamin free) pig plasma obtained from pigs housed in our facility. Quantitation and detection were based on a four point calibration curves covering the range of plasma HOCbl and CNCbl concentrations found 10–40 min after HOCbl administration. HOCbl calibration standards were prepared at 100, 500, 1000 and 2000 μmole/L, and CNCbl standards at 50, 100, 250, and 500 μmole/L. Plasma HOCbl and CNCbl calibrators were also prepared at 1.0–10.0 μmole/L to determine the LOD and LLOQ. The calibrator standards, controls, and plasma blanks were stored at 5 ± 3 °C until analyzed.

2.4. Plasma protein precipitation procedure

Plasma proteins were precipitated by adding 100 μL of each plasma calibrator or plasma test sample and 100 μL of the protein precipitating reagent (acetonitrile:10% formic acid, 98:2, v/v) to 1.5 mL pre-labeled microcentrifuge tubes. The samples were capped and vortex-mixed for approximately 10 s and then centrifuged in an Eppendorf Minispin Plus centrifuge (Model 5453 with F45-12-12 rotor, Hamburg, Germany) at 14,500 rpm for 15 min. The supernatant phase of each sample was then transferred

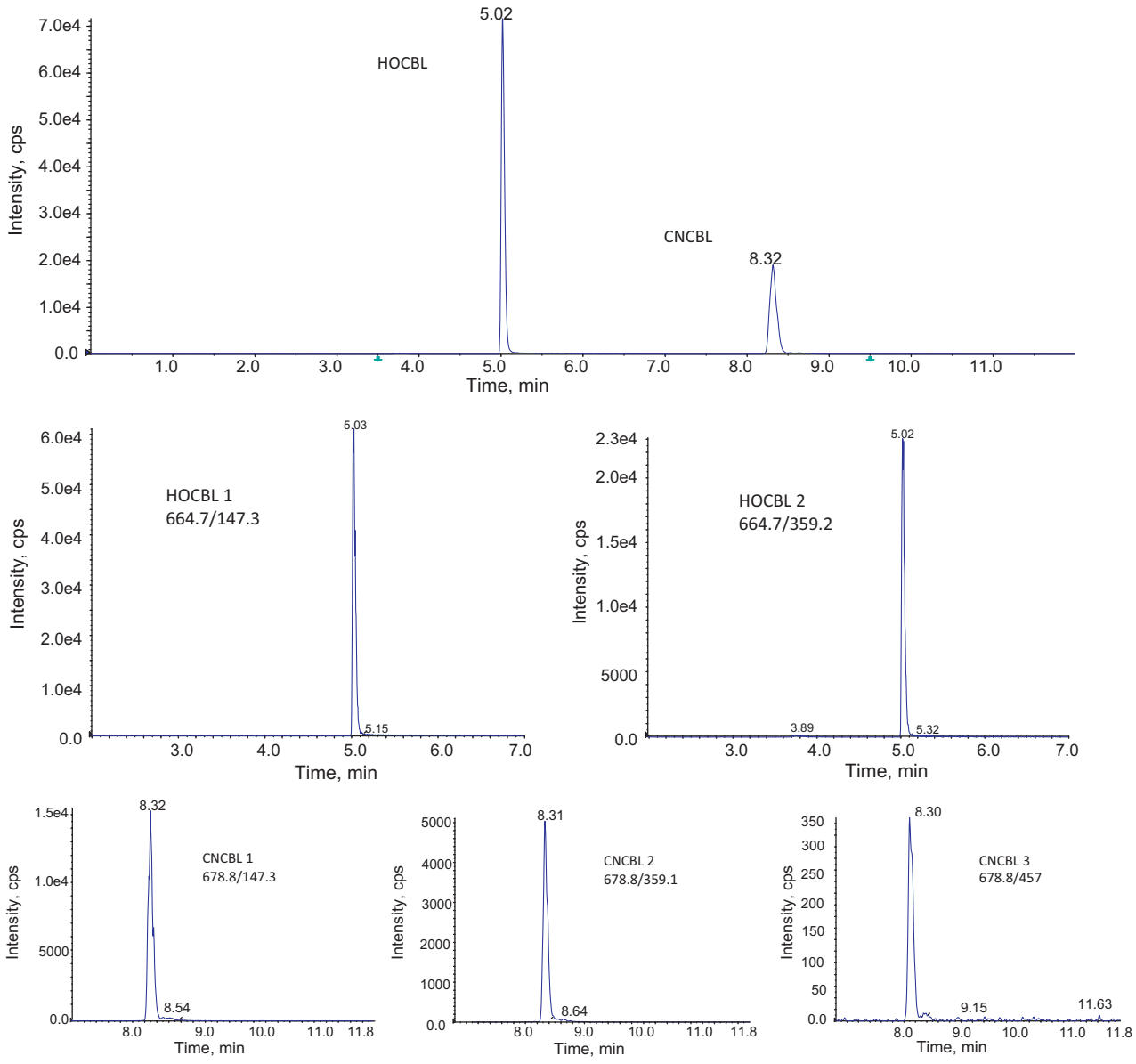


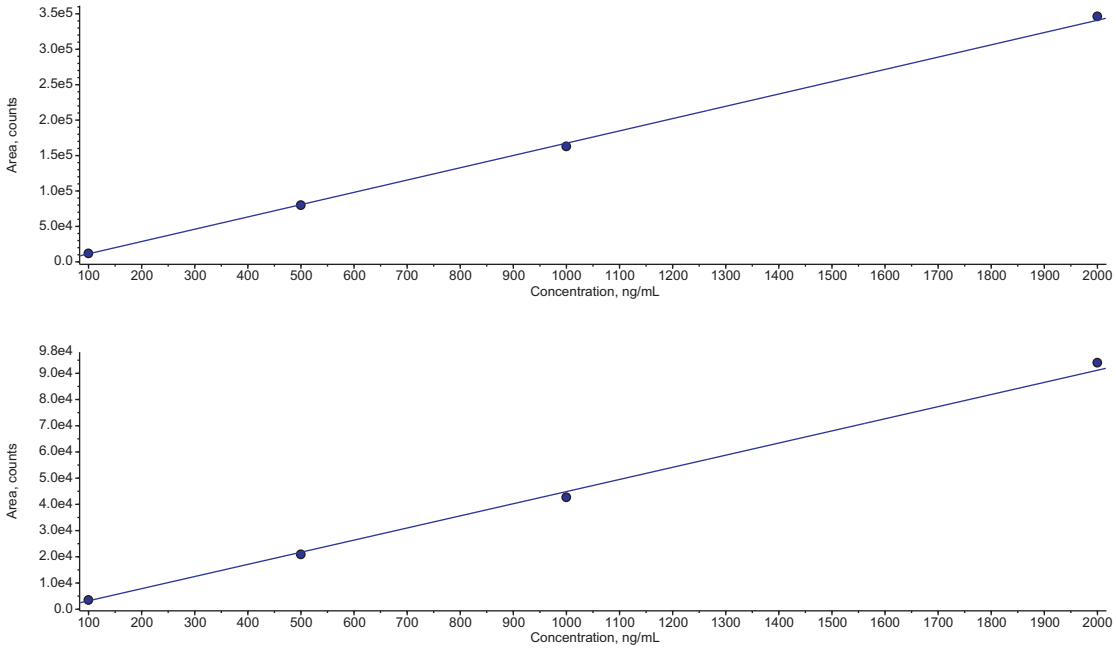
Fig. 2. LC/MS/MS chromatograms of plasma HOCBL and CNCBL calibrators.

to a separate 1.0 mL glass autosampler vial and injected onto the LC/MS/MS system.

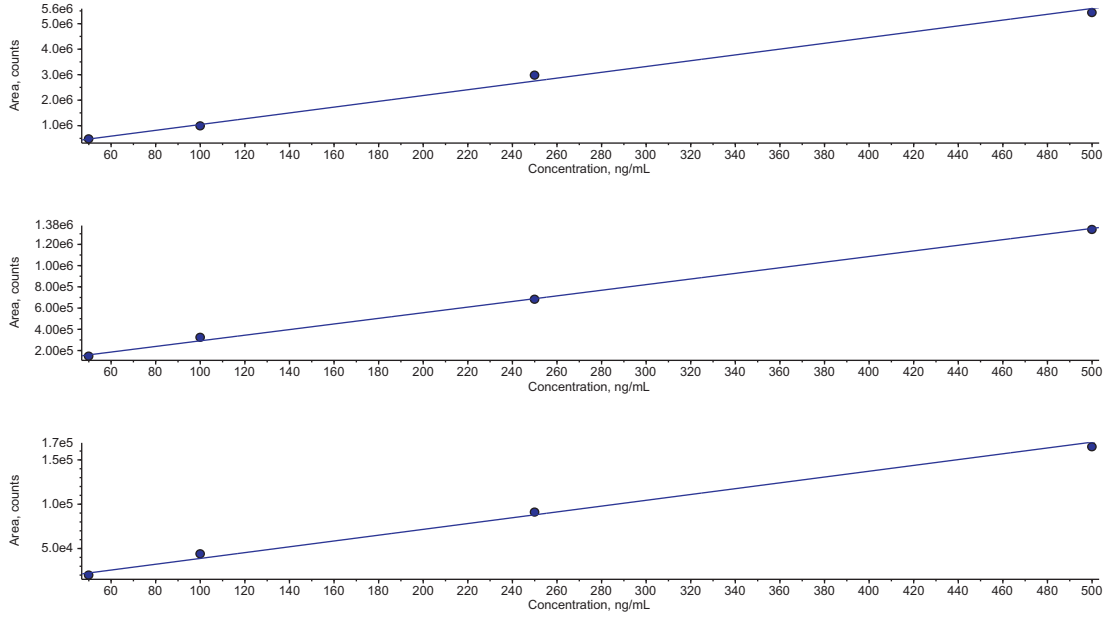
2.5. LC/MS/MS analysis

LC conditions (Agilent Technologies 1200 Series Liquid Chromatographic System). A two pump configuration with a 6 port column switching valve was used to perform on-line sample extraction. On the first pump, Millipore water and 5 μL injected sample were introduced onto a 2.1 mm i.d. × 20.0 mm, 25 μm Waters Oasis HLB on-line extraction column at 1 mL/min. HOCbl and CNCbl were trapped on the extraction column and the proteins were eluted to waste. At 2 min the valve was switched to allow pump two mobile phase to back flush the analytes of interest onto a 2.0 mm i.d. × 150 mm, 5 μm, C18 (2) Phenomenex Luna analytical column at 0.3 mL/min. The gradient mobile phase on pump 2 consisted of solvent A: 2.5 mM Ammonium acetate in 50:50 methanol:water, pH 4.0 and solvent B: 10% acetonitrile in water. The timed events used were as follows: 0.00–3.00 min, 5% solvent A and 95% solvent B; at 4 min, 95% solvent A and 5% solvent B; and at 11.0–12 min, back to starting condition, and valve switches to extraction column to waste.

Mass spectrometric analysis was performed on an Applied Biosystems 4000 Q TRAP™ LC/MS/MS System (Applied Biosystems, Foster City, CA). The LC/MS/MS operating parameters were as follows: polarity mode; ESI positive; scan type: MRM; curtain gas: 20, CAD: high, GS1: 30, GS2: 20, temperature: 750; ion spray: 4500; declustering potential: 130; exit potential: 10. MRM transitions (m/z) monitored were 664.7 → 147.3 and 664.7 → 359.2 for HOCbl and 678.8 → 147.3, 678.8 → 359.1 and 678.8 → 457.1 for CNCbl. Collision energies for HOCbl 1, HOCbl 2, CNCbl 1, CNCbl 2, and CNCbl 3 were 60, 35, 55, 35, and 40 eV, respectively. Ion ratios derived from MRM transitions ions for the analytes in controls and subject samples had to be within ±20% of the ion ratio calculation; the calculated range was based on the average of ratio ranges obtained for the six standards. Quantitative values were obtained from transition 1; transition 2 had to meet acceptance criteria [S/N] ≥ 3) and was used to calculate the ion ratio. The ratios were calculated by dividing the area of transition 1 by the area of transition 2.



Calibration curves at 100, 500, 1000 and 2000 µmole/L for the 147.3 and 359.3 HOCBL transitions that were monitored.



Calibration curves at 50, 100, 250 and 500 µmole/L for the 147.3, 359.2, and 457.1 CNCBL transitions that were monitored.

Fig. 3. Representative LC/MS/MS calibration curves of plasma HOCBL and CNCBL.

3. Results and discussion

3.1. Method development and validation

HOCbl and CNCbl chromatographic peaks based on precursor and product ions (MRM transition ions) are shown in Fig. 2. The ion transitions were 664.7/147.3 and 664.7/359.2 for HOCbl and 678.8/147.3, 678.8/359.2, 678.8/457.1 for CNCbl. The retention times of the HOCbl and CNCbl peaks were approximately 5.02 and 8.32 min, respectively.

Standard curves for HOCbl and CNCbl were based on a four point calibration curve (Fig. 3). The HOCbl calibrator concentrations

were 100, 500, 1000 and 2,000 μmole/L and CNCbl concentrations were 50, 100, 250 and 500 μmole/L. The between-day linear regression coefficients (R^2) for HOCbl and CNCbl were used to calculate the minimum acceptable R^2 value for a given calibration curve. Based on 6 separate calibration curves, the minimum acceptable *r*-squared (R^2) for HOCbl and CNCbl was found to be 0.992.

The linear range for this procedure was based on the analysis of 6 injections of plasma HOCbl and CNCbl calibrators. The linear range for HOCbl was shown to be 100–2000 μmole/L and 50–500.0 μmole/L for CNCbl. Values were considered within acceptable range if the measured amount was within ±20% of target concentration and ±20% of ion ratio calculation. The second transition for each analyte was used in the calculation of ion ratios for each concentration; the ion ratio range was based on the average of ratio ranges obtained for the six standards. Within-run precision and between-run CVs were 4.3 and 6.4% for plasma HOCbl at 500 μmole/L ($n = 6$) and 5.5 and 5.7% for CNCbl at 100 μmole/L ($n = 6$), respectively. Plasma HOCbl and CNCbl standard curves were also found to be linear at 1.0–10.0 μmole/L ($R^2 \geq 0.992$, $n = 6$).

The sensitivity of the method was established by determining the limit of detection (LOD) and lower limit of quantitation (LLOQ). The LOD (acceptable ion ratios, presence of all MRM transitions and signal-to-noise ratio [S/N] ≥ 3) was 1.0 μmole/L and 0.1 μmole/L for plasma HOCbl and CNCbl, respectively. The LLOQ was based on the lowest concentration that plasma HOCbl and CNCbl concentrations could be determined with an acceptable level of precision and accuracy for which the ion ratios fell within ±20% of the established range and the S/N ratios were ≥10.0 times baseline. The LLOQ was 1.0 μmole/L for HOCbl and 0.5 μmole/L for CNCbl. No efforts were made to increase the sensitivity by injecting larger volumes of the supernatant phase, altering the ratio of the plasma to precipitating agent volumes, or further purifying the supernatant phases.

Recoveries and matrix effects were determined on plasma samples obtained from 10 different pigs. The plasma samples were prepared at a HOCbl and CNCbl concentration of 5.0 μmole/L. All plasma HOCbl and CNCbl samples and standards underwent protein precipitation and on-column extraction as previously described. Recoveries of plasma HOCbl and CNCbl were based on HOCbl and CNCbl standards prepared in mobile phase at 1.0, 2.5, 5.0, 7.5, and 10.0 μmole/L. Recoveries of plasma HOCbl and CNCbl were 64.2 ± 0.43 and 106 ± 0.58% (mean ± SD), respectively.

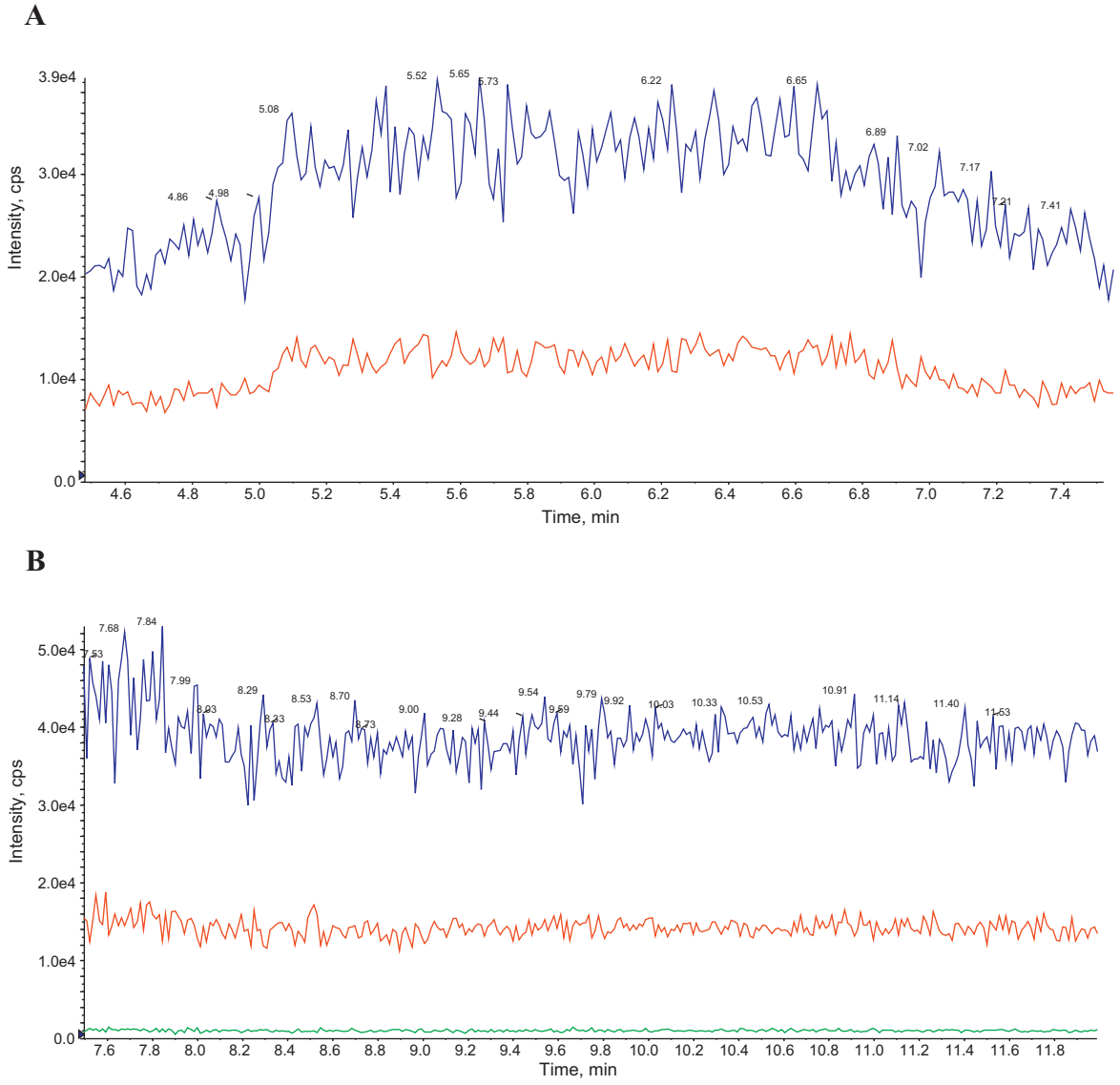


Fig. 4. Ion suppression evaluation. Infusion of (A) HOCBL (MRM transitions 664.7/147.3 and 664.7/359.2) and (B) CNCBL (MRM transitions 678.8/147.3, 678.8/359.2, 678.8/457.1) at 100 μmole/L while injecting blank matrix (cobalamin negative plasma).

The specificity of the method was determined by analyzing pre-dose plasma samples from 13 different pigs. We did not see any extraneous peaks that might interfere with the analysis of HOCbl or CNCbl nor did we see any extraneous peaks in any of the plasma HOCbl calibrators or post-dose test samples. Likewise, we analyzed six random cobalamin and cyanide free plasma samples and found no peaks that would interfere with the HOCbl or CNCbl analyses. Since matrix effect can influence the extent of analyte ionization, an ion suppression experiment was conducted. Cobalamin negative pig plasma was treated in the same manner as test samples to best mimic matrix complexity. A 100 μmole/L solution of the analytes in acetonitrile was prepared and was loaded into the infusion pump syringe. The infusion pump was connected post-column via a tee connector; the solution was infused at 5 μL/min. Once the baseline was stable, injections of cobalamin-free plasma were made in the same manner as standard acquisitions. No ion suppression was seen at the retention times of interest for any of the analytes. Fig. 4 demonstrates a typical ion suppression pattern for a blank plasma sample. Carry-over was evaluated by injection of a blank sample (mobile phase) or known cobalamin negative plasma following high concentration samples (2000 μmole/L HOCbl, 1000 μmole/L CNCbl). Carry-over was seen in blank and negative plasma samples; therefore, blank injections were made following each sample injection and up to three blank injections were made following high concentration samples.

The stability of HOCbl and CNCbl plasma samples were tested after storage at 2–8 °C for one week, at −10 °C for 3 weeks and at −70 °C for 3 weeks. No differences were found in plasma HOCbl and CNCbl concentrations between any of the samples stored at the three different temperatures ($p > 0.05$). Stability of prepared plasma samples while sitting on the autosampler for 6 h was evaluated. Plasma samples containing 100 and 250 μmole/L HOCbl and CNCBL were prepared as described in Section 2.4 and were placed in the autosampler and immediately analyzed. The samples were allowed to sit on the autosampler and re-analyzed after 6 h. Data obtained from the re-analyzed samples were compared with the initial analysis by calculating the percent of the initial analysis. The results from the studies showed that the samples analyzed 6 h later were ±5.5 and ±12.6% of the initial concentrations for HOCBL and CNCBL, respectively.

A limitation of the method is that deuterated forms of hydroxocobalamin and cyanocobalamin were not available for use as internal standards. Since the deuterated forms of hydroxocobalamin and cyanocobalamin are not available, quantitation could possibly be based on the use of methylcobalamin. The two other HPLC [22] and LC/MC/MS [23] methods likewise did not use internal standards for their analyses of plasma samples and pharmaceuticals. All of the methods, however, had reasonably good accuracies and precisions.

3.2. Application of the method

The method has been used to determine the HOCbl and CNCbl concentrations following HOCbl treatment in pigs with acute cyanide poisoning. The HOCbl and CNCbl concentrations at time 0, 10, 20, 30, and 40 min after an intravenous injection of HOCbl are shown in Fig. 5. HOCbl peaked at 10 min for all animals and the HOCbl concentrations ranged from 478 to 1430 μmole/L. As shown, CNCbl formation occurred rapidly after administration of HOCbl.

The LC/MS/MS method described in this study permits the analysis of both plasma HOCbl and CNCbl concentrations. The method has a relatively low within-day and between-day coefficient of variation for both HOCbl and CNCbl. The method also has a LLOQ for HOCbl that is adequate for pharmacokinetic studies. The LLOQ of HOCbl is lower than the 10.0 μmole/L reported for the HPLC ultraviolet method [22] and lower than the 25.0 μmole/L limit

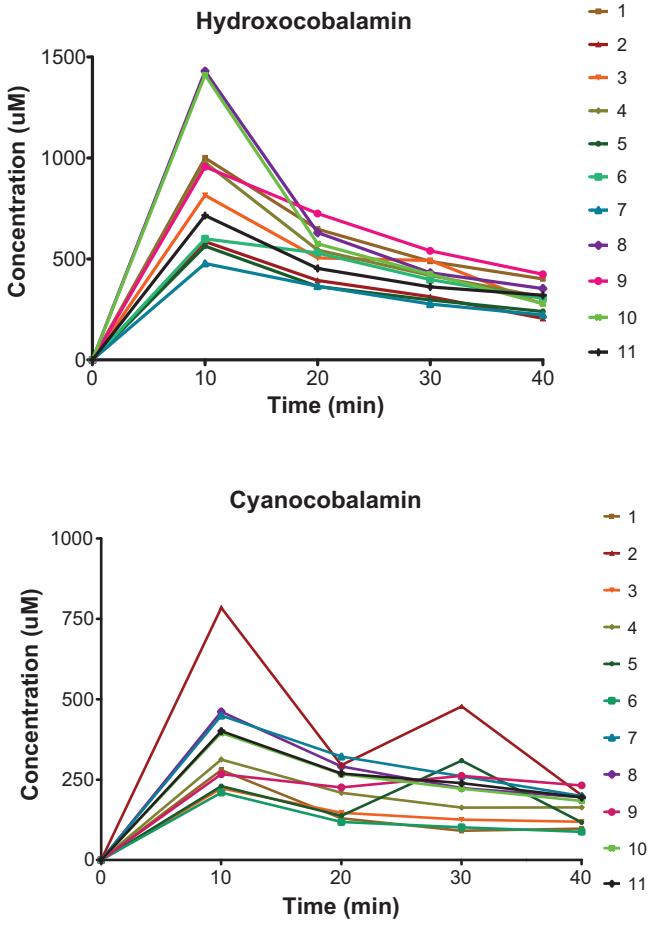


Fig. 5. Pharmacokinetic profile of plasma HOCbl and CNCbl in pigs ($n = 11$) after treatment with 150 mg/kg of hydroxocobalamin (HOCbl).

described for the previous LC/MS/MS method [23]. In addition, the plasma HOCbl and CNCbl concentrations measured at 10, 20, 30, and 40 min after intravenous administration of a standard dose of HOCbl were found to be well within the sensitivity limits of the method.

The LC/MS/MS method described here differs from that of the previously published LC/MS/MS method in several ways [23]. The previous LC/MS/MS method was developed for the analysis of HOCbl in pharmaceutical preparations. Therefore, further method validations would have to be performed before it could be used for the analysis of plasma HOCbl and CNCbl concentrations. Our method also differs from this LC/MS/MS method in that we used the secondary transition ions to quantitate the plasma levels of HOCbl and CNCbl whereas they used the parent ions to quantitate the HOCbl concentrations.

Plasma HOCbl and CNCbl concentrations have also been analyzed by HPLC with ultraviolet detection [22]. Like our method, it used on-column extraction followed by analytical column separation. It has been used to determine the pharmacokinetics of HOCbl and CNCbl in one individual who was being treated with HOCbl after fire smoke inhalation. The results of that pharmacokinetic study differ from our study in that they infused hydroxocobalamin for 30 min whereas we used an intravenous bolus injection of hydroxocobalamin. There are several advantages of our LC/MS/MS method over the HPLC methods. The LC/MS/MS method described in this report is very specific and less likely to encounter interferences than the methods based on ultraviolet detection [19–22]. Since both HOCbl and CNCbl have similar ultraviolet absorption maxima, the two compounds have to be completely separated

on the chromatographic columns and this was effectively done in one of the previous studies [22]. The LC/MS/MS method described here is also more sensitive than the HPLC methods using ultraviolet detection [19–21]. Furthermore, a number of the methods have been developed for the analysis of hydroxocobalamin but not cyanocobalamin [19,21] or for the analysis of cyanocobalamin but not hydroxocobalamin [20]. Only two of the methods used serum or plasma for analysis [20,22].

The validated LC/MS/MS method described here was successfully applied to a pharmacokinetic study of HOCbl and CNCbl after HOCbl treatment of cyanide-induced poisoning in pig animal models [24]. The method can be applied to pharmacokinetic studies of HOCbl and CNCbl in other animal models as well as in more detailed human studies. Such studies can help establish the most effective dose and the optimum timing of HOCbl administration for treating individuals with cyanide poisoning. With slight modifications, this method can be used to analyze other cobalamins in body fluids as well as the stability of vitamin B12 in various drug formulations.

4. Conclusions

In summary, the LC/MS/MS method was developed and validated for the analysis of plasma HOCbl and CNCbl concentrations using on-column extraction and LC/MS/MS analysis. The method demonstrated acceptable sensitivity, precision, and accuracy. The method was successfully applied to the study of plasma HOCbl and CNCbl concentrations following HOCbl treatment for acute cyanide poisoning. Further more detailed pharmacokinetic studies are needed and should provide needed information that can be used to optimize HOCbl therapy.

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