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Simultaneous determination of 3-mercaptopyruvate and cobinamide in plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

The current suite of Food and Drug Administration (FDA) approved antidotes (i.e., sodium nitrite, sodium thiosulfate, and hydroxocobalamin) are effective for treating cyanide poisoning, but individually, each antidote has major limitations (e.g., large effective dosage or delayed onset of action). To mitigate these limitations, next-generation cyanide antidotes are being investigated, including 3-mercaptopyruvate (3-MP) and cobinamide (Cbi). Analytical methods capable of detecting these therapeutics individually and simultaneously (for combination therapy) are essential for the development of 3-MP and Cbi as potential cyanide antidotes. Therefore, a liquid chromatography–tandem mass-spectrometry method for the simultaneous analysis of 3-MP and Cbi was developed. Sample preparation of 3-MP consisted of spiking plasma with an internal standard ($^{13}C_3$-3-MP), precipitation of plasma proteins, and derivatizing 3-MP with monobromobimane to produce 3-mercaptopyruvate-bimane. Preparation of Cbi involved denaturing plasma proteins with simultaneous addition of excess cyanide to convert each Cbi species to dicyanocobinamide ($Cbi(CN)_2$). The limits of detection for 3-MP and Cbi were 0.5 μM and 0.2 μM, respectively. The linear ranges were 2–500 μM for 3-MP and 0.5–50 μM for Cbi. The accuracy and precision for 3-MP were 100 ± 9% and <8.3% relative standard deviation (RSD), respectively. For $Cbi(CN)_2$, the accuracy was 100 ± 13% and the precision was <9.5% RSD. The method presented here was used to determine 3-MP and Cbi from treated animals and may ultimately facilitate FDA approval of these antidotes for treatment of cyanide poisoning.

1. Introduction

Although the current suite of Food and Drug Administration (FDA) approved cyanide (HCN and CN^-, inclusively represented as CN) antidotes (i.e., sodium thiosulfate, sodium nitrite, and hydroxocobalamin (Cbl)) are effective for treating cyanide poisoning, each has major limitations [1–3]. Therefore, next generation cyanide antidotes, are being developed. Two of these next generation antidotes, cobinamide (Cbi) and sulfanegen (i.e., dimer of 3-mercaptopyruvate (3-MP)), have shown promise in remedying the limitations of currently approved antidotes. For example, although Cbl (i.e., the current FDA approved direct cyanide binding treatment [4–6]) works well to detoxify cyanide [7–9], and its potential adverse effects are generally mild [10,11], it is very large and can only sequester one CN ion per Cbl molecule. Therefore, very large doses of Cbl must be administered intravenously over minutes, making it a poor antidote in mass casualty situations [11]. To address the disadvantages of Cbl, Cbi is being developed as an alternative direct cyanide binding agent. Cbi is the penultimate precursor in the biological production of Cbl and is naturally found in human serum and bile [2,12]. Cbi works by sequestering cyanide from its active site (i.e., the heme A site in cytochrome C oxidase). Structurally, Cbi lacks Cbl's dimethylbenzimidazole ribonucleotide tail [12,13]. Consequently, it has a smaller mass, greater overall affinity towards cyanide, ($K_f \approx 10^{22}$) [14,15], higher water solubility, and the ability to bind two cyanide ions [12,14–17]. Cbi has been shown to have 3–10 times more potency than hydroxocobalamin, depending on the formulation [2,18]. The main difference between

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Cbi formulations is the ligand identity, with the current formulation using nitrite ions, to improve absorption during intramuscular administration [15].

Another treatment strategy for cyanide poisoning is conversion of cyanide to the much less toxic thiocyanate (SCN^-, represented herein as SCN) with sulfur donors and native sulfurtransferase enzymes as catalysts. Sodium thiosulfate is currently the only FDA approved sulfur donor for treating cyanide toxicity. Although sodium thiosulfate is effective, it has a slow onset of action attributed to slow entry into cells and the mitochondria [1]. This necessitates its use in combination with faster acting therapeutics, typically nitrites. Also, sodium thiosulfate utilizes the enzyme rhodanese, which is primarily found in the liver and kidneys [19,20], leaving the heart and central nervous system less protected [19].

Because of the disadvantages of thiosulfate, alternate sulfur donors are being investigated, including 3-MP [3,16,19]. The conversion of CN to SCN by 3-MP is catalyzed by 3-MP sulfurtransferase (3-MST) [3,20,21]. This conversion occurs primarily in blood or tissue areas near blood. The highest concentrations of 3-MST are found in the liver and kidneys, with heart, brain and lungs also supporting the enzyme [20,22–24]. In contrast to rhodanese, 3-MST is distributed in both the cytosol and mitochondria, allowing the reaction of 3-MP with CN to occur primarily in the cytosol, with the remaining CN distributing into the mitochondria where it is metabolized by rhodanese. Although 3-MP is an effective sulfur donor, it readily degrades in the blood. Therefore, several prodrugs of 3-MP (i.e., chemical compounds that convert to 3-MP upon administration), inclusively called sulfanegen, were developed. In multiple studies, intramuscularly administered sulfanegen was highly effective in reversing cyanide toxicity [3,19,22,25,26].

Although both Cbi and 3-MP have advantages as individual antidotes, they work by distinct mechanisms. Therefore, the combination of these two compounds has been suggested to provide greater protection than the compounds alone [16]. In general, the main advantages of combination therapeutics are (1) lowering the dose of the individual components, which potentially leads to minimized side effects, and (2) allowing drugs with distinct pharmacokinetic profiles to be administered simultaneously, potentially extending the therapeutic window of individual treatments (e.g., combining a fast-acting quickly metabolized compound with a longer acting therapeutic). As a combination therapy, Cbi and sulfanegen have been administered intramuscularly to treat cyanide poisoning in mice [16] with additive protection against cyanide toxicity.

In order to determine the stability and pharmacokinetic properties of a 3-MP and Cbi combination therapy, and to ultimately obtain FDA approval, a validated analytical method is essential. Although previous analytical methods have been developed to individually determine 3-MP or various Cbi species [12,27–36], there is a need to develop an analytical technique to simultaneously determine Cbi and 3-MP in order to reduce effort, analysis costs, and minimize necessary sample volumes. Therefore, the focus of this project was to develop an analytical method to simultaneously determine and quantify dicyanocobinamide $Cbi(CN)_2$ and 3-MP using LC–MS-MS.

2. Experimental

2.1. Reagents and standards

All solvents were LC–MS grade unless otherwise noted. Ammonium formate and 3-mercaptopyruvate (3-MP; $HSCH_2COCOOH$) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetone (HPLC grade, 99.5%) was purchased from Alfa Aesar (Ward Hill, MA, USA). Cobinamide (C_{48} H_{72} CoN_{11} O_8; 989.76 g/mol) was obtained from the Department of Medicine, University of California, San Diego (La Jolla, CA) [17]. Potassium cyanide, sodium hydroxide, and Millex® tetrafluoropolyethylene syringe filters (0.22 μm, 4 mm, Billerica, MA, USA) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Isotopically-labeled 3-MP ($HS^{13}CH_2{}^{13}CO^{13}COOH$) was synthesized and provided by the Center for Drug Design, University of Minnesota (Minneapolis, MN, USA) [3]. Monobromobimane (MBB, 3-(bromomethyl)-2,5,6-trimethyl-1*H*,7*H*-pyrazolo[1,2-*a*]pyrazole-1,7-dione; $C_{10}H_{11}BrN_2O_2$) was obtained from Fluka Analytical (Buchs, Switzerland) and a standard solution (500 μM) was prepared in LC–MS grade water and stored at 4 °C. 3-MP calibration and QC standards were prepared from a 3-MP stock solution (10 mM) by serial dilution with swine plasma. The internal standard solution was prepared from a stock solution of 1 mM isotopically-labeled 3-MP in LC-MS grade water and stored at 4 °C. The $Cbi(NO_2)_2$ was prepared by adding excess sodium nitrite (4:1 NO_2^-:$Cbi(H_2O)(OH)^+$) to ensure two nitrite ligands were bound to the Cbi [15]. The $Cbi(CN)_2$ was prepared by adding 3x the concentration of cyanide to $Cbi(H_2O)(OH)^+$ in water. *Note: Cyanide is toxic and is released as HCN in neutral and acidic solutions. Therefore, all solutions were prepared in a well-ventilated hood, and aqueous standards were prepared in ≥10 mM NaOH.*

2.2. Biological fluids

Animal plasma was obtained from four sources: two commercial vendors, a study to evaluate the effectiveness of sulfanegen in rabbits, and a separate study to evaluate Cbi as a cyanide therapeutic in rats. Rat plasma (with Na Heparin as an anti-coagulant) was purchased from Innovative Research (Novi, MI) and stored at −80 °C until used. Rabbit plasma (EDTA anti-coagulated) and swine plasma (EDTA anti-coagulated), were purchased from Pel-Freeze Biological (Rogers, AR, USA) and stored at −80 °C until used. Swine plasma was initially chosen as the method development matrix because at the time of major development of the analytical method, Cbi/sulfanegen combination therapy studies were predominantly carried out in swine. Before the method was fully validated, animal models of efficacy testing for Cbi and 3-MP were transitioned to rats and rabbits, respectively. Therefore, evaluation of the method's ability to determine 3-MP and Cbi from treated animals was carried out from rabbit and rat studies, respectively. Rabbit plasma from a sulfanegen efficacy study was gathered at SRI International in Menlo Park, California. The rabbits (New Zealand, 2.2-4.0 kg) had blood drawn pre-dose. Sulfanegen (136 mg/kg) was administered intramuscularly and blood was drawn at 10 min, 30 min, 1, 2, 4, and 8 h into heparin collection tubes. Plasma was immediately separated from blood and frozen at −70 °C. The samples were then shipped on dry ice to South Dakota State University for analysis. Upon arrival, the plasma samples were stored at −80 °C until analyzed.

Rat plasma from a cobinamide/dimethyl trisulfide (DMTS) efficacy study was gathered at the Department of Chemistry at Sam Houston State University, Huntsville, Texas. Male rats (Charles River Breeding Laboratories, 315–320 g) had a catheter implanted on the jugular vein and a DMTS solution (960 μL, 50 mg/mL in 15% Polysorbate 80) was injected into the right leg muscle. Nitrocobinamide (43 μL, 15 mg/kg) was injected into the left leg muscle. Blood was then drawn into heparinized microcentrifuge tubes at 3, 10, 20, 35, and 90 min after cobinamide administration and plasma was immediately separated from blood by centrifugation (4 °C, 13,500 rpm) for 10 min. Plasma samples were then frozen at −80 °C and shipped on dry ice to South Dakota State University for analysis. Upon arrival, the plasma samples were stored at −80 °C until analyzed.

All animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals [37] and accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. The Institutional Animal Care and

Use Committee (IACUC) at the respective institution approved the experiment.

2.3. One-pot sample preparation of total Cbi

Simultaneous analysis of total Cbi and 3-MP was initially attempted using a one-pot sample preparation method. Total Cbi was determined by denaturing plasma proteins, converting each Cbi species to $Cbi(CN)_2$ using excess cyanide and then analyzing the concentration of $Cbi(CN)_2$. Multiple protein denaturing schemes were evaluated in an attempt to produce quantitative recovery of Cbi, while not degrading 3-MP, by varying base concentration, cyanide concentration, and temperature. Initially, separate volumes of swine plasma (in a 2 mL centrifuge vial) were spiked with $Cbi(CN)_2$, $Cbi(NO_2)_2$, and/or $Cbi(H_2O)(OH)^+$ (50 μL, 1 mM), 3-MP (50 μL, 1 mM), and aqueous 3-MP internal standard (100 μL, 500 μM). Aqueous potassium cyanide and strong base solutions (100 μL each) were then spiked into the plasma, followed by vigorous mixing. To convert the Cbi species to $Cbi(CN)_2$, two concentrations of CN were evaluated, 1.5 mM and 5 mM, which correspond to 3× and 10× molar equivalent of cyanide to the highest concentration of Cbi calibration standard tested. To denature plasma proteins and release bound Cbi species, two concentrations of NaOH (300 μM and 1 mM) were evaluated. When elevated temperatures were investigated for denaturing proteins, samples were heated on a heat block (VWR International, Radnor, PA, USA) in a hood at 80 °C for 15 min. At the conclusion of all denaturing protocols, acetone (600 μL) was added to precipitate plasma proteins. Samples were subsequently cold-centrifuged (4 °C) for 15 min at 13,100 RPM (16,500 × *g*). The supernatant (700 μL) was transferred to a 4 mL glass vial and dried under N_2.

2.4. Quasi one-pot sample preparation of 3-MP and total Cbi

Because the one-pot sample preparation scheme gave undesired results, a quasi one-pot sample preparation scheme was devised (i.e., samples were initially split and then recombined for the duration of sample preparation). To a 2 mL centrifuge vial, separate aliquots of 3-MP (50 μL in plasma), a 3-MP internal standard (100 μL of 15 μM aqueous 3-MP-$^{13}C_3$), and $Cbi(CN)_2$ (50 μL in plasma) were added and mixed. (Note: the addition of $Cbi(CN)_2$ prior to 3-MP internal standard caused 3-MP degradation in prepared samples.) The samples were split evenly (100 μL each) into 2 mL centrifuge vials designated as sub-samples A (i.e., total Cbi analysis) and B (i.e., 3-MP determination). To sub-sample A, cyanide (10 mM KCN in 0.1 M NaOH) was added and samples were heated on a heat block at 80 °C for 15 min. While sub-sample A was being heated, acetone (300 μL) was added to sub-sample B to precipitate proteins. Sub-sample B was then cold-centrifuged (4 °C) for 15 min at 13,100 RPM (16,500 × g) (Thermo Scientific Legend Micro 21R centrifuge, Waltham, MA, USA) and placed in the refrigerator. After sub-sample A was removed from heat, acetone (600 μL) was added to precipitate plasma proteins and the sample was centrifuged. Afterwards, the supernatants (600 μL from A, and 300 μL from B) were recombined in a 4 mL glass vial and dried under N_2.

2.5. Sample preparation for LC–MS-MS

For both the one-pot and quasi one-pot sample preparation schemes, monobromobimane (100 μL 500 μM) and 5 mM aqueous ammonium formate with 10% methanol (100 μL) were added to the dried samples. The resulting solution was heated on a block heater at 70 °C for 15 min to convert 3-MP to a 3-mercaptopyruvate-bimane (3-MPB) complex [28]. Samples were filtered with a 0.22 μm tetrafluoropolyethylene membrane syringe filter into autosampler vials fitted with 150 μL deactivated glass inserts for LC–MS-MS analysis. It should be noted that when the number of samples were above the maximum limit of the sample apparatus (e.g., the centrifuge), the samples that were not being actively prepared were stored in a standard refrigerator (4 °C) to impede degradation of 3-MP.

2.6. LC–MS–MS analysis of $Cbi(CN)_2$ and 3-MPB

For LC-ESI-MS–MS analysis a Shimadzu UHPLC (LC 20A Prominence, Kyoto, Japan) coupled to an electrospray ionization quadrupole ion trap QTRAP 5500 LC–MS/MS System (AB Sciex, Framingham, MA, USA) was used. The low-energy collision dissociation tandem mass spectrometric analysis (CID-MS/MS) was conducted with the LINAC Collision Cell of the triple quadrupole/linear ion trap (LIT) instrument. Separation was performed on a Phenomenex Synergy Max RP column (50 × 2.0 mm, 4 μm 80 Å) with an injection volume of 10 μL from samples stored in a cooled autosampler (15 °C) and a flow rate of 0.25 mL/min. Mobile phase solutions consisted of 5 mM aqueous ammonium formate with 10% methanol (Mobile Phase A) and 5 mM ammonium formate in 90% methanol (Mobile Phase B). A gradient of 0 to 100% B was applied over 3 min, held constant for 0.5 min, then reduced to 0% B over 1.5 min. The gradient produced a total run-time of 5.1 min and retention times of about 2.60 min for 3-MPB and 2.9 min for $Cbi(CN)_2$. The turbo ion-spray interface was heated at 500 °C with zero air nebulization at 90 psi in positive ionization mode with drying and curtain gasses pressures of 60 psi each. The ion-spray voltage, declustering potential, collision cell exit potential, and channel electron multiplier voltage were 4500, 121, 10, and 2600 V, respectively. Low-energy CID-MS/MS using multiple reaction-monitoring (MRM) transition scans of precursor ion → product ion were used for analysis. The precursor → product ions were 311 → 223.1 and 311 → 192.2 *m/z* for 3-MPB and 314 → 223.1 and 314 → 192.2 m/z for the internal standard–bimane complex with collision energies of 30.5 and 25 V, respectively. The $Cbi(CN)_2$ transitions of 1015.9 → 930.5 and 1015.9 → 988.5 were utilized with collision energies of 75.91 and 50.70 V and declustering potentials of 249.42 and 237.79 V, respectively. These transitions correspond to a precursor ion of $Cbi(CN)^+$, which includes both the monocyanocobinamide and dicyanocobinamide species [32]. The 1064 *m/z* transition of $Cbi(CN)_2$ was not used because the 1015.9 *m/z* transition offered greater sensitivity, lower limits of detection (LODs), and accounts for potentially incomplete conversion of Cbi species to $Cbi(CN)_2$. The dwell time was 100 ms for all transitions. Analyst software (Applied Biosystems version 1.5.2) was used for data acquisition and analysis.

2.7. Calibration, quantification and limit of detection

For validation of the analytical method, we generally followed the FDA bioanalytical method validation guidelines [38]. The lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) were defined using the following inclusion criteria: (1) calibrator precision of <15% relative standard deviation (RSD), and (2) accuracy of ±15% of the nominal calibrator concentration back-calculated from the calibration curve. It is important to note that calibration curves must be prepared each day for $Cbi(CN)_2$ because of daily variation in instrumental sensitivity and sample preparation with no internal standard to correct for these errors. Calibration standards were initially prepared from 1 to 1000 μM (1, 2, 5, 10, 20, 50, 100, 200, 500, 1000 μM) for 3-MP, with the range later decreased to 2–500 μM. Calibration curves for $Cbi(CN)_2$ were prepared from 0.5–500 μM (0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500 μM) and later decreased to 0.5–50 μM.

To determine the accuracy and precision of the method, QCs ($N = 5$) were prepared at three concentrations not included in the

calibration curve: 1.5 and 7.5 μM (low QCs for $Cbi(CN)_2$ and 3-MP, respectively), 7.5 and 30 μM (medium QCs for $Cbi(CN)_2$ and 3-MP, respectively), and 35 and 150 μM (high QCs for $Cbi(CN)_2$ and 3-MP, respectively). The 3-MP internal standard was prepared daily and added to each sample, calibration standard and QC during sample preparation. Calibration curves and QCs, were prepared fresh each day during intra-assay (daily) and inter-assay (over three separate days, within seven calendar days) analyses to calculate the intra-assay and inter-assay accuracy and precision.

The LOD was determined by analyzing multiple concentrations of 3-MP (0.2, 0.5, 1, and 2 μM) and $Cbi(CN)_2$ (0.2, 0.5, 1, 2, 3, 4, and 5 μM) below the LLOQ and determining the lowest concentration that reproducibly produced a signal-to-noise ratio (S/N) of 3. For 3-MP, noise was measured as the peak-to-peak noise directly adjacent to the 3-MP peak since it is inherently present in the plasma of mammals [20,21]. $Cbi(CN)_2$ is not endogenously present in plasma, so noise was measured as peak-to-peak noise from blank plasma over the full elution time of $Cbi(CN)_2$.

2.8. Stability, recovery, and matrix effects

To evaluate the stability of the two analytes, low and high QCs were stored at various temperatures (room temperature (RT), 4 °C, −30 °C, and −80 °C) and analyzed at multiple storage times. As stability samples were prepared for analysis, 3-MP internal standard was added to help correct for sample preparation and instrumental variability. The stability of $Cbi(CN)_2$ and 3-MP were calculated as a percentage of the initial concentration (standards were prepared each day of analysis), with the sample considered stable if the concentration of the stored sample was within 10% of the standard. The stability of only $Cbi(CN)_2$ was determined, as the stability of the other Cbi species of interest ($Cbi(H_2O)(OH)^+$ and $Cbi(NO_2)_2$) are well known [15,32]. Long-term stability was conducted at three storage temperatures (4 °C, −30 °C, and −80 °C). The samples were analyzed in triplicate after 1, 6, 10, 15, and 30 days. Autosampler stability of 3-MPB and $Cbi(CN)_2$ was determined after typical sample preparation of low and high QCs and storage in a cooled autosampler (15 °C) for approximately 2, 4, 8, 12, and 24 h. Short term stability of 3-MP and $Cbi(CN)_2$ was conducted at RT, after 0, 2, 4, 8, 12, and 24 h. For freeze–thaw stability of 3-MP and $Cbi(CN)_2$, each set of QCs (low and high) were stored at −80 °C for 24 h, thawed unassisted at RT, and a single set of QCs were prepared and analyzed. The remaining QCs were then placed in the −80 °C freezer. This procedure was repeated for a total of three freeze–thaw cycles. A set of QC standards (low and high) for 3-MP and Cbi were prepared fresh each day. Stability was determined by comparing QCs to standards.

To determine the recovery of $Cbi(CN)_2$ and 3-MP, five aqueous QCs (low, medium, and high) were prepared, analyzed and compared with equivalent concentrations of QCs in plasma. Recovery was calculated as a percentage by dividing the analyte plasma concentration with the calculated aqueous equivalent QC concentration. Matrix effects were evaluated by comparing aqueous and plasma calibration curves.

3. Results and discussion

3.1. Determination of $Cbi(CN)_2$ and 3-MP from swine plasma

Multiple species of Cbi are likely to exist in plasma samples after its administration. For example, if the current formulation of $Cbi(NO_2)_2$ [15] is administered intramuscularly to a victim of cyanide poisoning, $Cbi(NO_2)_2$, $Cbi(CN)_2$, $Cbi(CN)(NO_2)$, $Cbi(H_2O)(OH)^+$, Cbi(CN)(OH), and other Cbi species may be present. Therefore, in order to determine the total Cbi in a plasma sample, all Cbi species were converted to $Cbi(CN)_2$ by the addition of excess cyanide [18,32,39]. This approach is based on the strong affinity cyanide has for the cobalt atom of Cbi, allowing cyanide to out-compete other Cbi ligands and create one form of cobinamide. Once all the Cbi species are converted to $Cbi(CN)_2$, the total Cbi can be analyzed as $Cbi(CN)_2$. Another difficulty in analyzing Cbi is its affinity for haptocorrin proteins [2,32]. Because it is necessary to precipitate plasma proteins during analysis, any protein-bound Cbi would decrease the amount of free Cbi available for analysis, consequently decreasing recovery. Therefore, it was necessary to denature the plasma proteins to release bound Cbi. Denaturing the plasma proteins was accomplished using the combination of base and heat. Simultaneously, CN was used to convert Cbi species to $Cbi(CN)_2$, which has the added advantage of lower affinity for plasma proteins than other Cbi species [18]. Others have used this approach successfully to analyze total Cbi from plasma spectrophotometrically [18].

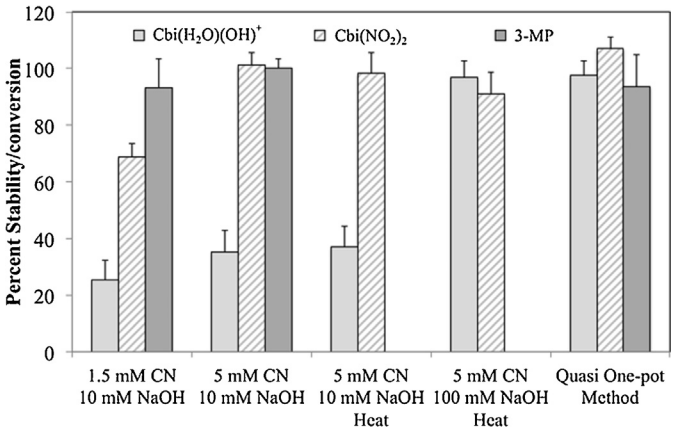

Fig. 1. Various attempts to fully convert Cbi species ($Cbi(H_2O)(OH)^+$) and $Cbi(NO_2)_2$) to $Cbi(CN)_2$, while monitoring 3-MP stability. The use of 1.5 or 5 mM CN and base (10 mM) with or without heat (80 °C) to denature plasma proteins did not fully convert $Cbi(H_2O)(OH)^+$ to $Cbi(CN)_2$. The use of 5 mM CN with base (100 mM) and heat (80 °C) fully converted all Cbi species to $Cbi(CN)_2$ but degraded 3-MP. The quasi one-pot method allowed full conversion of Cbi species to $Cbi(CN)_2$ and protection of the 3-MP.

It is known that 3-MP degrades quickly in plasma at room temperature [28], so it was inferred that it would be highly unstable when denaturing proteins at elevated temperatures. Therefore, no heat was used to denature proteins when initially converting Cbi species to $Cbi(CN)_2$ with excess cyanide. Two concentrations of cyanide were evaluated, 1.5 and 5 mM (i.e., 3× and 10× the highest concentration $Cbi(CN)_2$ calibration standard tested) under mildly basic conditions (10 mM NaOH). As shown in Fig. 1, the use of 1.5 and 5 mM cyanide produced ~70% and ~100% conversion $Cbi(NO_2)_2$, respectively. Although 100% of the $Cbi(NO_2)_2$ was converted to $Cbi(CN)_2$ using 5 mM CN, only about 30% of the $Cbi(H_2O)(OH)^+$ was converted to $Cbi(CN)_2$ at both CN concentrations tested. Although it was expected that excess CN would degrade 3-MP, it was found that 3-MP signals remained consistently 100 ± 10%. This may be due to the rapid reactivity of CN with Cbi and multiple other components of blood, inclusively minimizing the reaction of CN with free 3-MP.

The inability to fully convert $Cbi(H_2O)(OH)^+$ to $Cbi(CN)_2$ under ambient temperatures indicated that the conditions were too mild to fully release the $Cbi(H_2O)(OH)^+$ from plasma proteins. Therefore, a harsher denaturing protocol was added to the sample preparation procedure, where both higher concentrations of base and elevated temperatures were evaluated. Fig. 1 shows that 5 mM KCN, a higher concentration of base (0.1 M NaOH), and heating at 80 °C converted both $Cbi(NO_2)_2$ and $Cbi(H_2O)(OH)^+$ completely to $Cbi(CN)_2$ (100 ± 10%). However, as expected, 3-MP was not stable

at this temperature. Since the conditions to prepare 3-MP and Cbi were not fully compatible, we evaluated a quasi one-pot sample preparation procedure.

For the quasi one-pot procedure, the samples were initially split in half and prepared separately until precipitation of proteins occurred, then the prepared samples were re-combined for the duration of the analysis. As shown in Fig. 1, full conversion of Cbi to $Cbi(CN)_2$ was successful and 3-MP was stable with the quasi one-pot procedure. Therefore, the quasi one-pot sample preparation scheme was adopted for plasma sample preparation in order to analyze 3-MP and $Cbi(CN)_2$ simultaneously.

Utilizing the quasi one-pot sample preparation, Fig. 2 shows the chromatograms of 3-MP (A) and $Cbi(CN)_2$ (B), eluting at 2.6 and 2.9 min, respectively. Fig. 2A shows four 3-MP chromatograms that are plotted from samples prepared with multiple Cbi species. Each is essentially identical, indicating the presence of the Cbi species do not interfere with the analysis of 3-MP. Fig. 2B, shows chromatograms of $Cbi(CN)_2$ after conversion from $Cbi(H_2O)(OH)^+$ and $Cbi(NO_2)_2$, each in the presence of 3-MP. The chromatograms are essentially identical to the $Cbi(CN)_2$ standard, indicating that full conversion of $Cbi(H_2O)(OH)^+$ and $Cbi(NO_2)_2$ was accomplished, and that 3-MP does not interfere with Cbi analysis. The final combined 3-MP/Cbi method shows excellent selectivity with no interfering peaks observed in the blank. In fact, in swine plasma, no other peaks were observable for either 3-MPB or $Cbi(CN)_2$.

3.2. Linear range, limit of detection, and sensitivity

The linear range, limit of detection, and sensitivity of the method were evaluated to ensure the applicability of the method for combined 3-MP and Cbi analysis. The linear range for 3-MP was 2–500 μM using a $1/x^2$ weighted curve. Of the original concentrations tested, 1 and 1000 μM were excluded because they did not meet the accuracy and/or precision inclusion criteria. The linear range of the current method for 3-MP was actually greater than our previous study, where we analyzed 3-MP individually [28]. The linear dynamic range for $Cbi(CN)_2$ was 0.5–50 μM using a $1/x^2$ weighted calibration curve. Cbi calibrators at concentrations of 100, 200, and 500 μM were excluded because they did not meet the accuracy and/or precision inclusion criteria.

The LODs of $Cbi(CN)_2$ and 3-MP were 0.2 μM and 0.5 μM, respectively. The LOD of 3-MP was similar to our previous analysis of 3-MP alone, 0.1 μM [28], but was significantly higher than the 1 nM LOD found for an HPLC method with fluorescence detection [27] utilized by Ogasawara et al. The LOD for $Cbi(CN)_2$ was also higher than a spectrophotometric method reported by Ma et al., which generated a LOD of 30 nM [31]. Although the LODs for the method reported here were higher than both the HPLC and spectrophotometric methods, they are acceptable for translational studies of 3-MP and Cbi, because plasma concentrations of 3-MP and Cbi from treated animals and humans are expected to be well above 1 μM [15,19,25,26,28].

The LLOQs for 3-MP and for $Cbi(CN)_2$ were 2 μM and 0.5 μM, respectively. The ULOQs were 500 μM for 3-MP and 50 μM for $Cbi(CN)_2$. Boss et al. reported a ULOQ of 10 μM with a linear range of 2.5 orders of magnitude. Ogasawara et al. reported a linear range from 0.2 to 40 μM for 3-MP, and our LC–MS-MS previous method for 3-MP alone had a linear range from 0.5 to 100 μM. Similar to the LODs, although previous methods had lower linear ranges, our linear range covers at least 2 orders of magnitude and should be very useful with therapeutic studies where large concentrations are administered.

3.3. Accuracy and precision

Accuracy and precision were determined by quintuplicate analysis of low, medium, and high QCs (7.5, 30, and 150 μM for 3-MP and 1.5, 7.5 and 35 μM for $Cbi(CN)_2$) on three different days (within 7 calendar days; Table 1). The intra-assay accuracy and precision for 3-MP were 100 ± 9% and <5.5% RSD, respectively. For $Cbi(CN)_2$, the intra-assay accuracy was 100 ± 13% and the intra-assay precision was <9% RSD. The inter-assay accuracy for 3-MP was 100 ± 9% and 100 ± 10% for $Cbi(CN)_2$. The inter-assay precision for 3-MP was <8.5% and <9.5% for $Cbi(CN)_2$. 3-MP had better accuracy and precision due to the internal standard that could correct for daily instrumental variations. Even without an internal standard for $Cbi(CN)_2$, the precision and accuracy for the method were excellent and well within the FDA method validation guidelines for both analytes.

Our previous method for 3-MP analysis produced an accuracy and precision of 100 ± 9% and <7% RSD, respectively, in aggregate (both intra and inter assay values are included) [28]. Ogasawara et al. [27] reported a better precision of <4.8%, but no accuracy was presented. The precision for the analysis of total cobinamide using the Ma et al. multiwavelength spectrometry method was similar, <7.6% for all QCs [31]. The likely cause of the slightly worse precision for our method compared to other similar methods for single species, is differences in the instruments used and the more involved sample preparation requirements for the simultaneous analysis of 3-MP and Cbi.

3.4. Stability, recovery, and matrix effects

The long term stability of $Cbi(CN)_2$ and 3-MP in swine plasma (i.e., both $Cbi(CN)_2$ and 3-MP were added to each plasma sample for stability studies) was evaluated at 4, −30, and −80 °C, with short-term stability evaluated at room temperature for 24 h. The low and high 3-MP QCs were stable at −80 °C for at least 30 days, but both degraded rapidly at 4 °C. While the high 3-MP QC was stable at −30 °C for at least 30 days, the low QC was only stable for 6 days. $Cbi(CN)_2$ was extremely stable, with the low and high $Cbi(CN)_2$ QCs stable for at least 30 days under each long-term storage condition. For freeze–thaw stability, 3-MP and $Cbi(CN)_2$ were both stable for all three cycles. This result differs from our previous 3-MP analysis, where 3-MP was only stable for one freeze–thaw cycle [28]. To determine if Cbi had a stabilizing effect on 3-MP during freeze–thaw cycles, freeze–thaw stability was evaluated with 3-MP alone. The 3-MP was still stable for 3 freeze–thaw cycles, indicating that $Cbi(CN)_2$ did not affect the 3-MP stability. In our previous method, although 3-MP was reported as stable for only one freeze–thaw cycle (i.e., within 90% of the initial analysis), it was within 80% of the initial analysis for all three days [28]. Therefore, it is likely that improved sample preparation and handling during the current study increased the stability of 3-MP for the second and third freeze–thaw cycles. For short-term stability (at RT), 3-MP was stable for less than 4 h for both the high and low QCs. The 3-MP was again more stable than in our previous work [28]. The $Cbi(CN)_2$ was extremely stable at RT, with concentrations for both QCs within ±10% for the entire study. In the autosampler, both the prepared 3-MP, as 3-MPB, and $Cbi(CN)_2$ were found stable for at least 24 h. The results from the stability studies, suggest that if storage is necessary, plasma samples with 3-MP and $Cbi(CN)_2$ should be frozen and immediately stored at −80 °C. Once samples are thawed, they should be prepared immediately and refrozen if future analysis is necessary. Samples can also be stored on a cooled autosampler for at least 24 h after preparation.

The recovery of 3-MP for the low, medium and high QCs were 104%, 99% and 99%, respectively, and no matrix effects were observed for 3-MP. Recoveries of 3-MP in previous methods were

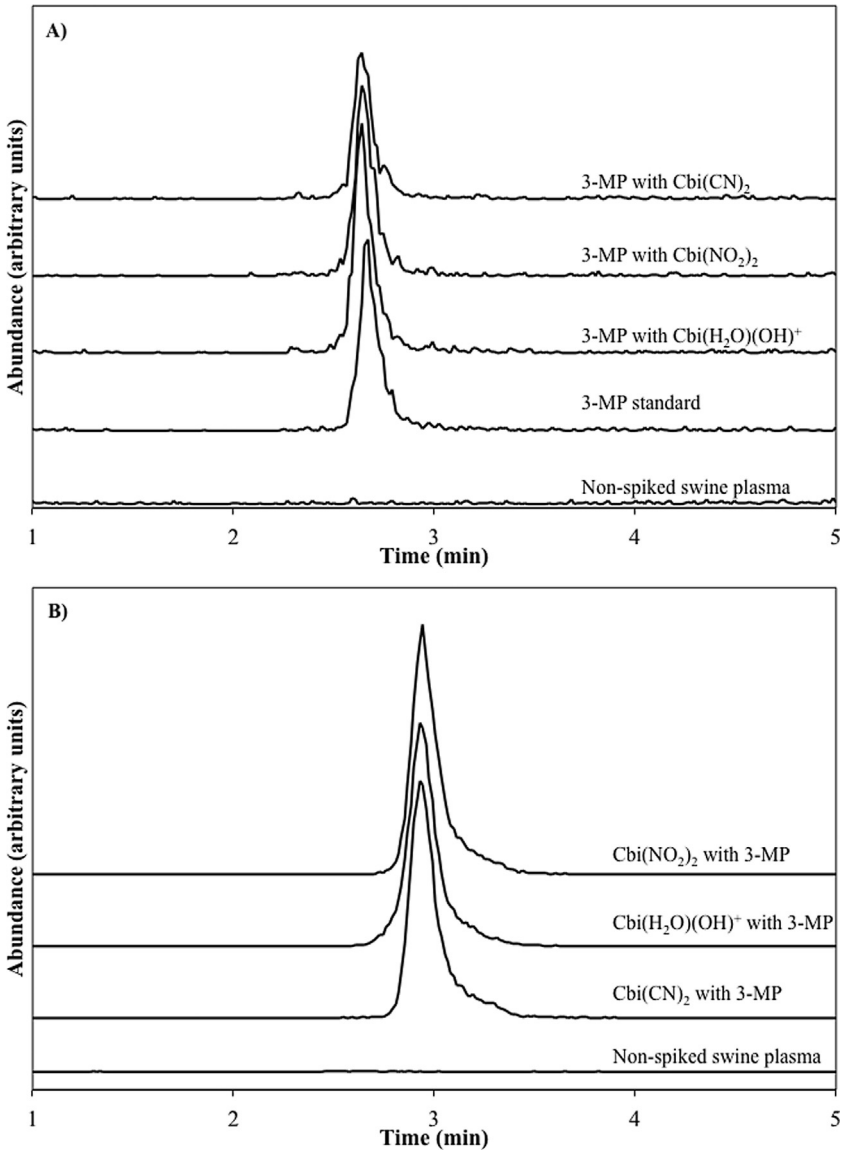

Fig. 2. LC–MS-MS analysis of 3-MP and $Cbi(CN)_2$ analytes in swine plasma. (A) The chromatograms (MRM 311 → 223 *m/z*) represent the signal response of 3-MP, eluting at 2.65 min, prepared from plasma with varying Cbi species and excess cyanide. (B) Chromatograms of multiple species of Cbi with 3-MP and excess cyanide after conversion to $Cbi(CN)_2$. The $Cbi(CN)_2$ elutes at 2.91 min. The chromatograms represent the signal response of the MRM transition of 1015.9 → 930.5 *m/z*.

Table 1
The accuracy and precision of 3-mercaptopyruvate and $Cbi(CN)_2$ analysis spiked in swine plasma by LC–MS-MS.

Analyte	Concentration (μM)	Intra-assay accuracy[a] (%)	Intra-assay precision[a] (%RSD)	Inter-assay accuracy[b] (%)	Inter-assay precision[b] (%RSD)
3-MP	7.5	100 ± 4	5.24	100 ± 5	8.22
	30	100 ± 9	2.57	100 ± 9	5.83
	150	100 ± 5	5.12	100 ± 6	8.34
$Cbi(CN)_2$	1.5	100 ± 7	7.96	100 ± 10	8.43
	7.5	100 ± 10	2.73	100 ± 9	9.47
	35	100 ± 13	8.99	100 ± 7	6.48

[a] QC method validation ($N = 5$) for day 1.
[b] QC mean from three different days of method validation ($N = 15$).

75–81% [28] and 79–85% [27]. Again, improved sample handling is likely the reason for improved 3-MP recoveries in this study. The apparent initial recoveries for $Cbi(CN)_2$ were 29%, 29%, and 33% for the low, medium, and high QCs, respectively. The recoveries were highly reproducible both with and without 3-MP. To determine if the apparently low recoveries for Cbi were truly due to low recovery or due to matrix effects, calibration curves for $Cbi(CN)_2$ were produced in aqueous solution and in plasma. The slope of the $Cbi(CN)_2$ plasma calibration curve was 2–3 times less than the slope of the aqueous calibration curve, clearly indicating matrix effects were present for $Cbi(CN)_2$ analysis from plasma. Therefore, one-half to one-third of the aqueous signal is produced when the plasma matrix is present. In aggregate the extent of the matrix effects, results from the stability studies, and the ability to fully convert all Cbi species to $Cbi(CN)_2$ (Fig. 1), indicates that the apparently low recovery was caused by matrix effects. Because an internal

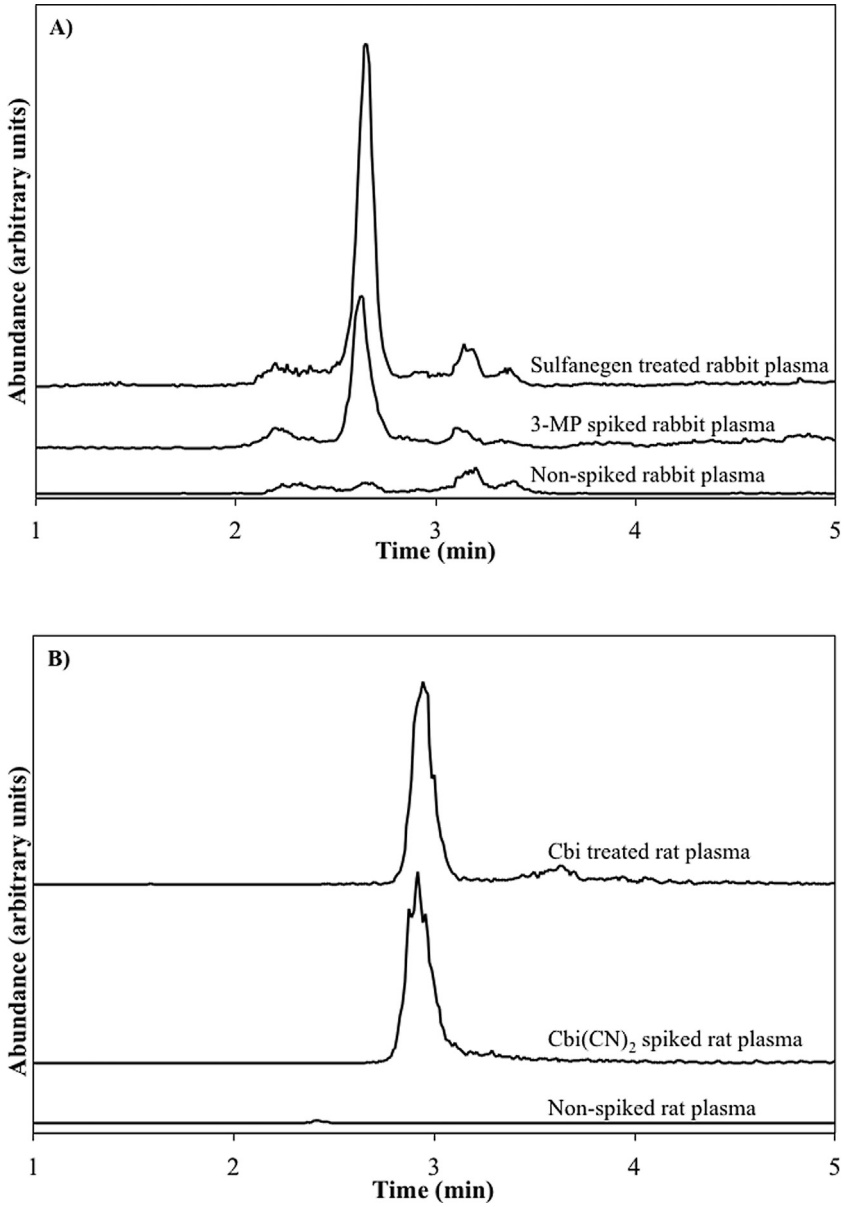

Fig. 3. (A) The LC–MS-MS chromatograms from sulfanegen treated rabbits compared to 3-MP spiked rabbit and swine plasma, and blank plasma with all 3-MP samples eluting at 2.65 min. (B) Cbi treated rats compared to spiked $Cbi(CN)_2$ swine and rat plasma and blank plasma, with $Cbi(CN)_2$ eluting at 2.9 min. Treated rats with Cbi and spiked plasma have the same retention time of $Cbi(CN)_2$. Non-spiked and spiked 3-MP or $Cbi(CN)_2$ swine can be seen in Fig. 2.

standard for $Cbi(CN)_2$ would likely correct for matrix effects, the addition of an internal standard for $Cbi(CN)_2$ may be an area of further improvement of the method.

3.5. Analysis of Cbi and sulfanegen-exposed animals

The validated LC–MS-MS method was applied to the analysis of plasma gathered from rabbits and rats treated with sulfanegen and cobinamide, respectively. The LC–MS-MS analysis of sulfanegen treated rabbit and Cbi treated rat plasma is shown in Fig. 3. The sulfanegen treated rabbit plasma contains greatly elevated 3-MP concentrations compared to untreated rabbits. Unlike 3-MP swine samples, another peak was observed in rabbit plasma (eluting at 3.15 min), but was fully resolved from the 3-MP peak ($R_s = 1.91$). Similarly, elevated Cbi concentrations were observed with Cbi treated compared to untreated rats. Overall, Fig. 3 confirms that the validated method has the ability to detect 3-MP and Cbi in the plasma of sulfanegen and cobinamide treated animals. In the near future, a pharmacokinetic study that utilizes sulfanegen and Cbi as a combination therapeutic for cyanide poisoning will be performed using the LC–MS-MS method presented here.

4. Conclusion

An LC–MS-MS method for the simultaneous detection of $Cbi(CN)_2$ and 3-MP was developed featuring simple sample preparation, rapid analysis, excellent accuracy and precision, excellent limits of detection, and linear ranges of at least 2 orders of magnitude. The method presented here has the ability to simultaneously analyze 3-MP and Cbi from the plasma of treated animals, which will facilitate the study of sulfanegen and Cbi as a combination therapy for cyanide poisoning.

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References

[1] A.H. Hall, J. Saiers, F. Baud, Which cyanide antidote? Crit. Rev. Toxicol. 39 (2009) 541–552.
[2] K.E. Broderick, P. Potluri, S. Zhuang, I.E. Scheffler, V.S. Sharma, R.B. Pilz, G.R. Boss, Cyanide detoxification by the cobalamin precursor cobinamide, Exp. Biol. Med. 231 (2006) 641–649.
[3] H.T. Nagasawa, D.J.W. Goon, D.L. Crankshaw, R. Vince, S.E. Patterson, Novel, orally effective cyanide antidotes, J. Med. Chem. 50 (2007) 6462–6464.
[4] M. Sutter, N. Tereshchenko, R. Rafii, G.P. Daubert, Hemodialysis complications of hydroxocobalamin: a case report, J. Med. Toxicol. 6 (2010) 165–167.
[5] J. Hamel, A review of acute cyanide poisoning with a treatment update, Crit. Care Nurse 31 (2011) 72–82.
[6] D.J. O'Brien, D.W. Walsh, C.M. Terriff, A.H. Hall, Empiric management of cyanide toxicity associated with smoke inhalation, Prehosp. Disaster Med. 26 (2011) 374–382.
[7] S.W. Borron, F.J. Baud, P. Barriot, M. Imbert, C. Bismuth, Prospective study of hydroxocobalamin for acute cyanide poisoning in smoke inhalation, Ann. Emerg. Med. 49 (2007) 794–801.e2.
[8] A.H. Hall, R. Dart, G. Bogdan, Sodium thiosulfate or hydroxocobalamin for the empiric treatment of cyanide poisoning? Ann. Emerg. Med. 49 (2007) 806–813.
[9] C.A. DesLauriers, A.M. Burda, M. Wahl, Hydroxocobalamin as a cyanide antidote, Am. J. Ther. 13 (2006) 161–165.
[10] C.E. Becker, N.L. Benowitz, J.C. Forsyth, A.H. Hall, P.D. Mueller, J. Osterloh, Hydroxocobalamin as a cyanide antidote: safety, efficacy and pharmacokinetics in heavily smoking normal volunteers, J. Toxicol.: Clin. Toxicol. 31 (1993) 277–294.
[11] C. Brunel, C. Widmer, M. Augsburger, F. Dussy, T. Fracasso, Antidote treatment for cyanide poisoning with hydroxocobalamin causes bright pink discolouration and chemical-analytical interferences, Forensic Sci. Int. 223 (2012) 10–12.
[12] K.E. Broderick, L. Alvarez, M. Balasubramanian, D.D. Belke, A. Makino, A. Chan, V.L. Woods, W.H. Dillmann, V.S. Sharma, R.B. Pilz, T.D. Bigby, G.R. Boss, Nitrosyl-cobinamide, a new and direct nitric oxide-releasing drug effective in vivo, Soc. Exp. Biol. Med. 232 (2007) 1432–1440.
[13] V.S. Sharma, R.B. Pilz, G.R. Boss, D. Magde, Reactions of nitric oxide with vitamin B12 and its precursor, cobinamide, Biochemistry 42 (2003) 8900–8908.
[14] G. Hayward, H. Hill, J. Pratt, N. Vanston, R. Williams, 1196. The chemistry of vitamin B12. Part IV. The thermodynamic trans-effect, J. Chem. Soc. (1965) 6485–6493.
[15] A. Chan, J. Jiang, A. Fridman, L.T. Guo, G.D. Shelton, M. Liu, C. Green, K.J. Haushalter, H.H. Patel, J. Lee, Y. David, T. Burney, D. Mukai, S.B. Mahon, M. Brenner, R.B. Pilz, G.R. Boss, Nitrocobinamide, a new cyanide antidote that can be administered by intramuscular injection, J. Med. Chem. 58 (2015) 1750–1759.
[16] A. Chan, D.L. Crankshaw, A. Monteil, S.E. Patterson, H.T. Nagasawa, J.E. Briggs, J.A. Kozocas, S.B. Mahon, M. Brenner, R.B. Pilz, T.D. Bigby, G.R. Boss, The combination of cobinamide and sulfanegen is highly effective in mouse models of cyanide poisoning, Clin. Toxicol. 49 (2011) 366–373.
[17] M. Brenner, S.B. Mahon, J. Lee, J. Kim, D. Mukai, S. Goodman, K.A. Kreuter, R. Ahdout, O. Mohammad, V.S. Sharma, W. Blackledge, G.R. Boss, Comparison of cobinamide to hydroxocobalamin in reversing cyanide physiologic effects in rabbits using diffuse optical spectroscopy monitoring, J. Biomed. Opt. 15 (2010) 017001.
[18] 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. 48 (2010) 709–717.
[19] M. Brenner, J.G. Kim, J. Lee, S.B. Mahon, D. Lemor, R. Ahdout, G.R. Boss, W. Blackledge, L. Jann, H.T. Nagasawa, S.E. Patterson, Sulfanegen sodium treatment in a rabbit model of sub-lethal cyanide toxicity, Toxicol. Appl. Pharmacol. 248 (2010) 269–276.
[20] G.E. Isom, J.L. Borowitz, S. Mukhopadhyay, Sulfurtransferase Enzymes Involved in Cyanide Metabolism, In: C. McQueen, (Ed.), Comprehensive Toxicology, (2010), pp. 485–500.
[21] A. Spallarossa, F. Forlani, A. Carpen, A. Armirotti, S. Pagani, M. Bolognesi, D. Bordo, The rhodanese fold and catalytic mechanism of 3-mercaptopyruvate sulfurtransferases: crystal structure of SseA from *Escherichia coli*, J. Mol. Biol. 335 (2004) 583–593.
[22] N. Nagahara, T. Ito, M. Minami, Mercaptopyruvate sulfurtransferase as a defense against cyanide toxication. Molecular properties and mode of detoxification, Histol. Histopathol. 14 (1999) 1277–1286.
[23] M. Wróbel, H. Jurkowska, L. Śliwa, Z. Srebro, Sulfurtransferases and cyanide detoxification in mouse liver, kidney, and brain, Toxicol. Mech. Methods 14 (2004) 331–337.
[24] A.A. Al-Qarawi, H.M. Mousa, B.H. Ali, Tissue and intracellular distribution of rhodanese and mercaptopyruvate sulphurtransferase in ruminants and birds, Vet. Res. 32 (2001) 63–70.
[25] S.E. Patterson, A.R. Monteil, J.F. Cohen, D.L. Crankshaw, R. Vince, H.T. Nagasawa, Cyanide antidotes for mass casualties: water-soluble salts of the dithiane (sulfanegen) from 3-mercaptopyruvate for intramuscular administration, J. Med. Chem. 56 (2013) 1346–1349.
[26] K.G. Belani, H. Singh, D.S. Beebe, P. George, S.E. Patterson, H.T. Nagasawa, R. Vince, Cyanide toxicity in juvenile pigs and its reversal by a new prodrug, sulfanegen sodium, Anesth. Analg. 114 (2012) 956–961.
[27] Y. Ogasawara, T. Hirokawa, K. Matsushima, S. Koike, N. Shibuya, S. Tanabe, K. Ishii, A novel method for the analysis of 3-mercaptopyruvate using high-performance liquid chromatography with fluorescence detection, J. Chromatogr. B 931 (2013) 56–60.
[28] M.W. Stutelberg, C.V. Vinnakota, B.L. Mitchell, A.R. Monteil, S.E. Patterson, B.A. Logue, Determination of 3-mercaptopyruvate in rabbit plasma by high performance liquid chromatography tandem mass spectrometry, J. Chromatogr. B 949–950 (2014) 94–98.
[29] R.H. Allen, S.P. Stabler, Identification and quantitation of cobalamin and cobalamin analogues in human feces, Am. J. Clin. Nutr. 87 (2008) 1324–1335.
[30] Y. Men, E.C. Seth, S. Yi, T.S. Crofts, R.H. Allen, M.E. Taga, L. Alvarez-Cohen, Identification of specific corrinoids reveals corrinoid modification in dechlorinating microbial communities, Environ. Microbiol. (2014) 1–12.
[31] J. Ma, P.K. Dasgupta, W. Blackledge, G.R. Boss, Cobinamide-based cyanide analysis by multiwavelength spectrometry in a liquid core waveguide, Anal. Chem. 82 (2010) 6244–6250.
[32] J. Ma, P.K. Dasgupta, F.H. Zelder, G.R. Boss, Cobinamide chemistries for photometric cyanide determination. A merging zone liquid core waveguide cyanide analyzer using cyanoaquacobinamide, Anal. Chim. Acta 736 (2012) 78–84.
[33] S.H. Ford, A. Nichols, M. Shambee, The preparation and characterization of the diaquo-forms of several incomplete corrinoids: cobyric acid, cobinamide, and three isomeric cobinic acid pentaamides, J. Inorg. Biochem. 41 (1991) 235–244.
[34] S.H. Ford, J. Gallery, A. Nichols, M. Shambee, High-performance liquid chromatographic analysis of the (cyanoaquo) stereoisomers of several putative vitamin B 12 precursors, J. Chromatogr. A 537 (1991) 235–247.
[35] A. Astier, F.J. Baud, Simultaneous determination of hydroxocobalamin and its cyanide complex cyanocobalamin in human plasma by high-performance liquid chromatography. Application to pharmacokinetic studies after high-dose hydroxocobalamin as an antidote for severe cyanide poisoning, J. Chromatogr. B: Biomed. Sci. Appl. 667 (1995) 129–135.
[36] F. Blanche, D. Thibaut, M. Couder, J.-C. Muller, Identification and quantitation of corrinoid precursors of cobalamin from *Pseudomonas* denitrificans by high-performance liquid chromatography, Anal. Biochem. 189 (1990) 24–29.
[37] Guide for the Care and Use of Laboratory Animals, 8th ed., The National Academies Press, Washington, D.C., (2011).
[38] Food and Drug Administration Guidance for industry bioanalytical method validation, Rockville, MD, (2001).
[39] K. Zhou, F. Zelder, One-step synthesis of α/β cyano-aqua cobinamides from vitamin B12 with Zn (II) or Cu (II) salts in methanol, J. Porphy. Phthalocyanines 15 (2011) 555–559.