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Cobinamide is superior to other treatments in a mouse model of cyanide poisoning

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Abstract

Context—Cyanide is a rapidly acting cellular poison, primarily targeting cytochrome c oxidase, and is a common occupational and residential toxin, mostly via smoke inhalation. Cyanide is also a potential weapon of mass destruction, with recent credible threats of attacks focusing the need for better treatments, since current cyanide antidotes are limited and impractical for rapid deployment in mass casualty settings.

Objective—We have used mouse models of cyanide poisoning to compare the efficacy of cobinamide, the precursor to cobalamin (vitamin B₁₂), to currently approved cyanide antidotes. Cobinamide has extremely high affinity for cyanide and substantial solubility in water.

Materials and Methods—We studied cobinamide in both an inhaled and intraperitoneal model of cyanide poisoning in mice.

Results—We found cobinamide more effective than hydroxocobalamin, sodium thiosulfate, sodium nitrite, and the combination of sodium thiosulfate-sodium nitrite in treating cyanide poisoning. Compared to hydroxocobalamin, cobinamide was 3 and 11 times more potent in the intraperitoneal and inhalation models, respectively. Cobinamide sulfite was rapidly absorbed after intramuscular injection, and mice recovered from a lethal dose of cyanide even when given at a time when they had been apneic for over two minutes. In range finding studies, cobinamide sulfite at doses up to 2000 mg/kg exhibited no clinical toxicity.

Discussion and Conclusion—These studies demonstrate that cobinamide is a highly effective cyanide antidote in mouse models, and suggest it could be used in a mass casualty setting, because it can be given rapidly as an intramuscular injection when administered as cobinamide sulfite. Based on these animal data cobinamide sulfite appears to be an antidote worthy of further testing as a therapy for mass casualties.

Keywords

Antidote; Poisoning management; Poisoning; Hydroxocobalamin

Introduction

Cyanide is an extremely potent and rapidly acting cellular poison. Cytochrome c oxidase appears to be its primary intracellular target, although cyanide binds to other metalloenzymes¹. Hydrogen cyanide (HCN) gas, the cyanide form present under

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physiological conditions, reacts with purified cytochrome c oxidase in two steps: (i) relatively rapid formation of an enzyme-HCN intermediate; and (ii) slow conversion of the intermediate to a stable product, possibly an enzyme-cyanide ion complex that blocks mitochondrial electron transport^{2,3}. The LD₅₀ of potassium cyanide (KCN) for animals is in the range of 2–8 mg/kg, with as little as 50 mg fatal to humans².

Cyanide appears to have been used as a weapon dating back to ancient Rome⁷. Because it is easy and inexpensive to make, it is a potential weapon of mass destruction, either as HCN gas in an enclosed space or as potassium or sodium cyanide added to water or food supplies. It was used in the Nazi concentration camps during the Holocaust as Zyklon B, a stabilized form of cyanide. The Jonestown Massacre in 1978 is the most recent mass cyanide poisoning, and, in 2003, United States intelligence authorities learned of a credible al-Qaeda plot to use cyanide in the New York subway system⁸.

Current treatments for cyanide poisoning are hydroxocobalamin, sodium nitrite, and sodium thiosulfate, all of which must be given by intravenous injection. We have shown that cobinamide is superior to hydroxocobalamin as a cyanide antidote in cultured cells, *Drosophila melanogaster*⁹, and in a sublethal rabbit model¹⁰. When combined with sodium sulfite, intramuscular cobinamide rapidly and effectively reverses cyanide toxicity in the rabbit model¹¹. We now show that cobinamide is superior to the current treatments for cyanide poisoning in two lethal mouse models, and is highly effective by intramuscular injection when used with sodium sulfite.

Materials and Methods

Materials

Male C57BL/6J mice, 6–12 weeks old, were from Jackson Laboratories (Bar Harbor, ME), and were fed Teklad 7001 standard diet from Harlan Laboratories (Madison, WI) *ad libitum*. All studies were performed according to NIH Guidelines for the Care and Use of Laboratory Animals, and approved by the Institutional Animal Care and Use Committee of the Veterans Administration San Diego Healthcare System. Potassium cyanide (Fisher Scientific Inc; Waltham, MA) was dissolved immediately before use in 0.1 M NaOH for the inhalation model, and in 10 mM Na₂CO₃ for the intraperitoneal injection model; the pK_a of HCN is 9.3, and thus in these alkaline solutions cyanide is present as a non-volatile salt. A 4.3 L gas chamber constructed of acrylic glass (Plexiglas®) was maintained at 30° C using a heated-air circulation system regulated by a feedback loop controller (Watlow, Winona, MN) (please see full details and Figure 1 in the supplement). Aquohydroxocobinamide, referred to as cobinamide throughout the text, was prepared from hydroxocobalamin (Wockhardt LTD, Mumbai, India) under mild alkaline conditions using cerium hydroxide¹². The cobinamide product was isolated on a weak cation exchange column eluted with a NaCl gradient, and was desalted on a C₁₈ reversed phase column. The final product was concentrated on a rotary evaporator and by lyophilization. By high performance liquid chromatography analysis, the cobinamide used in these studies was > 95% pure, with the major contaminant being hydroxocobalamin carried through unhydrolyzed. All other chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO) and were of the highest purity available.

Choice of the Animal Model and Study Conditions

Mice were chosen for these studies because they are the smallest mammal in which the proposed work could be conducted. C57BL/6 mice were used because they are a well-characterized, in-bred mouse strain used in prior studies of cyanide toxicity. Cyanide treatment is classified as a USDA Pain and Distress Category E condition, and the IACUC

of the VA San Diego and the investigators deemed the study acceptable only if the mice were anesthetized. The investigators realized this might have impacted the outcome, but concluded that without the use of anesthesia the proposed work was inhumane.

Exposure of Mice to Inhaled Cyanide

Mice were anesthetized with isoflurane (Baxter Healthcare Co., Deerfield, IL) in the airtight gas chamber described above using an amount of liquid isoflurane calculated to deliver 2% when fully evaporated. This led to a surgical plane of anesthesia within 5 min, which was maintained throughout the experiment. Once the mice were anesthetized, HCN gas was generated in the chamber by injecting 100 mM KCN into a glass beaker containing 10 ml of 1 M sulfuric acid. Mice were exposed to the gas for 30 min, and were observed for the onset of respiratory arrest. The HCN concentration in the chamber was stable over the duration of exposure, when measured in gas samples using previously described methods^{9, 13}.

Mice were given cyanide antidotes or saline solution by intraperitoneal injection 15 min prior to being placed into the cyanide gas chamber. They were observed for survival during the 30 min interval of exposure and for the following three days. In all cases, at least five animals were studied per condition.

Intraperitoneal Injection of Mice with Cyanide

Mice were anesthetized with 3% isoflurane in an induction chamber, and maintained at 2% isoflurane using a nose cone; core temperature was kept at 36.5° C using a temperature-controlled warming table. The mice were then administered antidotes or saline solution intravenously via lateral tail vein in a volume of 100 µl. Immediately following antidote administration, 20 mM KCN was injected into the peritoneal cavity in 200 µl. The antidotes and cyanide were given via different routes to avoid possible direct interaction prior to systemic delivery to the animal. Animals were observed for 1 h for the onset of death, defined as apnea without further respiratory effort or movement, or palpable cardiac pulsation. In all circumstances, at least five animals were studied per condition.

Measurement of Red Blood Cell Cyanide Concentration

Cyanide in blood is bound almost exclusively to methemoglobin in red blood cells (RBCs); thus, blood cyanide can be measured by separating RBCs from plasma, and acidifying the RBCs to release cyanide as HCN gas¹⁴. Heparinized whole blood was collected by intracardiac puncture at the time of sacrifice. It was centrifuged and the pelleted RBCs were lysed in ice-cold water. The lysates were placed into glass tubes sealed with stoppers holding plastic center wells (Kontes Glass Co., Vineland, NJ) containing 0.1 M NaOH. A volume of 10% trichloroacetic acid equal to the lysate was added through the septum of the stopper, and the tubes were shaken at 37° C for 60 min. After cooling to room temperature, cyanide trapped in the NaOH was measured in a spectrophotometric assay following its reaction with *p*-nitrobenzaldehyde and *o*-dinitrobenzene at 560 nm^{13, 15}. Concentrations were determined from standard curves using freshly prepared KCN dissolved in 0.1 M NaOH.

Measurement of Mouse Plasma and Urinary Thiocyanate Concentrations

Plasma was obtained as described above, and urine was collected after sacrifice by intravesical puncture. Samples were placed into the stoppered tubes containing plastic center wells described above, and thiocyanate was oxidized to cyanide at 37°C using acidified KMnO₄¹⁶. Ethanol was injected through the stopper after 3–5 min to quench the reaction. The resultant HCN gas trapped in the NaOH in the center wells was measured as described above for measuring RBC cyanide.

Intramuscular Injection of Cobinamide

We administered 0.2 mmol/kg (200 mg/kg) of cobinamide or cobinamide sulfite (cobinamide mixed with equimolar sodium sulfite) in 50 μ l into the gastrocnemius muscle of mice. To examine the kinetics of cobinamide, these animals were rapidly sacrificed at 2.5, 5, 10, 60 and 360 min after the intramuscular injection. To examine the efficacy of these preparations when administered intramuscularly, the intramuscular injection was preceded 3 min by an intraperitoneal injection of 0.16 mmol/kg of KCN (a lethal dose) and the animals were observed for death as an end point. In all cases, at least five animals were studied per condition.

Measurement of Plasma Cobinamide Concentration

Blood samples were heparinized and plasma was separated by centrifugation. Cobinamide in the plasma was converted to dicyanocobinamide by adding KCN to a final concentration of 5 mM. Protein in the plasma was denatured by heating the samples to 80° C for 15 min in a chemical fume hood, followed by adding an equal volume of acetonitrile. The samples were vortexed for 5 min, and centrifuged at 10,000 rpm for 15 min at 4° C. The supernatants were dried by rotary vacuum, re-constituted in 0.2 ml water, and clarified through a 0.20 μ m filter. The samples were analyzed on a high performance liquid chromatography system using a C₁₈ reversed phase column eluted with a gradient from 20 mM potassium phosphate, pH 4.6 containing 0.16 mM KCN (solvent A) to 60% methanol/water (solvent B; one minute to 40% B, 11 min to 50% B, and 1 min to 100% B; flow rate 1 ml/min). Dicyanocobinamide eluted at 16 min, and was detected by spectral absorption at 366 nm; it was quantified by comparison to authentic dicyanocobinamide (Sigma-Aldrich Chemicals; St Louis, MO) standards over a 60-fold concentration range.

Data Analysis

Survival curves were analyzed by the log-rank (Mantel-Cox) test. Dose-response curves were analyzed by log transformation of the dose followed by non-linear regression analysis with reporting of the LD₅₀ or ED₅₀ and the 95% confidence interval. Studies measuring cyanide or thiocyanate concentrations were analyzed by repeated measures analysis of variance with a Bonferroni post-hoc test for multiple comparisons. These data are reported as the mean $\pm$ standard error of the mean. Simple means (two samples) were analyzed using an unpaired Student's *t*-test. All analyses were performed using Prism software, version 5 (GraphPad Software, San Diego, CA). Differences were considered significant when $p \leq 0.05$.

Results

Determination of Lethal and Sub-Lethal Doses of Inhaled and Injected Cyanide

To establish the lethal concentration (LC)₅₀ and LC₁₀₀ of inhaled cyanide gas during a 30 min exposure or the lethal dose (LD)₅₀ and LD₁₀₀ of intraperitoneal administration of KCN, the up-and-down procedure for acute toxicity testing was used¹⁷. In response to 534 ppm HCN (LC₁₀₀) mice would become apneic and die within 30 min. The LC₅₀ was found to be 451 ppm (95% confidence interval (CI) of 424 to 480; $n = 5$) (Supplemental Figure 2, supplement), which is higher than previously reported in the literature^{2, 18}. However, these earlier studies were performed in a different mouse strain, and without general anesthesia. General anesthesia appears to decrease toxicity by preventing the hyperventilation that occurs in awake animals in response to inhaled cyanide¹⁹. Intraperitoneal injection of KCN at 0.16 mmol/kg induced apnea and death within 5–9 min. The observed LD₅₀ was 0.144 mmol/kg (10 mg/kg)(95% CI of 0.090 to 0.232; $n = 5$)(Supplemental Figure 3, supplement), which is similar to prior studies^{2, 20}.

Cyanide Distribution and Metabolism

To study cyanide distribution and clearance, mice were exposed to 260 ppm HCN gas for 30 min, and then allowed to recover. Red blood cell cyanide, and plasma and urine thiocyanate were measured prior to cyanide exposure, immediately after exposure, and at 2 and 6 h post exposure (Supplemental Figure 4, online data supplement). The RBC cyanide concentration peaked immediately after exposure and then decayed over the ensuing 6 h. The urine thiocyanate concentration increased as the RBC cyanide decreased. No change was observed in the plasma thiocyanate concentration, which remained low throughout the study, indicating that thiocyanate was freely excreted in the urine.

Efficacy of Antidotes in Inhaled Model of Cyanide Poisoning

Cyanide antidotes available in the United States were compared to cobinamide at the LC₁₀₀ of inhaled cyanide. Hydroxocobalamin, sodium thiosulfate, and sodium nitrite were used at doses of 0.2, 2.6, and 1.3 mmol/kg, respectively^{2, 7}. These doses are the maximal recommended clinical dose when calculated on a mg/kg basis, and exceed the recommended human dose when calculated on a mg/m² basis⁷. Cobinamide was used at the same molar dose as hydroxocobalamin. When used at these doses, only cobinamide and sodium thiosulfate resulted in survival (Figure 1)($p < 0.0001$). To further compare the efficacy of these two agents, the cyanide concentration was increased to 801 ppm; all cobinamide-treated animals survived, whereas only 60% of sodium thiosulfate-treated animals survived ($p = ns$). However, at this cyanide concentration, the combination of sodium thiosulfate and sodium nitrite, a recommended clinical treatment, was fully effective. To compare cobinamide to the combination of sodium thiosulfate and sodium nitrite, animals were challenged with a cyanide dose of 908 ppm. All cobinamide-treated animals survived, whereas only 20% of the sodium thiosulfate and sodium nitrite-treated animals survived ($p < 0.015$). Plotting survival against the inhaled cyanide concentration increased the LC₅₀ for cyanide to 803, 901, and significantly greater than 908 ppm for sodium thiosulfate, sodium thiosulfate and sodium nitrite, and cobinamide, respectively (Figure 1) when the data were log transformed and analyzed by non-linear regression. Thus, cobinamide was superior to these established treatments for cyanide poisoning.

To more accurately compare the efficacy of cobinamide to hydroxocobalamin, we administered a range of doses of the two compounds. A dose as low as 0.1 mmol/kg (100 mg/kg) of cobinamide was fully effective, with 100 % survival (Figure 2)($p < 0.0002$). Hydroxocobalamin was significantly less effective with a dose 0.4 mmol/kg (553 mg/kg) required to obtain 100% survival (Figure 2)($p < 0.03$). The dose that produced 50% survival (ED₅₀) was 0.029 (29 mg/kg) (95% CI of 0.025 to 0.033) and 0.301 mmol/kg (416 mg/kg) for cobinamide and hydroxocobalamin, respectively. Therefore, cobinamide was about 10-fold more potent than hydroxocobalamin in this inhaled model of cyanide poisoning.

To compare biochemical evidence of efficacy of cobinamide and hydroxocobalamin, we measured red blood cell cyanide, as well as plasma and urine thiocyanate levels immediately, and 2 and 6 h after a sub-lethal dose (260 ppm) of inhaled cyanide. We found that red blood cell cyanide increased 50-fold immediately in response to inhaled cyanide (24.9 ± 6.2 vs 0.5 ± 0.03 $\mu\text{g/g}$ protein; HCN alone vs control), but did not differ significantly from controls in cobinamide-treated animals at any time (3.0 ± 0.4 vs 0.5 ± 0.03 $\mu\text{g/g}$ protein; Cbi vs control) (Figure 3A). Plasma thiocyanate was reduced significantly at 2 h in cobinamide-treated animals (15.9 ± 3.3 vs 23.5 ± 5.0 $\mu\text{g/g}$ protein; Cbi vs HCN alone), whereas it was not reduced significantly in hydroxocobalamin-treated animals compared to those treated with HCN alone (26.8 ± 1.0 vs 23.5 ± 5.0 $\mu\text{g/g}$ protein; OHC vs HCN alone) (Figure 3B). Urine thiocyanate slowly increased in all HCN-treated animals, but in both cobinamide- and hydroxocobalamin-treated animals it never rose to levels observed in those

treated with HCN alone 477.7 ± 93.8 vs 151.0 ± 24.4 vs 237.8 ± 41.1 $\mu\text{g/g}$: HCN alone vs OHC vs Cbi)(Figure 3C). Thus, the increased potency of cobinamide compared to hydroxocobalamin as a cyanide antidote was reflected in cyanide and thiocyanate blood concentrations.

Comparison of Cobinamide and Hydroxocobalamin in an Intraperitoneal Model of Cyanide Poisoning

We next compared cobinamide and hydroxocobalamin in a parenteral cyanide model. Mice were given either cobinamide or hydroxocobalamin intravenously at various doses immediately before they were given an intraperitoneal injection of 0.24 mmol/kg (16 mg/kg) of KCN (about 6 μmol per mouse). Survival was the observed endpoint (Figure 4). With cobinamide, 100% survival was observed at 0.16 mmol/kg (160 mg/kg), whereas 0.32 mmol/kg (442 mg/kg) of hydroxocobalamin was required for 100% survival. The data were plotted as a dose-response curve, and the dose log transformed. The data were then subjected to non-linear regression analysis. The calculated ED_{50} was 0.054 (95% CI 0.041 to 0.072)(54 mg/kg) and 0.175 (95% CI 0.162 $\pm$ 0.189)(242 mg/kg) mmol/kg for cobinamide and hydroxocobalamin, respectively (Figure 4). Thus, cobinamide was again more potent than hydroxocobalamin, and the difference in relative potencies of the two compounds between the inhaled and injection models is considered in the Discussion.

Intramuscular Injection of Cobinamide

Cyanide is an extremely rapid metabolic poison, and cyanide-poisoned victims may be unconscious, hypotensive, and die rapidly if untreated. Therefore, intramuscular injection may be the most expeditious, viable route, especially in a setting of mass casualties. We found that cobinamide was absorbed slowly, and we noticed that some of the animals injected with cobinamide developed paresis of the injected limb. Cobinamide reacts with nitric oxide (NO)^{21, 22} and we hypothesized that cobinamide was inducing localized ischemia by consuming NO, thereby retarding its own absorption. To prevent this possible sequence of events, we added sodium sulfite to cobinamide. Sulfite binds to cobalamin with a reasonably high affinity²³. We showed that cobinamide sulfite was absorbed more effectively than cobinamide (Figure 5A) with peak absorption occurring at 5 and 60 min respectively. Cobinamide sulfite had a half-life of 32.3 (95% CI 27.2 to 39.6; $n = 5$) min using a one-phase decay model. Cobinamide measurements did not adequately fit this model because of its slow absorption phase, but the half-life was estimated to be greater than 6 h. We compared these two preparations in their ability to prevent death in the intraperitoneal KCN injection model (Figure 5B). In both cases, 0.2 mmol/kg (200 mg/kg) of cobinamide was administered intramuscularly. Only cobinamide sulfite resulted in 100% survival.

Efficacy of Cobinamide Sulfite Post-Poisoning

Pre-poisoning treatment models are not always useful in predicting efficacy of antidotes. Therefore, we further examined the effectiveness of cobinamide sulfite relative to the timing of KCN poisoning. Mice were given intramuscular cobinamide sulfite, either before or up to 4 min after a lethal dose of intraperitoneal KCN (Figure 6). The cobinamide sulfite was 100% effective up to 3 min post cyanide injection ($p \leq 0.0001$; $n = 5$), which was at a time when the mice had been apneic for over two minutes.

Range-finding Toxicity Studies

We performed general range-finding toxicity studies of cobinamide in mice, assessing clinical parameters and survival. We administered cobinamide at increasing amounts by intraperitoneal injection, and the animals were observed for up to seven days for adverse effects. Animals injected with 0.2 mmol/kg (200 mg/kg) tolerated this dose without

observable abnormalities. However, 0.4 mmol/kg (400 mg/kg) reduced spontaneous activity, which was followed by respiratory distress, hunched posture, piloerection, and ultimately death by 36 h (Figure 7). Log transformation of the dose and linear regression analysis revealed an LD₅₀ of 0.32 mmol/kg (320 mg/kg). We postulated that this adverse effect could be from cobinamide binding endogenous NO, and, therefore, tested cobinamide sulfite. At doses up to 2.0 mmol/kg (2000 mg/kg), cobinamide sulfite induced no clinical signs of toxicity with animals surviving for at least seven days (Figure 7; only doses up to 0.8 mmol/kg (800 mg/kg) are shown in the figure for clarity). We did not try doses higher than 2000 mg/kg because the United States Food and Drug Administration considers 2000 mg/kg an appropriate limit dose in rodent toxicity studies ²⁴.

Discussion

In the United States, three cyanide antidotes are available: nitrites (e.g., sodium nitrite and amyl nitrite), sodium thiosulfate, and hydroxocobalamin (vitamin B_{12a}) ²⁵. Nitrites generate met(ferric)hemoglobin, which has a high affinity for cyanide, but can no longer bind oxygen; thus, nitrites can exacerbate the carbon monoxide-induced reduction in oxygen-carrying capacity in smoke inhalation victims. Moreover, nitrites can induce vasodilatation, causing hypotension ². Sodium thiosulfate acts as a sulfur donor for the enzyme rhodanese, which detoxifies cyanide by converting it to thiocyanate, but rhodanese is limited both in cellular amount and tissue distribution. Hydroxocobalamin binds cyanide with a relatively high affinity ($K_A \sim 10^{12} \text{ M}^{-1}$) ²⁶, but 5–10 grams are required for cyanide poisoning.

We have shown previously that cobinamide is effective as a cyanide scavenger in cultured cells ⁹, a fly model ²⁷, and nitroprusside-induced cyanide toxicity in mice ²⁸. We now show that cobinamide is effective in two lethal mouse models of cyanide poisoning, and demonstrate it is superior, in our models, to currently available treatments. Although cobinamide was absorbed poorly, cobinamide sulfite was rapidly absorbed from an intramuscular site, and protected mice from cyanide-induced death, even when administered after cyanide. Evans previously showed that cobinamide neutralizes cyanide in mice and rabbits, but he administered it by intravenous injection and did not strictly compare it to other cyanide antidotes ²⁹. With the exception of amyl nitrite, currently approved drugs for cyanide poisoning are available only as intravenous preparations, limiting their usefulness in a mass casualty setting. The time required to start intravenous lines and administer relatively large fluid volumes would be prohibitively long in treating many cyanide-poisoned persons in the field. Therefore, an intramuscular preparation that is rapidly absorbed would be highly desirable.

To develop a model of cyanide inhalation, we needed to construct a suitable exposure chamber. Cyanide gas (hydrogen cyanide) is not commercially available, and, therefore, a flow-through exposure system with accurately-controlled cyanide concentrations is not feasible. Requirements of a sealed chamber are that gases must equilibrate rapidly, and the chamber must be maintained above the boiling point of HCN (26° C). We found that our chamber generated reproducible, stable concentrations of cyanide with a sustained level of anesthetic gas throughout the exposure period (at least 30 min). The lethal LC₅₀ we observed for cyanide (451 ppm for 30 min) was higher than previously reported in mice ^{18,30}. Three factors may contribute to this difference: 1) mouse strains vary in their sensitivity to cyanide and C57BL/6 mice are relatively resistant; 2) previous reports used measured concentrations of cyanide gas that tend to underestimate the concentration of cyanide because of gas loss or condensation at room temperature; and 3) the earlier studies were performed in awake mice that likely hyperventilated on initial cyanide exposure ^{18,30}, whereas our studies were performed with anesthetized mice.

Cobinamide is the penultimate compound in cobalamin biosynthesis, lacking the dimethylbenzimidazole nucleotide tail coordinated to the cobalt atom in the lower axial position²¹. Whereas cobalamin has only a free upper ligand binding site, cobinamide has free upper and lower binding sites; moreover, the dimethylbenzimidazole group has a negative *trans* effect on the upper binding site, thereby reducing cobalamin's affinity for ligands²². The net effect is that cobinamide binds two cyanide ions, and has a greater affinity for cyanide than hydroxocobalamin, with a K_A overall of $\sim 10^{22} \text{ M}^{-1}$ ²⁶. In addition, cobinamide is at least five times more water-soluble than hydroxocobalamin. These three chemical differences translate into smaller volumes of administration of cobinamide than hydroxocobalamin, and we calculate that 5 ml of a 200 mM cobinamide solution should neutralize one human LD₅₀ of cyanide (5 ml can be given intramuscularly in the gluteal region).

We found that cobinamide is considerably more effective than hydroxocobalamin, and that the difference is more pronounced in the inhaled than the parenteral model of cyanide poisoning. Thus, cobinamide is 11 times more effective in the inhaled model and 3 times more effective in the parenteral model. Although there are several possible explanations for these differences in efficacy, the most plausible are the kinetics of the two models. Because of the time needed to absorb cyanide gas into the circulation, the inhaled model leads to a slower onset of toxicity, whereas, in the intraperitoneal model, cyanide is absorbed rapidly and distributes into the vascular system at a rate approaching that of intravenous injection. Other small molecules administered to mice by intraperitoneal injection are rapidly absorbed³¹. A difference between two compounds is, therefore, more likely to be seen in the inhaled model where these compounds have a longer time to act. We should note that the inhaled model is more representative of real-life circumstances, in which people are likely to be exposed to cyanide gas.

We have shown previously that cobinamide has high affinity for nitric oxide (NO)^{21, 22}. Binding NO *in vivo* may lead to systemic hypertension as occurs with intravenous hydroxocobalamin³², and to localized vasoconstriction when given by intramuscular injection. Initial studies with intramuscular cobinamide supported this impression, because post-mortem examination of injected animals demonstrated significant quantities of residual cobinamide at the injection site (TDB, unpublished observations). We found that cobinamide sulfite does not bind NO *in vitro*, and that it is rapidly absorbed and highly effective. Moreover, it exhibited no clinical toxicity, even at a dose of 2000 mg/kg (2.0 mmol/kg).

Although the precise LD₅₀ for cyanide is not known in humans, lethal poisoning has occurred with as little as 50 mg (~ 0.5 to 1 mg/kg). Mice are more resistant to the effects of cyanide than humans with the LD₅₀ for KCN reported to be between 2–8 mg/kg, depending on the strain and mode of cyanide exposure^{20, 33}. We found that C57BL/6 mice are particularly resistant, with an LD₅₀ for cyanide of 9.75 mg/kg (0.15 mmol/kg). Thus, the amounts of cobinamide required to rescue humans from a lethal cyanide dose is likely to be considerably less than that required in this study.

The current study has several limitations. First, anesthetized animals do not approximate real-life exposures. However, we felt that studies in awake animals would be inhumane, and current animal care guidelines strongly discourage and limit the use of awake animals in studies of toxins such as cyanide^{34, 35}. Anesthetics might bias our data by inducing hypotension, which could increase the susceptibility of the animal to the cardio-depressant effects of cyanide. Alternatively, in the inhaled model, anesthetics could protect animals through decreased minute ventilation or by preventing hyperventilation in response to cyanide gas¹⁹. Second, in the parenteral model, the onset of death was very rapid, leaving

only a narrow window for intervention. Third, the studies were not conducted in a randomized fashion. However, all animals were the same inbred strain from the same supplier, and the observed effects were highly reproducible. And, fourth, the studies were not blinded because of the complexity of doing this, and the nature of the antidotes (cobinamide and hydroxocobalamin are both intensely colored).

Conclusion

Cyanide, in practical terms, cannot be regulated. It is used in countless industrial applications, is cheap to make, and is abundant. Hundreds of thousands tons of cyanide are manufactured each year in the United States ⁷. The National Institutes of Health and the Department of Defense have both emphasized the need for new, more effective, and less toxic treatments for cyanide poisoning that can be deployed rapidly in a mass casualty setting ^{7, 36}. We conclude that cobinamide may be an agent that satisfies these requirements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

List of Abbreviations

Cbi	Aquohydroxocobinamide, cobinamide
Cbi-SO₃	cobinamide sulfite
CI	confidence interval
ED	effective dose
HCN	hydrogen cyanide
OHC	hydroxocobalamin, vitamin B _{12a}
LC	lethal concentration
LD	lethal dose
KCN	potassium cyanide
KMnO₄	potassium permanganate
Na₂CO₃	sodium carbonate
NaNO₂	sodium nitrite
Na₂S₂O₃	sodium thiosulfate

Acknowledgments

Declarations of Interest

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Figure 1. Comparison of Available Cyanide Antidotes against Inhaled Cyanide

A) Maximal clinical doses of currently available cyanide antidotes were compared to cobinamide (0.2 mmol/kg; 200 mg/kg) in C57BL/6 mice exposed to 534, 801, and 908 ppm of cyanide gas for 30 min (LC₁₀₀). These included hydroxocobalamin (0.2 mmol/kg; 200 mg/kg), sodium thiosulfate (2.6 mmol/kg), and sodium nitrite (1.3 mmol/kg) ($n = 5-6$ per condition). All cobinamide-treated animals survived, whereas 80%, 60%, and none of sodium thiosulfate treated animals survived when treated with 534, 801, and 908 ppm, respectively. The combination of sodium thiosulfate and sodium nitrite was fully effective except at 908 ppm cyanide was administered, in which only 20% of animals survived ($n = 5$). The data were plotted and log transformed for non-linear regression analysis. We determined and LC₅₀ inhaled cyanide of 803 ppm and 901 ppm for sodium thiosulfate and the combination of sodium thiosulfate/sodium nitrite, respectively. For cobinamide, the LC₅₀ was significantly in excess of 908 ppm. (Cbi = cobinamide; OHC = hydroxocobalamin; Na₂S₂O₃ = sodium thiosulfate; NaNO₂ = sodium nitrite)

Figure 2. Dose-Response to Cobinamide and Hydroxocobalamin in the Inhaled Model

Mice were injected with vehicle, cobinamide (Cbi) or hydroxocobalamin (OHC) 15 min before being placed in an exposure chamber and exposed to cyanide gas at 534 ppm for 30 min. Data were plotted in dose-survival curves and the dose log transformed for non-linear regression analysis. The effective dose (ED)₅₀ regression analysis was 0.029 (29 mg/kg) (95% CI of 0.025 to 0.033) and 0.301 mmol/kg (416 mg/kg) for cobinamide and hydroxocobalamin, respectively ($n = 5$ per condition).

Figure 3. Measurement of RBC Cyanide, and Plasma and Urine Thiocyanate

Mice were injected in the peritoneal cavity with 0.2 mmol/kg of hydroxocobalamin (OHC), cobinamide (Cbi), or buffer. Fifteen minutes later they were anesthetized in the exposure chamber and exposed to 260 ppm of cyanide gas for 30 min, and then sacrificed immediately (time zero), or at 2 or 6 h after removal from the chamber. Mice not exposed to cyanide were sacrificed at the time other animals were placed in the chamber (shown as Control). Red blood cell cyanide (panel A), and plasma (panel B) and urine thiocyanate (panel C) were measured as described in Methods ($n = 3$ per condition).

Figure 4. Dose-Response to Cobinamide and Hydroxocobalamin in the Intraperitoneal Model
 A) Mice were given varying doses of cobinamide (Cbi) or hydroxocobalamin (OHC) intravenously immediately before receiving an intraperitoneal injection of 0.24 mmol/kg of KCN. Data were plotted as dose response curves and the ED₅₀ calculated by log transformation of the dose and non-linear regression analysis of the data. The calculated ED₅₀ was 0.054 (95% CI 0.041 to 0.072)(54 mg/kg) and 0.175 (95% CI 0.162 ± 0.189)(242 mg/kg) mmol/kg for cobinamide and hydroxocobalamin, respectively (*n* = 5 for each condition).

Figure 5. Intramuscular Cobinamide

A) Kinetics of intramuscular absorption of cobinamide. Cobinamide in a volume of 50 μl was injected intramuscularly into the gastrocnemius muscle. Solutions included cobinamide and cobinamide sulfite. Animals were sacrificed quickly at noted times, and blood was obtained by intracardiac puncture. Cobinamide in plasma was measured as described in Methods by HPLC. Cobinamide sulfite had a half-life of 32.3 (95% CI 27.2 to 39.6; $n = 5$) min using a one-phase decay model. Cobinamide measurements did not adequately fit this model because of its slow absorption phase, but the half-life was estimated to be greater than 6 h ($n = 3$). B) Effectiveness of cobinamide intramuscular preparations. Cobinamide or cobinamide-sulfite (0.2 mmol/kg; 200 mg/kg) was injected intramuscularly 3 min after mice

received a lethal intraperitoneal injection of KCN (0.16 mmol/kg). A Kaplan-Meier survival curve is shown (n = 5 for each condition).

Figure 6. Post-Cyanide Exposure Treatment

Cobinamide sulfite (0.2 mmol/kg; 200 mg/kg) was administered either 10 min before, simultaneously, or up to 4 min after a 0.16 mmol/kg dose of intraperitoneal KCN. Control animals were treated with an identical amount of sodium sulfite. Animals were protected by cobinamide sulfite if administered up to 3 min after the intraperitoneal KCN ($n = 5$).

Figure 7. Range Finding Toxicity Studies of Cobinamide and Cobinamide Sulfite

General range-finding toxicity studies of intraperitoneal cobinamide (Cbi) or cobinamide sulfite (Cbi-SO₃) were performed. Animals were observed for up to seven days. B) Data were plotted as dose response curves and the LD₅₀ by log transformation of the dose and non-linear regression analysis. The LD₅₀ for cobinamide was 0.32 mmol/kg (320 mg/kg). Although only doses up to 0.8 mmol/kg (800 mg/kg) are shown, no mortality or adverse effect was observed with any dose of cobinamide sulfite up to and including a dose of 2 mmol/kg (2000 mg/kg) ($n = 5$ for each dose).