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Detection and prevention of aggregate formation of cobinamide as an antidote for cyanide poisoning

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Detection and prevention of aggregate formation of cobinamide as an antidote for cyanide poisoning

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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2012
The Thesis of Megan Elizabeth Glasheen is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

Chair

University of California, San Diego

2012
DEDICATION

I would like to dedicate this thesis to my family and boyfriend, Erik. Their unconditional love and support makes achieving my goals possible.
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ABSTRACT OF THE THESIS

Detection and prevention of aggregate formation of cobinamide as an antidote for cyanide poisoning

by

Megan Elizabeth Glasheen

Master of Science in Biology

University of California, San Diego, 2012

Professor Gerry Boss, Chair
Professor James Golden, Co-Chair

Cobinamide, a vitamin B12 precursor, has a high affinity for cyanide and is an effective antidote for cyanide poisoning in animal models. However, when given as an intramuscular injection at high concentrations, the compound forms aggregates that both inhibit its absorption and damage the surrounding muscle tissue. The objective of this study is to investigate aggregate formation in terms of aggregate number and thermodynamics of formation as a means to prevent aggregate formation in the drug formulation. Cobinamide solutions ranging in concentrations from 5µM to 1mM were
scanned from 400nm to 600nm at 25°C, 35°C, 45°C, and 65°C using a spectrophotometer. The spectrum within this range was representative of aggregate formation as visible changes were observed with increasing concentrations and temperatures. A variety of compounds were tested for their ability to prevent aggregate formation. We analyzed the effect of temperature on the stability of the aggregated compounds. Our results show that cobinamide forms very strong aggregates that can be largely prevented using malic acid.
INTRODUCTION

Cyanide Poisoning

Cyanide poisoning poses a very real threat as it is found in a wide variety of materials and locations, as well as its potential use as a weapon of terrorism. Cyanide is present in the industrial world in chemical synthesis, insecticides, agriculture, manufacture of paper and plastics, and photography. It can also be found in the waste products of mining, as well as nitrogen-containing household materials such as nylon and plastic products. In the event of a fire, these products have the capability to release hydrogen cyanide during combustion, which can easily lead to cyanide poisoning caused by smoke inhalation\textsuperscript{1}, currently the most common cause of cyanide poisoning\textsuperscript{2}, responsible for 5000-10000 deaths in the United States per year\textsuperscript{3}. Cyanide has dangerous potential as chemical warfare by terrorists due to its ease of accessibility and handling, and it has many mediums through which it can be delivered. Mass casualties could occur were hydrogen cyanide to be delivered to a closed space in which large numbers of people were present, such as a large building or subway station\textsuperscript{2}. This threat is very real as U.S. intelligence uncovered terrorist plans to attack the New York City subway system using cyanide in 2003, a transportation system that carries nearly 5 million people per day\textsuperscript{4}. A lethal dose for humans could be as little as 50mg.

The mechanism of cyanide poisoning occurs by inhibiting oxidative phosphorylation. Oxidative phosphorylation is the process through which the
energy released by the oxidation of glucose is used to produce adenosine triphosphate (ATP), which is then used to fuel essential cellular functions. It takes place in mitochondria via a series of enzymes located in the inner mitochondrial membrane called the electron transport chain. The penultimate step involves the reduction of oxygen to water when electrons are transferred to oxygen from cytochrome oxidase a₃.⁵ Cytochrome oxidase a₃ is composed of two heme A groups and two copper ions. The cyanide molecule binds to the ferric ion in the heme component of the oxidized form of the enzyme, altering its structure. This occurs in two steps, the first being the formation of an HCN-enzyme intermediate, followed by the binding of cyanide to the heme iron, producing the enzyme-cyanide product. The altered structure of the enzyme inhibits its normal function, while other cellular metabolic functions like glycolysis continue. Since the non-functioning cytochrome oxidase a₃ can no longer reduce oxygen to produce ATP, cellular metabolism shifts from aerobic to anaerobic. The pyruvate produced by glycolysis is then reduced to lactate, as it has become the replacement terminal electron acceptor, and metabolic acidosis results.⁶ It is hypothesized that other enzymes are affected as well, namely metalloenzymes, and can lead to cardiac shock through pulmonary arteriolar and coronary artery vasoconstriction⁶.

**Current Treatments**

There are currently three classes of cyanide antidotes: methemoglobin generators, sulfur donors, and direct binding agents⁷. The methemoglobin
generators include sodium nitrite, amyl nitrite, and 4-Dimethylaminophenol (4-
DMAP). The exact mechanism of these drugs is not known, but it is
hypothesized that they work to detoxify cyanide by inducing methemoglobin
formation; the ferrous (Fe$^{2+}$) atom in the heme portion of hemoglobin is
oxidized to the ferric (Fe$^{3+}$) atom. Methemoglobin has a higher affinity for
cyanide than cytochrome c oxidase, so it will liberate the enzyme from cyanide
and allow it to resume its function in cellular respiration. However, this
mechanism can be extremely dangerous, especially in the case of smoke
inhalation victims, because it also disrupts oxygen transport to cells as oxygen
bound to methemoglobin cannot be released. Smoke inhalation victims may
also suffer from some degree of carbon monoxide poisoning, so the oxygen
carrying capacity of their blood is already compromised; administration of
these drugs will exacerbate their condition and could potentially be fatal. An
additional risk posed by nitrites is the possibility of hypotension caused by vasodilation$^1$.

The sulfur donor treatment usually utilizes sodium thiosulfate, which is
typically administered intravenously after amyl nitrite or sodium nitrite$^6$. Minute
levels of cyanide are both consumed and produced by respiration and
metabolic processes in mammals, and there is a mitochondrial enzyme called
rhodanese that has evolved to detoxify cyanide. A sulfur transferase,
rhodanese is a catalyst in the reaction that converts cyanide into the harmless
thiocyanate; sodium thiosulfate acts as a sulfur donor to rhodanese, triggering
this catalytic reaction. A major issue with this antidote is that it has a relatively slow onset, as it takes time for the compound to make its way into the mitochondria, where the rhodanese is located, when rapid action is needed to sequester the cyanide away from cytochrome c oxidase.

Direct binding agents include hydroxocobalamin and dicobalt edetate. The principle behind these compounds is the chelation of cyanide to form a less harmful product that can be safely excreted as waste. Dicobalt edetate is not an ideal antidote, however, as it can result in cobalt toxicity due to the presence of cobalt ions in the solution. Cobalamin (Cbl), also known as vitamin B12, is an essential water-soluble vitamin that is necessary for successful erythrocyte production and proper functioning of the central nervous system. The molecule can only be synthesized by a few select microorganisms in nature. It is a member of a corrinoid family of molecules that contain a cobalt atom coordinated by a corrin ring via four nitrogen atoms. It is asymmetric, made so by the substitution of different chemical groups on the perimeter of the macrocycle. These side chains include methyl, acetamide, propionamide, and a dimethyl-benzimidole ribonucleotide tail in the lower axial position (Fig. 1B). The upper axial position of cobalt is typically occupied by a methyl, hydroxyl, or adenosyl, depending on a cell’s requirement for its activity as a cofactor. It is involved in cellular methyltransferase and mutase activity.
As a cyanide antidote, hydroxocobalamin is the form that is used\(^9\). The cyanide molecule displaces the hydroxyl group in the upper axial position, forming cyanocobalamin, which is then harmlessly excreted in the urine. Hydroxocobalamin has a much higher affinity for cyanide than cytochrome c oxidase, so the cyanide molecule readily dissociates from the enzyme and allows it to regain normal function. However, due to the single binding site available to cyanide, one molecule of hydroxocobalamin is required for each molecule of cyanide\(^6\). This imparts a major drawback in its use as a cyanide antidote due to the fact that such large doses, in the amount of 5-10 grams, are required for it to be effective. *While large doses have not proven to be toxic, it does reduce the efficiency of the drug*\(^11\).

Given the prevalence and high mortality of cyanide poisoning, and the major drawbacks to the many existing antidotes, there is a clear need for a more effective cyanide antidote that has rapid action and simple administration. Cobinamide is a molecule that will be able to effectively fill this role.

**Cobinamide**

Cobinamide, the precursor to cobalamin, has proven to be a superior cyanide antidote to any current agent used. The structure of cobinamide (Fig. 1A) differs from cobalamin (Fig. 1B) in only that it lacks the dimethylbenzimidazole nucleotide tail that is used to coordinate the cobalt atom in the lower axial position\(^11\). This structural difference results in two
available binding sites for cyanide in cobinamide, as opposed to the one in cobalamin. It has been shown that the dimethylbenzimidazole group has a negative trans effect on the upper binding site that reduces cobalamin’s affinity for ligands\textsuperscript{11}. This is evident in the fact that cobinamide has a higher association constant ($K_A$) of $10^{22}$ M$^{-1}$, whereas cobalamin has a $K_A$ of $10^{12}$ M$^{-1}$. Cobinamide is also approximately five times more soluble in water than cobalamin\textsuperscript{11}. The combination of the higher binding affinity for cyanide, two binding sites for cyanide, and higher water solubility support the fact that cobinamide is both more effective as a cyanide antidote than cobalamin, and can be given in much smaller doses. This is desirable because it will make administration and storage of the drug simpler. In addition to binding cyanide, cobinamide has also been shown to bind nitric oxide (NO)\textsuperscript{12} and superoxide radical with high affinity (Ali and Boss, unpublished). The high affinity for NO has the possibility of causing systemic hypertension and localized vasoconstriction at the site of injection\textsuperscript{13}.

The desired method for administering cobinamide is as an intramuscular injection at a concentration of around 300 mM. The current agents used for cyanide poisoning all require intravenous administration by trained personnel\textsuperscript{6,9}. Due to cobinamide’s proven stability at room temperature, high solubility in water, and rapid action to detoxify cyanide, it would be a great candidate for intramuscular injection, which could either be administered to a patient by themselves or by an untrained individual\textsuperscript{7}. 
Issue of Aggregation

While cobinamide has proven extremely effective via intravenous injection in animals\textsuperscript{3,9,13}, there has been some difficulty with the intramuscular formulation. At the desired concentration, the drug forms aggregates in the muscle tissue surrounding the site of injection. This aggregation results in decreased absorption and muscle toxicity. Muscle necrosis, along with infiltration of polymorphonuclear leukocytes into the muscle, has been observed when aggregated cobinamide is injected. Not only does the aggregated drug cause damage to the muscle tissue, it is not able to effectively detoxify cyanide in the patient. In order to prevent aggregation of the cobinamide molecules, determination of the mechanism of formation of aggregates is needed, which we attempted in the present work.

The field of supramolecular chemistry provides information on the types of bonds that may be responsible for the aggregation of cobinamide, as well as theories on the mechanism of formation and how they may be dissociated. This realm of chemistry is important in crystal engineering, the study of biological systems, and drug development\textsuperscript{14}. At the core of supramolecular chemistry is the number of intermolecular forces that can occur between molecules. The strongest possible bond is a covalent bond, which involves the sharing of pairs of electrons between atoms\textsuperscript{5}. Supramolecular chemistry focuses instead on the weaker intermolecular forces such as hydrogen bonding, coordination complexes, van der Waals forces, electrostatic forces,
and pi-pi stacking interactions, all of which are possibilities for the cause of the aggregation of cobinamide, due to its structure.

Hydrogen bonding is the noncovalent electrostatic interaction between an electronegative atom and a hydrogen atom that is covalently bound to another electronegative atom. The electronegative atom, such as oxygen, nitrogen, or fluorine, has a partial negative charge that attracts the partial positive charge of the hydrogen atom. This type of bond is common in biological systems, as in protein folding and nucleic acid structure\textsuperscript{15}. There are a number of atoms that are electronegative on each of cobinamide’s side chains, as well as a number of hydrogen atoms, that are possible candidates for hydrogen bonding between two or more cobinamide molecules.

Van der Waals forces occur when two molecules are very close together, causing their electron clouds to interact with one another. As a result, transient dipoles may be created that are weakly attracted to each other, and bring the nuclei closer together\textsuperscript{5}. The planar nature of cobinamide presents the likelihood that van der Waals interactions and electrostatic forces occur between a number of atoms on the molecules due to their orientation and proximity to one another in solution.

Coordination complexes involve molecules with metal cation centers that react with anions or other ligands. These complexes can then form coordination polymers, which are essentially repeating units of the coordination complexes. These structures can be one, two, or three
dimensional in nature, forming a plethora of geometries like rectangles, cyclic arrangements, or linear polymers. The dimensionality of the polymer is thus determined by the coordination number of the metal center, which is the number of ligands surrounding it, and the specific angles in which they are oriented. Coordination polymers make up a progressive field in chemistry in which molecules, namely metal-based compounds, are manipulated structurally to induce self-assembly for the purposes of nanotechnology. Many of the desirable structural components required for these self-assembled nanostructures are present in cobinamide, including nitrogen-containing heteroaryls, a transition metal (Cobalt), and polar side chains\textsuperscript{16}.

Pi-pi stacking interactions are another type of intermolecular occurrence that involves the strong attraction between aromatic rings. This is a common phenomenon that occurs in biological systems, and is responsible for the stacking of base pairs in DNA to form a double helix, the tertiary structure of some proteins, aggregation of porphyrin molecules, and complexation in host-guest systems\textsuperscript{17,18}. Studies on this type of interaction typically focus on aromatic amino acids and benzene as models, and the most common configurations are T-shaped and off-centered parallel displaced structures\textsuperscript{19}. The aromatic nature of the corrin ring that makes up cobinamide, as well as the side chains that project off of the macrocycle, provide an amplitude of opportunities for the molecule to aggregate with itself via pi-pi interactions, in addition to other intermolecular forces.
Understanding what types of intramolecular interactions are occurring between cobinamide molecules in solution to produce aggregates, as well as what the structures of the aggregates are, are both important when devising a strategy to prevent the aggregation from occurring. While cobinamide lacks the dimethylbenzimidazole tail the cobalamin has, the two molecules are nearly structurally identical and there have been studies on the aggregation of cobalamin\textsuperscript{20}. Based on NMR analysis, the proposed aggregate was a dimer that was potentially coordinated by overlap of the dimethylbenzimidazole tail and the corrin ring, and pi-pi stacking was evident. In addition, it was noted that the propensity to form dimers was highly dependent on the ligand bound to the cobalt atom; H\textsubscript{2}O, CN, and OH substituents bound to cobalt did not form dimers\textsuperscript{20}. Therefore, it may be possible to disaggregate cobinamide in solution by combining it with the appropriate molecule.

**Experimental Approach**

There are a number of methods in literature that have been used to study the aggregate formation of these and other types of molecules. These include nuclear magnetic resonance spectroscopy (NMR), infrared spectroscopy (IR), UV-visible spectroscopy (absorption), fluorescence emission, X-ray scattering\textsuperscript{21, 22, 23}. For conjugated molecules like cobinamide that absorb strongly in the visible spectrum, UV-visible spectroscopy may be used to detect structural changes in the molecule, and can provide a wealth of
information about the nature of aggregate formation and was used in our study.

UV-visible spectroscopy is a technique that utilizes the inherent nature of pi-electrons or non-bonding electrons to be excited from a lower energy occupied molecular orbital to a higher energy unoccupied molecular orbital by absorbing UV or visible light. The wavelength absorbed corresponds to the energy required for this excitation to occur, and this value is recorded by the spectrophotometer\textsuperscript{24}. For UV-vis radiation to be absorbed, chemical bonds referred to as $\pi$ bonds and $\sigma$ bonds, or nonbonding electrons referred to as $n$ electrons, must be available to do so. There are different types of transitions that can take place when these electrons are excited, but the most commonly used in UV-vis spectroscopy are the $\pi-\pi^*$ or $n-\pi^*$ transitions because they absorb in wavelengths in the 200nm-700nm range. Upon collection of the absorbance values collected by the spectrophotometer, mathematical techniques can be applied to extrapolate information about a molecule\textsuperscript{24}. 
MATERIALS AND METHODS

Experimental Design

The goal of the experiment was to study the aggregation of cobinamide by close analysis of changes in the absorption spectrum in the 400nm to 600nm range (Fig. 2A). This range was chosen due to drastic visible changes in this area of the spectrum in water with increasing concentrations, as the rest of the spectrum continued to follow Beer's law (Fig. 2B). In addition, increasing temperatures were studied as an attempt to derive further information about the nature of the aggregation of cobinamide with regards to the number or molecules involved, types of bonds, and quantitative estimates about the energy involved.

Cobinamide solutions ranging in concentration from 5µM to 1mM were prepared from solid hydroxocobinamide and water in combination with other compounds via serial dilutions. The eight concentrations of each solution that were prepared were 5µM, 10µM, 20µM, 50µM, 100µM, 300µM, 600µM, and 1mM. The solutions of concentrations from 5µM to 100µM were placed in 1cm quartz cuvettes and solutions of concentrations from 300µM to 1mM were placed in 1mm cuvettes. Lower concentrations than 5µM were not used as the sensitivity of the spectrophotometer was not high enough to produce accurate measurements. The solutions were warmed in four separate water baths at 25°C, 35°C, 45°C and 65°C until desired temperatures were reached, and the
temperatures of the solutions were verified using a thermometer. Each concentration was heated in succession beginning at room temperature. The measurement chamber in the spectrophotometer was connected to the water bath corresponding to the desired temperature via a water pump. The water was pumped through the hollow walls of the chamber with the intent to maintain the temperature throughout the duration of spectrophotometric measurements, which lasted about one minute. The cuvettes were quickly transferred from the water baths to the temperature controlled chamber in the Uvikon spectrophotometer for absorbance measurements. The solutions were then scanned from 400nm to 600nm at 200nm per second.

The initial measurements were conducted using cobinamide in water and cobinamide in 20% SDS solution. The water solutions were representative of aggregated cobinamide, and the 20% SDS solutions were representative of the monomeric form of cobinamide. These measurements were used as standards to compare the effectiveness of various compounds to disaggregate cobinamide. The variations of cobinamide solutions that were studied were aged cobinamide (cobinamide in water for longer than 24 hours), nitrite, acetate (stored at room temperature and 37°C), glutaric acid, malic acid, malonic acid, tartaric acid, chloroquine, and levofloxacin in NH₄CO₃. The concentrations of each compound used were equal to the concentration of cobinamide in each solution; the serial dilutions were made from an initial stock of 1mM cobinamide with 1mM of the disaggregating compound.
Data Analysis

Data was collected from the spectrophotometer in Excel, and was analyzed using OriginPro 8.5.1 software. The absorbance was then plotted against the wavelength, and a four peak analysis was done for each solution. The Lorentzian model was used to fit four peaks that would produce a best fit for the spectra. Each peak was designated 1, 2, 3 or 4, and this designation was used consistently for all spectra (Fig. 3). Peak 1 corresponded to wavelengths between 400nm to 405nm, Peak 2 corresponded to wavelengths between 465nm to 475nm, Peak 3 corresponded to 490nm to 500nm, and Peak 4 corresponded to wavelengths between 520nm to 530nm. The calculated absorbance of each peak was then plotted against either concentration or temperature. From these plots, the degree of aggregation could be estimated as a function of the trends of each of the four peaks. The peaks that displayed the greatest consistent degree of change by temperature or concentration were Peak 2 and Peak 3, so these were attributed the areas in the spectra that were most affected by aggregation. This information could ultimately be used as a guide to measure the effectiveness of various compounds to disaggregate cobinamide.
RESULTS

Initial Measurements

To assess the degree of aggregation of a cobinamide solution, we first had to perform spectrophotometric measurements that would provide the spectra of both the monomeric form and aggregated form of cobinamide. The solutions were only scanned from 400nm to 600nm as this region (Fig. 2B) was the location of the most significant visible change in the spectrum when aggregate formation occurred. Cobinamide in water alone formed aggregates at even the lowest concentration used, which was 5μM. Spectra at lower concentrations could not be obtained due to the lack of sensitivity of the spectrophotometer. There have been similar studies performed on dye molecules with some structural similarities to cobinamide using this spectrophotometric technique. In these studies, a polar solvent such as alcohol is used to obtain the spectrum of the monomer\textsuperscript{25,26}.

We chose to use a solution of 20% SDS as it has a similar effect as the alcohol solutions, which is to prevent aggregation of the molecules. SDS is an organic compound consisting of a long carbon chain connected to a sulfate group that is commonly used in biology for experimental techniques such as lysing cells and denaturing proteins\textsuperscript{5}. Just as it is used to denature proteins by disrupting noncovalent bonds, we hypothesized that it would inhibit cobinamide molecules from binding to each other to form aggregates. While
there was still some visible aggregation at the highest concentrations, there was a clear difference between the lower concentrations of cobinamide in the 20% SDS solution (Fig. 4A) compared to the water solutions (Fig. 4B). Specifically, there were dramatic changes in the amplitudes and shifts in the wavelengths of Peak 2 and Peak 3. This is a common phenomenon seen in the aforementioned dye studies, in which the highest intensity band usually decreases in intensity and new bands are seen at other wavelengths as concentration, and subsequently aggregation, increase\textsuperscript{25,26}.

Aside from the concentration range, a temperature range was also used to assess the degree of aggregation of the solutions. The temperature range from 25°C to 65°C provided additional information about the degree of aggregation of the molecules, and potentially could provide information about the number of aggregates and types of bonds involved. Generally in aggregated solutions, the absorbance of the maxima of Peak 2 decreased with increasing temperature, while Peak 1 and Peak 4 remained the same or slightly increased (Fig. 5). The slight increase in absorbance of Peak 1, Peak 3, or Peak 4 at increasing temperatures was hypothesized to be an increase in the monomer concentration as the aggregates disassembled at higher temperatures. The decrease in absorbance of Peak 2 or other peaks was attributed to a decrease in the concentration of the aggregated form of cobinamide.

**Selection of compounds**
In an attempt to disaggregate cobinamide in solution, a number of compounds were tested. One of the initial strategies was to combine cobinamide in solution with existing drugs that had structures that ideally could break up the aggregates simply by physically separating the molecules. These structures were bulky in nature, and consisted of some arrangement of aromatic rings. Aside from considering the how well the structure of the molecule could physically prevent aggregation, it was also important to use compounds that kept the solution at a pH of 5, at which cobinamide is most stable. In addition, the cobalt atom that lies in the center of the cobinamide molecule is in a +3 valency state; one of these positive charges is neutralized by the surrounding corrin ring, and the other is shielded by some other ligand such as an amine or water. This leaves one remaining positive charge that must be neutralized to maintain a stable solution, so we also had to consider compounds that would provide this counter-ion.

Chloroquine was the first of the drugs that was tested, made up of a quinolone group and a branching carbon chain (Fig. 6A). It has been used since the 1940s as an anti-malarial drug, with very mild adverse effects that usually only occur when high doses are administered. The malarial parasite enters red blood cells and acquires vital amino acids by breaking down hemoglobin, and protects itself from destruction by forming hemozoin crystals from the free heme that is left in the process. Chloroquine acts by preventing the aggregation of the heme molecules, ultimately leading to destruction of the
parasite. This made the compound an ideal candidate for the disaggregation of cobinamide.

Solutions of diammine-cobinamide (DACO) and an equimolar concentration of chloroquine in water were measured and compared to the monomer and aggregate forms of cobinamide. The results were somewhat promising, but there were only minor changes in the spectra at each concentration (Fig. 6C). Peak 2 did decrease at a lower rate with regards to temperature compared to the aggregated form, suggesting that some level of disaggregation had occurred (Fig. 6E).

Levofloxacin, an antibiotic that also contains a quinolone group (Fig. 7A), as well as more aromatic rings that make it a more bulky molecule than chloroquine, was also tested. This antibiotic is widely prescribed and presents a lower risk of toxicity than chloroquine, so it was thought to be a better candidate than the chloroquine. An equimolar concentration of levofloxacin was combined with DACO, but was measured in a NH₄HCO₃ solution rather than water alone, producing spectra that contained more details than even the monomer standard (Fig. 7B, 7C). The NH₄HCO₃ was used to maintain the stable pH of 5 and to provide an anion, as the amine group bound to cobinamide was lost when the pH of the solution was brought down to 5. These spectra displayed minimal aggregation, as very slight decreases in Peak 2 were observed with increasing temperature, even at higher concentrations (Fig. 7D, 7E).
Another set of compounds that were tested were a group of carboxylic acids that included acetate, malate, glutaric acid, tartaric acid, and malonic acid (Fig. 8A). This group of compounds was hypothesized to be effective at both lowering the pH to 5 while also providing the necessary counter ion for cobinamide. A 1X concentration of each compound was combined with cobinamide and water and individually tested. The multi-peak analysis revealed that each of these compounds were almost equally as effective at aggregation prevention, as Peak 2 either decreased or stayed the same with increasing temperature (Fig. 8D, 8E). Compared to the aggregated scans of cobinamide, the acids appeared to significantly reduce aggregation, but did not work to eliminate it entirely (Fig. 8B, 8C).

One more condition that was examined was cobinamide that was prepared one day prior to the spectrophotometric measurements in water. This “aged” cobinamide was compared to a freshly prepared solution in water alone and appeared to be less aggregated than the fresh stock. The decrease in Peak 2 was comparable to that of the acids that were analyzed, and it was concluded that in the time between when the solution was prepared and the measurements were taken, the aggregates had disassembled to some extent (Fig. 9).

**Data Analysis**

Each set of spectra was subjected to a multi-peak analysis in the OriginPro 8.5.1 software. The curve function that was selected was the
Lorentzian function, as it produced the best fit curve in a series of trial and error comparing the Lorentzian function to the Gaussian function.

The Lorentz equation used was: \[ y = y_0 + \frac{2A}{\pi} \frac{w}{4(x-x_c)^2 + w^2} \], where \( y_0 \) is the offset of the curve, \( A \) is the area under the curve, \( w \) is the width of the curve, and \( x_c \) is the center of the curve. The adjusted R-squared value for each fitting was above 0.99. Upon calculating the best fit curve for each spectra, the OriginPro software provided the maxima (absorbance) for each of the 4 peaks, which we then used to produce graphs of the absorbance versus temperature. We looked at the relative changes in each of the peaks as the temperature of each solution was increased by setting the maxima at 25°C at zero, and normalizing the values for the remaining three temperatures. This provided a clear image of the changes that each peak experienced as the temperature increased.
DISCUSSION

Our results show that we were unable to completely prevent the aggregation of cobinamide, but were able to identify a compound suitable to use in the intramuscular formulation, which was malic acid. Of the compounds that we tested, the carboxylic acids proved to be most successful; specifically, cobinamide solutions with malic acid displayed the least amount of aggregation (Fig. 8). However, while complete disaggregation was not achieved, it has since been revealed that aggregation alone was not interfering with intramuscular absorption of cobinamide and an effective formulation has been reached. In addition, when dilute and aggregated cobinamide solutions were administered intravenously to rabbits, it was shown that aggregation does not reduce the efficacy or have any adverse physiological effects.

In other studies done by the lab, it was determined that one of the two high affinity binding sites on cobinamide had to be occupied by some ligand in order for proper intramuscular absorption to occur. It was hypothesized that cobinamide interacts with anions and proteins that are present in the extracellular space, interfering with the drug’s absorption into muscle tissue, as cobinamide carries a positive charge and these molecules are negatively charged. After eliminating a number of other ligands, nitrite was ultimately chosen as the most appropriate ligand to be used in the cobinamide
formulation. Ammonia, the ligand used throughout most of these aggregation experiments, was discarded because it only binds cobinamide at an alkaline pH. The current intramuscular formulation of cobinamide consists of two moles of sodium nitrite and one malic acid molecule per cobinamide molecule. This formulation has been proven successful in mice, rabbits, and pigs when administered via intramuscular injection.

We hypothesized that malic acid may serve to prevent aggregation to some degree because it requires less water molecules to completely dissolve in water, when compared to the chloride ion that was previously used in the form of hydrochloric acid to lower the pH of the solution. This essentially allows for more water molecules to be available to physically separate the cobinamide molecules in solution; this could disrupt some of the intermolecular forces that contribute to its self-aggregation. In addition, the molecule is not toxic in the concentrations used and it is a naturally occurring metabolite in all living organisms.

Another one of our main objectives when doing these experiments was to ultimately hypothesize what types of intermolecular forces contribute to the self-aggregation of cobinamide molecules. With the UV-spectrophotometric data that we have thus far, we are unable to make any conclusions with regards to the types of bonds or forces that are involved. Further experiments using solvents of different polarities would need to be completed in order to make any assumption of this nature. There are a number of such studies
described in literature, in which spectrophotometric measurements at a range of temperatures and solvents have been made\textsuperscript{28,29}. These studies are based on fundamental principles of UV-spectroscopy, namely the electronic transitions that take place when UV-Vis radiation is absorbed by a molecule. These electronic transitions require a specific amount of energy that, when a polar solvent is used, can either be increased or decreased depending on whether a $\sigma$ or $\pi$ bond, or $n$ electrons, are involved. The result of affecting the energy in this manner leads to shifts in wavelength that are commonly referred to as either red shifts or blue shifts\textsuperscript{30}. Determination of the nature of the specific intermolecular forces involved would greatly help in completely disaggregating cobinamide, but is not necessary to produce a safe an effective intramuscular formulation that can be used as a superior cyanide antidote to existing methods.
Figure 1. Structures of cobinamide (A) and cobalamin (B). Both molecules are made up of a central cobalt atom surrounded by a corrin ring. Cobinamide lacks the dimethylbenzimidole nucleotide tail that is used to coordinate the cobalt atom in the lower axial position. This results in two high affinity binding sites for cyanide in the cobinamide molecule.
Figure 2. (A) A representation of the spectral changes that occur in the 400nm-600nm range in an aggregated cobinamide solution compared to a solution containing minimal aggregates. (B) Full scans (200nm-600nm) of disaggregated and aggregated cobinamide. The most drastic changes in the spectrum occur in the 400nm-600nm range as the solution becomes more aggregated.
Figure 3. Representation of peak assignments for cobinamide spectra. The Lorentzian model was used for peak fitting of each solution to produce the best fit curve. Each peak was then assigned a number and plotted against either temperature or concentration, allowing for the estimation of the degree of aggregation. This information was then used as a guide to measure the effectiveness of various compounds to disaggregate cobinamide. with the ultimate goal of improving the intramuscular formulation of the drug as a cyanide antidote.
**Figure 4.** Representation of aggregated cobinamide (A) in water, and the monomer form of cobinamide (B) in 20% SDS. These were used as the standards by which to compare the effectiveness of the compounds tested to prevent the aggregation of cobinamide. There is a marked difference in the amplitudes and locations of the peaks in the aggregated form when compared to the monomer form.
Figure 5. A comparison of the peak trends as temperature increases between an aggregated solution of cobinamide (A), and the proposed monomer form of cobinamide (B). Peak 2 is thought to be the most representative of the degree of aggregation of a solution, and decreases as temperature increases.
Figure 6. (A) The structure of the antimalarial drug, chloroquine, containing a quinolone group. (B) A composite of the spectra the full range of concentrations for DACO, illustrating the changes in the spectra as the concentration (and degree of aggregation) increases. (C) A composite of the spectra the full range of concentrations for DACO + 1X chloroquine, illustrating the minor decrease in changes in the spectra as the concentration increases. (D) The temperature dependence of the peaks for DACO alone, displaying predicted trends in peaks for an aggregated solution. (E) The temperature dependence for a 600μM DACO + 600μM Chloroquine solution.
Figure 7. (A) Levofloxacin, containing quinolone group similar to chloroquine. (B) A composite of the spectra the full range of concentrations for DACO in NH$_4$HCO$_3$. (C) A composite of the spectra the full range of concentrations for DACO in NH$_4$HCO$_3$ +1X levofloxacin, illustrating the decrease in changes in the spectra as the concentration increases. (D) The temperature dependence of the peaks for DACO alone, displaying predicted trends in peaks for an aggregated solution. (E) The temperature dependence for 1mM DACO in NH$_4$HCO$_3$ +1mM levofloxacin.
Figure 8. (A) Structure of malic acid, representing the group of carboxylic acids that were tested. (B) A composite of the spectra the full range of concentrations for cobinamide in water. (C) A composite of the spectra the full range of concentrations for cobinamide + 1X malic acid, illustrating the decrease in changes in the spectra as the concentration increases. (D) The temperature dependence of the peaks for cobinamide alone, displaying predicted trends in peaks for an aggregated solution. (E) The temperature dependence for 600μM cobinamide + 600μM malic acid. There is minimal change in peaks over the range of temperatures, indicating minimal aggregation.
Figure 9. Composites of the spectra of a fresh cobinamide solution (A) and a cobinamide solution that was prepared at least 24 hours prior to measurements (B). (C) The temperature dependence of the peaks of a fresh cobinamide solution, displaying predicted trends in peaks for an aggregated solution. (D) The temperature dependence of the peaks of the aged cobinamide solution, with minute changes in the peaks as temperature increased, indicating minimal aggregation.
REFERENCES


