UC San Diego
UC San Diego Electronic Theses and Dissertations

Title

Development of a novel method to measure sodium azide using the vitamin B_{12} precursor cobinamide

Permalink

Author

Lin, Jeffrey

Publication Date

2012

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Development of a novel method to measure sodium azide using the vitamin B_{12}

precursor cobinamide

A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in

Biology

by

Jeffrey Lin

Committee in charge:

Professor Gerard R. Boss, Chair
Professor Gen-Sheng Feng, Co-Chair
Professor Madeline Butler

2012

The Thesis of Jeffrey Lin is approved, and it is acceptable

in quality and form for publication on microfilm and electronically:

_____

_____

_____

<div align="right">Chair</div>

University of California, San Diego

2012

DEDICATION

I dedicate this thesis to my father, mother, brother, Dr. Gerry Boss, and members

of the Boss/Pilz lab for their love and support.

Table of Contents

List of Figures and Tables

ACKNOWLEDGEMENTS

I would like to first express my appreciation for Dr. Gerard Boss for giving me the opportunity to join his lab. I would also like to take this chance to show my immense gratitude for his guidance, mentorship, and encouragement through my years working at the Boss/Pilz lab. He has not only bestowed me with the skills necessary for basic research but also taught me the value of persistence, patience, and passion required for successful scientific research.

Secondly, I would like to thank my father, mother, and my brother for providing me suggestions and support during this past year.

Lastly, I would like to thank the members of the Boss/Pilz lab for their support and assistance this past year. Every member contributed in one way or another to the success of my project.

ABSTRACT OF THE THESIS

Development of a novel method to measure sodium azide using the vitamin B_{12} precursor cobinamide

by

Jeffrey Lin

Master of Science in Biology

University of California, San Diego

Professor Gerard Boss, Chair

Professor Gen-Sheng Feng, Co-Chair
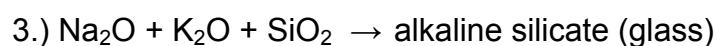
Professor Madeline Butler

Sodium azide is a toxic substance that has a high potential for abuse and is used in multiple industries ranging from laboratories to farms to automobile factories. The potentially deadly toxic has multiple routes of absorption, which make a fast and effective detection method extremely important. We developed a dynamic method of detection by observing the spectral changes when sodium azide binds to cobinamide, a vitamin B_{12} analogue. The K_a of sodium azide binding to cobinamide was found to be 6.10×10^{-5} 1/M. The detection limit and quantification limit of the cobinamide-based method performed in phosphate

buffer were 180 nM and 580 nM respectively. The method was sensitive and accurate enough to detect sodium azide at a concentration of 25 µM in blood samples. The absorbance values change upon addition of sodium azide in the 300 nm to 600 nm range. At 420 nm, the absorbance values increases linearly upon addition of sodium azide and decreases linearly at 491 nm upon increasing addition of sodium azide. The 420 nm: 491 nm wavelength ratio was chosen for accuracy and sensitivity. Our method has a lower detection limit and quantification limit when compared to the micro-diffusion assays described in literature. Although the cobinamide based method is less sensitive than ion chromatography or gas chromatography methods, the assay described in this paper has a potential higher throughput, is easier to process, and is sensitive enough to detect potentially toxic concentrations in biological samples. In this paper we describe a facile method of detecting the deadly chemical, sodium azide.

I:

Introduction

Two decades ago, when sodium azide was used mainly as a bactericide in laboratories, the compound didn't raise much concern because a couple kilograms of sodium azide per year was enough to meet the world's demand. However, with the advent of more efficient technology, sodium azide became the detonator for automobile "air" bags. With the increase in automobile needs, the demand for sodium azide skyrocketed from a few kilograms per year to thousands of metric tons. One metric ton will provide approximately 15,000 new cars with one airbag each (Kruszyna et. al., 1998). Briefly, airbags work by installing a sensor steel ball that is held in place by a stiff spring or magnet inside the airbag to differentiate between regular bumps and collisions. When a collision occurs, the steel ball moves forward and turns on an electric circuit subsequently setting off an electric impulse to ignite sodium azide and the following chemical reactions occur.

1.) $2NaN_3 \rightarrow 2Na + 3N_2$,

2.) $2Na + 2KNO_3 \rightarrow Na_2O + K_2O + N_2$

3.) $Na_2O + K_2O + SiO_2 \rightarrow$ alkaline silicate (glass)
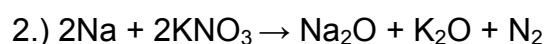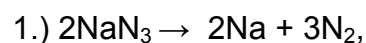
(Cassiday et. al., 2000)

Due to its rapidly acting, highly toxic qualities, azide is listed as a toxic industrial chemical (TIC) and could potentially be abused as a chemical agent for terrorism and used as a poison. The U.S. government and homeland security has recently discovered the reported interest in NaN_3 and it's acid counterpart, HN_3. Large amounts of the chemical compounds were retrieved in terrorist groups linked to Al' Queda in 2004 (Kosol, 2006). Moreover, in the late nineties,

Japan experienced the biggest chemical-terrorism events in the world and sodium azide was one of the compounds used for terrorism (Okumura et. al, 2003). Many cases of sodium azide poisoning from unknowingly consuming the compound have also been reported in the United States and Japan. Just last year, the Dallas County Health and Human Services reported several cases of acute-onset dizziness among patrons at a local restaurant. After five months of extensive laboratory testing, the CDC identified the toxic agents responsible for the poisoning to be sodium azide and hydrazoic acid (Center for Disease Control and Prevention [CDC], 2010). Other cases of attempted suicides and homicides involving sodium azide poisoning have been reported, which made azide a target for initial inquiry in unknown poisoning cases (Tsuge et. al, 2001). In Arizona, a woman was convicted of murdering her husband after severely poisoning him with sodium azide (Hacker, 2004).

Sodium azide is a colorless salt with the chemical formula NaN_3. The inorganic compound is acutely toxic and has an LD_{50} oral (rats) of 27mg/kg and an i.p. LD_{50} in mice is 0.57 mmol/kg, only five times greater than the notoriously deadly cyanide (Kruzyna et. al, 1998). According to the Hazardous Substance Data Bank (HSDB), non-lethal doses of 5 mg to 150 mg (1.63 μM to 47 μM) causes mild to severe symptoms and consumption of approximately 700mg (0.21mM) of the chemical have been reported to be fatal. The possibility of intoxication is compounded by the fact that aqueous sodium azide is readily hydrolyzed to yield hydrazoic acid (HN_3), a volatile substance that

partitions strongly to the gas phase. When ingested or inhaled, HN_3 is also highly toxic (Betterton, 2003).

The symptoms associated with sodium azide toxicity are comparable to those of cyanide poisoning. Established toxic or lethal dose of sodium azide are not available in literature, but reported cases of severe symptoms such as loss of consciousness, nausea, angina, dyspnea requires at least 80 mg to take effect. Although sodium azide causes systemic poisoning, it has its main effects on the cardiovascular system. Ingesting the chemical causes dilation of peripheral blood vessels, which consequently causes hypotension, tachycardia, dizziness, and collapse followed by respiratory distress, vomiting, and diarrhea. In fatal doses, people can suffer rapid loss of consciousness, metabolic acidosis or even coma before death. Ingestion of dust or solutions containing sodium azide can induce a wide variety of symptoms within minutes potentially resulting in the loss of consciousness and death. Inhaling the compound can cause irritation to the respiratory tract and mucous membrane causing a sore throat, coughing, dizziness, and shortness of breath. Other possible route of exposure to the toxic compound includes skin contact that can cause skin irritation, redness, and pain. Not only is sodium azide extremely toxic, it can combust or explode upon heating, shock, or friction.

Sodium azide can be produced in many different ways but the most common industrial preparation of sodium azide is by reacting nitrous oxide with sodium amide. The overall reaction chemistry is given below:

$$N_2O + 2NaNH_2 \rightarrow NaN_3 + NaOH + NH_3$$
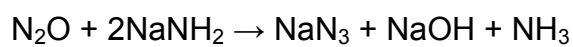
Sodium azide is a potent cytochrome c oxidase (complex IV of electron transport chain) inhibitor and causes ATP depletion and subsequent cell hypoxia (Bennett et al., 1996). Azide blocks cellular respiration at complex IV in the electron transport chain to prevent cytochrome c from contributing to the electron transport chain (ETC). The result of this inhibition causes chemical asphyxiation on cells and the depletion of cellular ATP. Azide inhibits cytochrome c oxidase by binding to the iron electron carrier of the heme prosthetic group in its oxidized ferric (Fe^{3+}) state. Interestingly, this is how azide works as a bactericide in laboratory reagents; azide prevents gram-negative bacteria from reproducing by inhibiting their cytochrome c oxidase (Bennet et. al., 1996). The azide anions also bind to other metalloenzymes such as catalases, peroxidases, and superoxide mutase (SOD), potentially inhibiting the enzyme's activity. However, the binding affinity of azide to these metalloenzymes depends greatly on the pH. In previous experiments described in literature, most metalloenzymes bind more favorably to the free acid rather than the anion (Bennett et al., 1996). Cytochrome c oxidase is one of the few exceptions to this rule and preferentially binds to the azide anion with higher affinity.

In addition to sodium azide being used as a bactericide, detonator in air bags, and poison, azide is also legitimately used as herbicides, fungicides, and soil fumigants (farm chemical handbook, 1999). Since the compound is highly soluble in water and doesn't easily decompose, the compound extends its toxicity to farmers, animals, and the general public. Furthermore, sodium azide is useful in organic synthesis as it is a way to introduce amide groups into compounds by

displacement of a halide group. While the individuals with the highest risk of exposure to sodium azide are automotive workers, wreckers, repairman, the potential abuse as a chemical terrorist weapon, and high occurrence of sodium azide makes a rapid, simple, and accurate method of detection to be extremely important. Not only would a facile method be extremely helpful to forensic investigations, the method can also be applicable to emergency medical investigations which would facilitate effective medical treatment.

Current methods to measure azide

Various methods for detecting and measuring the levels of sodium azide have been reported, including oxido-reductive titration, colorimetric test, spectrometry, gas chromatography-mass spectrometry, HPLC, and flow injection analysis. In laboratories, colorimetric tests are used to detect the presence of sodium azide indirectly. The procedure involves adding a drop of the solution of interest in the depression of a spot plate and then treating it with 1 or 2 drops of dilute hydrochloric acid. A drop of ferric chloride is added to the mixed solution and the spot plate is gently heated. A red spot indicates the presence of hydrazoic acid and thus the presence of sodium azide in the solution. This method has various limitations. First and foremost, the colorimetric method is only qualitative not quantitative and is not applicable to biological samples. Secondly, the method is tedious and requires multiple chemicals just to determine the presence of sodium azide. Lastly, the method is not very safe because it requires heating sodium azide, which can result in combustion or

explosion.

The most sensitive methods available now are GC/MS, ion chromatography, and flow injection analysis methods. While the current GC/MS method can detect to a lower detection limit of 1.25 µM, the procedure utilizes expensive and large equipment and is neither field deployable or practical in emergency situations. The ion chromatography method described in literature is also effective enough to detect sodium azide concentrations as low as 1.5 µM. Again, what ion chromatography gains in sensitivity, it lacks in practicality, throughput, and simplicity. The flow injection method provided in literature also has a low detection limit of 3 µM (Minakata et. al., 2012). While this is relatively more sensitive than a lot of detection methods available, the method requires a tedious protocol and expensive equipments. Therefore, taking into account the sensitivity and practicality of the methods, the cobinamide-based method proves to be better than the aforementioned methods.

In the early stages of a poisoning, the more quickly the toxic substances are identified from evidence samples, the more effective the applied medical treatment can be (Tsuge, et. al 2001). Taking into consideration the volatility of NaN_3, this narrows the selection of methods to spectrometry. The current spectrometric methods described in literature for sodium azide detection use either ferric perchlorate to detect the ferric azide complex or cerium ammonium nitrate to detect an oxido-reduction reaction. Amongst these established micro-diffusion spectrometric methods in literature, the modified and most sensitive method has a detection limit of 0.5 mM for azide (Tsuge et. al, 2001). This

detection limit is well above the projected lethal limit listed in the HSDB where approximately 700mg, or 0.230 mM, is lethal. According to the HSDB, serious symptoms begin to develop when one ingests more than 80mg (~26 µM) of sodium azide and we have proven that our method is sensitive enough to detect efficiently to concentrations as low as 25 µM (approximately 1mg/kg). The method also employs a two-part extraction method that involves taking the azide trapped in NaOH and then mixing it with either ferric trichloride (formation of ferric azide complex) or diammonium cerium (IV) nitrate (cerium reduction by azide). On the other hand, our method takes advantage of azide's high affinity to cobinamide and directly traps the azide in the Conway cells. On top of that, the reagents used in the aforementioned spectrometric processes are health hazards. On the other hand, cobinamide is not a toxic compound. Lastly, the current spectrometric methods have only tested sodium azide levels in neat standards only and have not been tested in biological samples.

Given the pros and cons of currently available detection methods of sodium azide, we aimed to develop a cheap, quick, and sensitive method of measuring sodium azide in standard and biological samples. We have developed a facile method to measure sodium azide based on characteristic spectral changes that occur upon sodium azide binding to cobinamide. We selected micro-diffusion spectrometry for its numerous advantages such as rapidity, accuracy, simplicity, and economy. Furthermore, the cobinamide-based method is efficient in detecting concentrations of azide well below the lethal limit which makes it applicable both as a preliminary screening method in forensic

investigation and emergency medical quantitation. Taking into consideration both the sensitivity and applicability of a detection method, we prove that the cobinamide-based method of sodium azide in aqueous and biological samples is superior to the current methods mentioned in literature.

Cobinamide

Figure 1. (a) cobalamin and (b) aquohydroxocobinamide
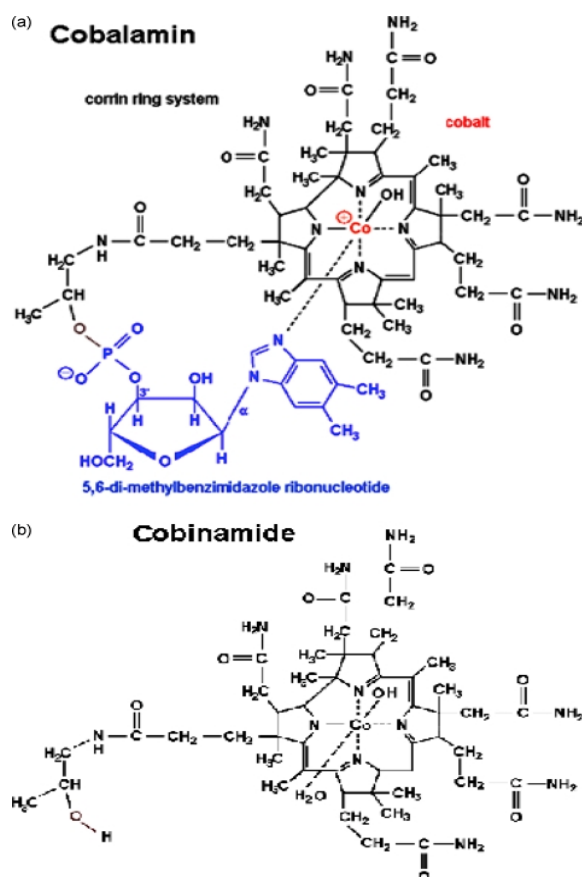
Hydroxocobalamin (vitamin B12) is a commercial form of the collective group of vitamins called cobalamins. Vitamin B_{12} is part of a group called bioogranometallic compounds, which are organic compounds that contain a

metal group. The reactive site of cobalamin lies in the cobalt center that can exist in the state (III) or reduced state (II) and surrounded by a corrin ring. The cobalt ion has six sites available for ligand binding, four of which are occupied by the corrin ring structure, and a fifth site is bound to the dimethylbenzimidazole (DBZ) ribonucleotide. The last binding site is the catalytic center responsible for the reactions associated with cobalamins.

Cobinamide is the penultimate precursor of cobalamin and has a chemical structure very similar to that of cobalamin. Some differences between the two compounds exist such as solubility, charges, and binding affinity. Cobinamide is relatively more soluble in water than cobalamin is and can exist in the oxidized (III) or reduced (II) form. All these differences can be attributed to cobinamide lacking the DBZ tail. First, without the bond to the DBZ tail, the steric hindrance at the trans site is eliminated and electrons within the corrin ring are allowed to move freely. Furthermore, instead of one reactive site, cobinamide now has another reactive site ready for ligand binding.

Using cobinamide to measure sodium azide

Determining whether cobinamide could be used to develop an accurate, field-deployable way of measuring sodium azide, a spectrophotometer will be used to obtain spectral scans of cobinamide with differing amounts of NaN_3 in the UV and visible wavelengths. Observation of the changes in the spectra will allow the determination of both the limit of detection (LOD) and limit of quantification (LOQ) of this spectrometric method. Furthermore, analysis of the spectra will

allow us to determine the best way to detect sodium azide. There are several methods of analysis that could identify the sodium azide concentration from the absorption spectra. Amongst them we have chosen two that seem the most straightforward: by measuring the change in a single wavelength or by taking the ratio of two wavelengths. Identifying an inexpensive, accurate and facile way of detecting and measuring sodium azide in the field could lead to faster, improved treatment of poisoned victims. Lastly, Ma (2011) has recently shown that cobinamide was efficient in measuring blood levels of cyanide in portable, point care applications. Proving that cobinamide can measure sodium azide levels will hopefully lead to a similar field deployable care device for sodium azide in the future.

II.

Materials and Methods

Cobinamide preparation

Cobinamide malate (Girindus AG) was weighed out and dissolved in 100mM monobasic phosphate buffer (pH: 4.7) to make a stock solution of 25mM cobinamide. The concentration and purity of the diaquocobinamide solution was determined spectrometrically using a molar extinction coeffcient of 2.8 x10^4 M/cm. The stock solution of cobinamide was stored at 4°C and diluted to the appropriate concentration with 100mM monobasic phosphate buffer (pH:4.7) daily before experiments. All other chemicals used were of analytical grade.

Spectrophotometer

A Kontron Instruments UVikon 860 spectrophotometer with 1-cm quartz cells was used for measuring and recording the spectrometric data. The scans were performed from 300-550 nm, at a sampling interval of 1 nm, and a speed of 120 nm/min.

Sodium azide

The 100mM sodium azide stock solution was prepared by dissolving sodium azide salt (Sigma Aldrich, 99.99% pure) in dibasic potassium phosphate buffer (Sigma Aldrich) at a pH of 8 and stored at 4°C. The work solutions were diluted down to the appropriate concentrations with 100mM monobasic potassium phosphate buffer daily before experiments.

Binding affinity calculations

To determine the binding affinity of cobinamide to sodium azide, increasing concentration of sodium azide, ranging from 1 μM to 95 μM were added to give a final concentration of 25 μM cobinamide. Individual solutions consisting 2 ml of 25μM cobinamide and the addition of 20 μl of increasing concentration of sodium azide made up in 100mM monobasic potassium phosphate buffer were combined until the absorbance spectra stopped changing indicating that the cobinamide solution has been fully saturated. The cobinamide with each concentration of sodium azide was placed into a quartz cuvette and scanned in the spectrophotometer.

Limit of detection and limit of quantification calculations

The limit of detection (LOD) and limit of quantification (LOQ) were calculated for each wavelength using standard deviations. Depending on whether the absorbance value increased or decreased, the LOD was calculated using these two equations.

1. LOD=average+(standard deviation x 3)

2. LOD=average− (standard deviation x 3)

The detection limit is the minimum point at which a sample can be distinguished from the absence of that substance (blanks or baselines). For wavelength 420

nm, where the absorbance values increased as increased concentration of sodium azide was added, the LOD is defined as the average of the baselines plus three times the standard deviation of the baselines. For wavelength 491 nm, where addition of sodium azide causes a decrease in absorbance values, the LOD is defined as baseline average minus three times the standard deviation of the baselines. The limit of quantification is the point at which a sample can be reasonably detected and quantified. The LOQ limits were calculated in the same way as the LOD but by adding or subtracting ten times the standard deviation instead of three times by the following two equations.

$$LOQ = average + (standard\ deviation\ x\ 10)$$

$$LOQ = average - (standard\ deviation\ x\ 10)$$

The LOD and LOQ were converted into moles by comparing the absorbance limits to the absorbance values of standard samples. Six baseline samples were used to obtain the averages and standard deviation.

Table 1. LOD and LOQ of the Cbi(III) method of detecting sodium azide at different wavelengths and wavelength ratio via direct addition of sodium azide to cobinamide solution. The ratios of absorbance values at 420 nm and 491 nm give the lowest LOD and LOQ.

	420 nm (µM)	491 nm (µM)	420 nm: 491nm (µM)
LOD	0.56	1.28	0.18
LOQ	1.87	4.27	0.58

Effect of pH

The pKa of sodium azide is 4.75 so the sodium azide stock solution was buffered at a pH of 8 to ensure that a large percentage of the compound was in the azide ion form and not in the acid form. When running standards, the addition of sodium azide to the cobinamide solution (pH: 4.7) converted some of the sodium azide to hydrazoic acid. Cobinamide was buffered at a pH of 4.7 to ensure that most of the cobinamide is in the diaquocobinamide form (pKa$_1$~6) (Fanchiang et. al, 1984). Cobinamide has a pKa$_1$ of 6.1 and a pKa$_2$ of 11. Therefore, at neutral pH cobinamide exists as aquohydroxycobinamide but at a pH<6 the compound exists mostly as diaquocobinamide and at a pH>11 it exists as dihydroxycobinamide. The cobinamide in the assays were prepared in pH 4.7 buffers to ensure that most of the cobinamide were in the diaquocobinamide form to ensure better binding since an aquo group is easier to displace than a hydroxy group. For the conway microdiffusion assays, 0.15 N of HCl was added to convert the sodium azide into hydrazoic acid in a 2:1 ratio which obtained a final solution with a pH of approximately 1.2 to make the compound volatile since the boiling point of hydrazoic acid is considerably lower (37°C instead of 300 °C).

Comparison assay

Anton and Terpinski's spectrometric method modified by Tsuge et al.

Sodium azide was measured following the formation of the azide ferric complex and the oxido-reduction using the cerium reagent described by Anton,

Dodd, Harvey, and Terpinski and modified by Tsuge et al. (This method is subsequently referred to as Tsuge's method). Briefly, the detection of sodium azide is observed via the formation of the ferric-azide complex and the oxido-reduction of azide and cerium ammonium nitrate. Conway diffusion cells (Fisher Scientific) were used for micro-diffusion. Two milliliters of 0.1 M sodium hydroxide (NaOH) was added to the center wall of the cell. One milliliter of the sample solution was added to one side of the outer well. A plastic lid containing grease was then placed over the cells, leaving a small opening on the side opposite of the sample, through which 0.5 milliliters of 10% (w/v) sulfuric acid (H_2SO_4) was added. The cells were closed rapidly, and the sample and acid were immediately mixed by gently tilting the cell. After the cells were incubated for 30 min at 40°C in an air incubator, the lid was removed and the extracted solution in the center cell was removed with a pipette. The extracted solution was then treated either via the spectrometric method no. 1 or spectrometric method no. 2 described below to determine the azide concentration.

Spectrometric method no. 1 (ferric azide complex formation)

20 µl of the extracted solution from the inner cell was extracted with a pipette and mixed with 0.18 ml of 50 mM ferric trichloride in 10mM hydrochloric acid (HCl) and subsequently pipetted into a 96-well microplate. After 5 min, the absorbance was determined at 450 nm using a microplate reader. The calibration standards (0-10mM) are also subjected to the same color reactions in parallel. The samples giving azide levels higher than 10mM were assayed again after

appropriate dilution of the inner cell solution with 0.1 M NaOH.

Spectrometric method no. 2 (cerium reduction by azide)

20 µl of the extracted solution from the inner cell was extracted and mixed with 0.18 ml of 600 µM diammonium cerium (IV) nitrate in 1.0 H_2SO_4 and then pipetted into a 96-well microplate. The absorbance was determined at 390 nm using a microplate reader. The calibration standards (0-6 mM) are also subjected to the same color reactions in parallel. The samples giving azide levels higher than 6 mM were assayed again after appropriate dilution of the inner cell solution with 0.1 M NaOH.

Measurement of sodium azide concentration using cobinamide Conway assay

Plastic Conway cells (outer cell, height 8mm, exterior diameter, 70 mm, interior diameter, 40 mm; center well, height 5mm, exterior diameter 34 mm; Fischer Scientific, were used for micro-diffusion. Two millimeters of 5 µM cobinamide was added to the center well. 400 µl of sample containing sodium azide in 100 mM phosphate buffer (pH 7.9) was added to the bottom of the outer well and 800 µl of TCA was added right beside the sample solution. The lid was immediately capped and the Conway cell was transferred to the 37°C room. The content in the outer well were allowed to mix thoroughly by gently tilting and swirling the cell. After a 45-minute incubation time, the lid was removed and the cobinamide solution was transferred from the center well to a microfuge tube with

a pipette. The solution was then transferred from the microfuge tube to a cuvette and scanned in the spectrophotometer to determine the azide concentration.

Measurement of azide in human blood

The concentration of azide in blood samples was measured using the cobinamide-based method and compared to the ion chromatography method published in literature. 2 ml of cobinamide was pipetted into the inner well of the Conway cell and 400 µl of human blood was added to the bottom of the outer well. 800 µl of TCA was added beside the blood sample and 25 µM of azide was added into the blood and the Conway cell was immediately capped and the cells were swirled gently to allow the blood and acid to mix. The samples were incubated in the warm room (37°C) for 45 minutes and the cobinamide was transferred into a cuvette and scanned in the spectrophotometer to determine the azide concentration.

Stability of sodium azide in blood

Human blood samples were spiked with 25 µM of sodium azide and incubated for 10 minutes, 1 hour, 3 hours, 24 hours, 48 hours, 72 hours at room temperature (25°C) and 4°C. The blood samples were then scanned by the spectrophotometer and observed for presence of the cbi-azide complex. The stability of the azide in blood was determined by comparing the 420nm:491nm wavelength ratios and calculating the percent recoveries relative to the standards. The percent recovery was calculated based on Conway cell

absorbance values and divided by standard absorbance values.

III.

Results

Determining the Affinity Constant

   To determine the binding affinity of sodium azide for cobinamide, 1 µM, 2 µM, 3 µM, 4 µM, 5 µM, 10 µM, 15µM, 20 µM, 25 µM, 27.5 µM, 30 µM, 32.5µM, 35 µM, 37.5 µM, 40 µM, 45 µM, 50 µM, 75 µM, 85 µM, 95 µM of sodium azide were added to 25 µM cobinamide and scanned in the spectrophotometer to determine that saturation had been achieved. The largest spectral change occurred at 325 nm, 348nm, 420nm, and 491nm. The 420 nm:491 nm ratio was chosen to calculate saturation since at this wavelength ratio the spectral changes were consistent with increased concentration of sodium azide. At this ratio of wavelength, the change in absorption between the samples with and without sodium azide was plotted against sodium azide concentration.

Figure 2. Increasing concentration of NaN_3 added to 25 µM of cobinamide until saturation is reached
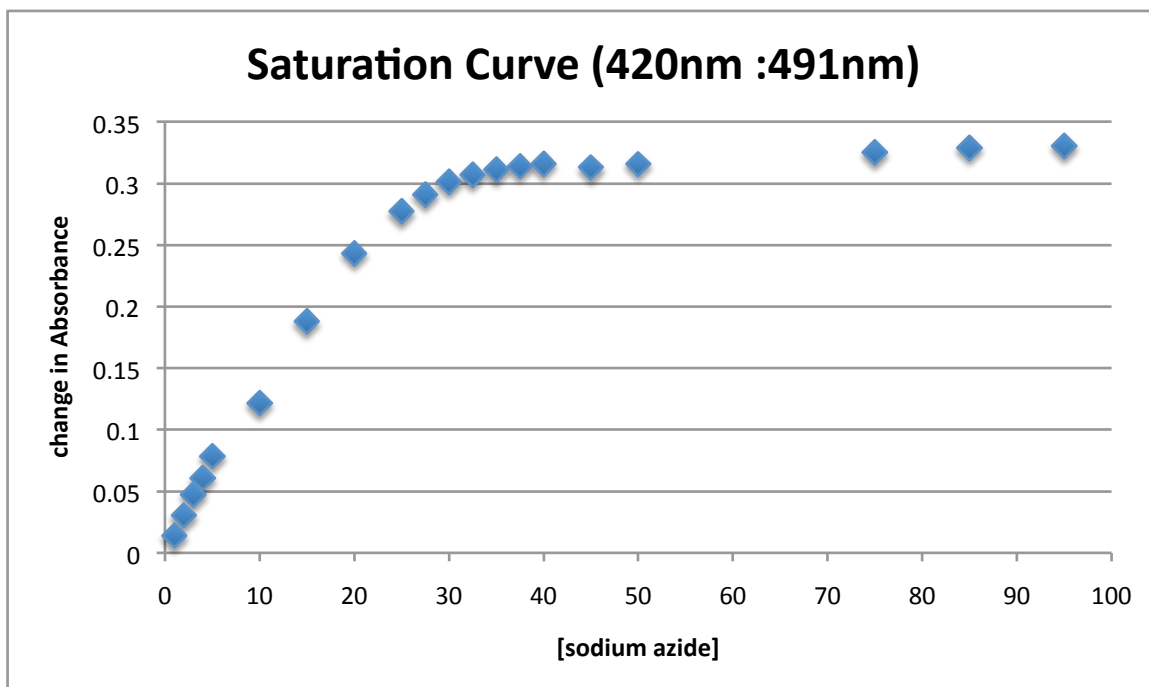
The inverse of the changes in absorption and sodium azide concentrations were taken to give a Lineweaver-Burke plot of the data. From the x-intercept of the best fit line, the binding affinity (Km) was calculated to be 6.10×10^{-5} M (LOG_{10} K= 4.21).

Figure 3. The Lineweaver-Burk plot of saturated cobinamide and increasing concentration of sodium azide
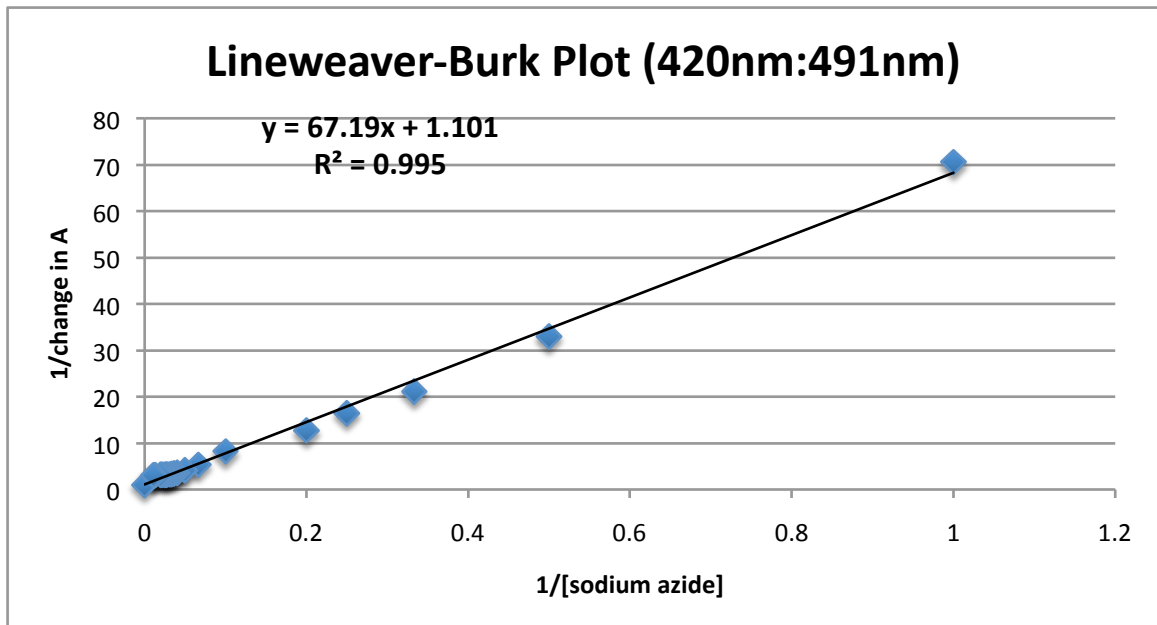
Azide can bind to cobinamide and change its spectrum

Cobinamide binds to sodium azide with 6.10×10^{-5} M affinity and therefore cobinamide can bind to sodium azide.

Figure 4. 5 µM of cbi (III) (blue line) with increasing addition of sodium azide until cobinamide solution is completely saturated

Cobinamide-azide complex absorbance spectrum
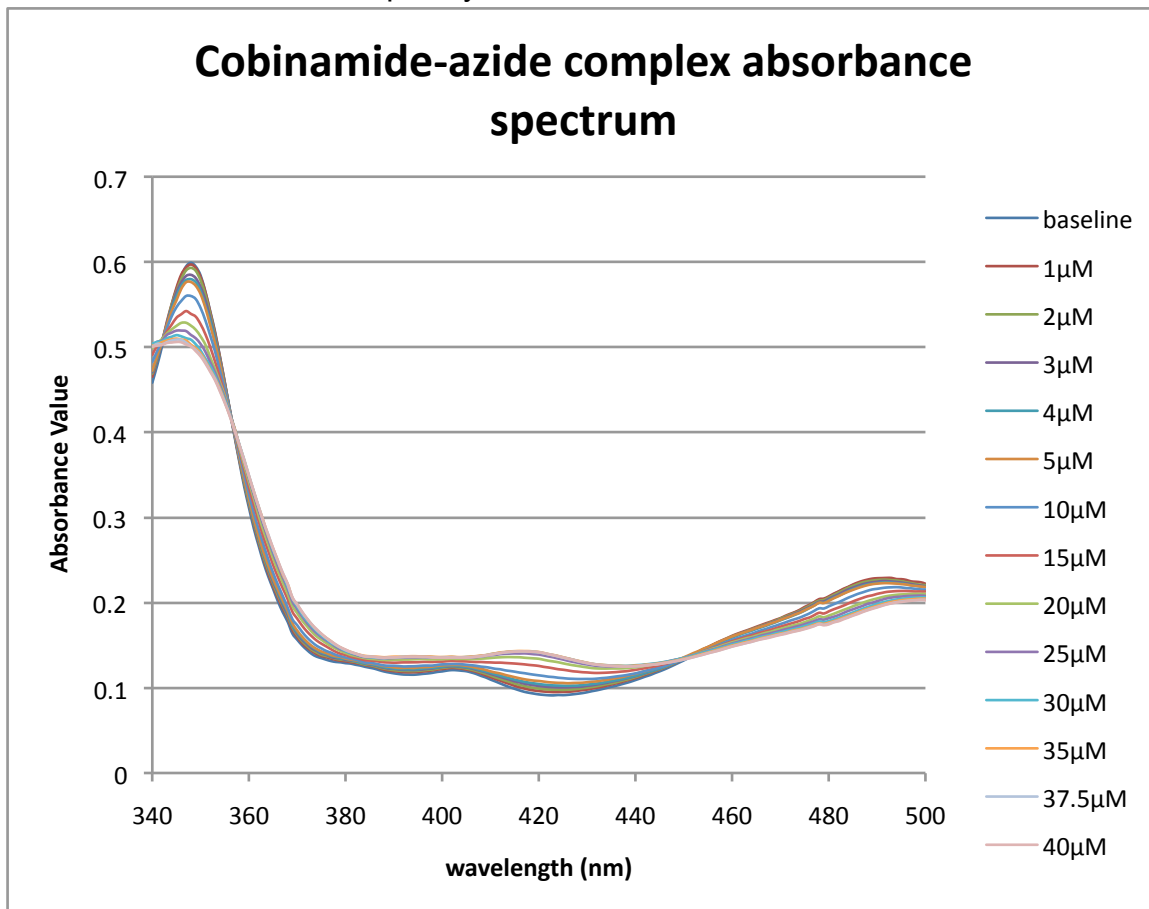
Decreasing amounts of sodium azide were added to cobinamide and scanned until no changes in the spectrum were observed. The samples with sodium azide were indistinguishable from baseline cobinamide when the amount of sodium azide reached 590 nmol. As the amount of sodium azide increases, the absorbance of cobinamide changes from 300 nm to 550nm, with the largest changes occured at 325nm, 348nm, 420nm, and 491 nm. We analyzed the absorbance values at 420 nm and 491 nm for this report due to the protein

interferences at lower wavelengths.

Determining the limit of detection and limit of quantification

Six baseline samples, containing only 25 μM cobinamide in 100 mM monobasic potassium phosphate buffer were scanned in order to calculate the LOD and LOQ. The LOD and LOQ were calculated at 420 nm and 491 nm and at the ratio of the wavelengths (420 nm: 491 nm). At 420nm, the baselines had an average absorption of 0.0926 absorbance units (AU) with a standard deviation of 0.0009 AU. Multiplying the standard deviation by 3 and 10 then adding it to the baseline averages gave a LOD absorbance value of 0.095327 AU and LOQ absorbance value of 0.1017 AU respectively. At 491nm, the baselines had an average absorbance of 0.2278 AU and a standard deviation of 0.0011 AU which gave a LOD of 0.2246 AU and an LOQ of 0.2172 AU. At the calculated wavelength ratio of 420nm: 491nm the average Absorbance values for the baselines were 0.4064 AU and a standard deviation of 0.0023 AU which obtained a LOD and LOQ of 0.4133 AU and 0.4294 AU respectively. All absorbance values increased or decreased linearly as the concentration of azide increased with the ratio of 420nm:491nm with the lowest LOD and LOQ.

Measurement of Sodium Azide Using a Standard Spectrometric Method

Both the ferric perchlorate method and the diammonium cerium (IV) nitrate method took an hour and gave a detection limit of 0.5 mM. The experiments were performed according to the protocol described in Tsuge's paper.

Measurement of Sodium Azide in Standard Solution

Using the previous cobinamide tests as standards, cobinamide was then

tested in Conway cells. Sodium Azide was measured following the formation of

the cobinamide-azide complex. The Conway assay was performed on sodium

azide amounts of 1μM, 2 μM, 5 μM, 10 μM, 20 μM, 25 μM. Just as with the

standards, increased amounts of sodium azide increase absorbance values from

300 nm to 342 nm, 356 nm to 451nm, 525 nm to 550 nm and decreases from

343 nm to 355 nm, 452 nm to 524 nm (figure 5). When compared to the

standards, the assay was sensitive enough to detect sodium azide down to 10

μM. However, after repeating the assay a couple times, the 10μM sodium azide

samples did not consistently give 100% recovery and thus the assay was

determined to be sensitive to 25 μM of sodium azide.

Measurement of Sodium Azide in Human Blood

The concentration of sodium azide in human blood was measured using

the cobinamide method and compared to the ion chromatography method

mentioned in literature. Data from the experiments indicate that the cobinamide-

based method was accurate in detecting as low as 25 μM of sodium azide in

blood. That is, in multiple experiments, the 420 nm: 491 nm wavelength ratios

obtained from the blood samples spiked with 25 μM of sodium azide had a high

correlation value (>99%) to cobinamide solutions directly spiked with 25 μM of

sodium azide.

Stability of Sodium Azide in Human Blood over time

We incubated 25 µM of sodium azide in 400 µl of blood under two different temperatures over 72 hours at six different time points. Contrary to what was described earlier in Krusyna's paper, we found that the half-life of azide in blood was approximately a week at room temperature. However, the azide in blood was noticeably more stable when stored at 4°C. The only limitation to this part of the experiment was that the blood used was 2 weeks old, which could have compromised azide metabolism in the blood.

Table 2. 25 µM of sodium azide incubated in blood at room temperature and 4°C over a period of 72 hours

Time (hours)	25°C (%)	4°C (%)
0	100	100
0.167 (10 mins)	95.5	95.5
1	97.5	98
3	97.4	96.4
24	95	97
48	90.1	95.1
72	77	94.9

IV.

Discussion

There are currently multiple methods utilized to measure the amount of azide in standard and biological samples. However, each method has its advantages and disadvantages and no one method is accepted as the universal go-to method to detect and measure sodium azide. Furthermore, none of the methods mentioned in literature are potentially field deployable. Thus, improving on current methods of detection can be useful especially if it offers a quick return in field or clinical settings. We have developed a new method to measure sodium azide, based on the extremely high affinity of azide to cobinamide. The assay can be used as a qualitative mode of detecting sodium azide and has a high throughput capacity. In addition to the high throughput capacity, other advantages of the cobinamide-based method are ease of use, stability of cobinamide, and application across a wide, dynamic range depending on the concentration of cobinamide used. 5 µM of cobinamide was used in the experiments to test the validity of the assay but usually a higher concentration of cobinamide (up to 100 µM) would be used in clinical settings since the lethal dose of sodium azide is much higher than 25 µM. We have shown in this paper that our method is orders of magnitude more sensitive than similar methods of detection found in literature. While gas chromatography and ion chromatography are more sensitive than the cobinamide-based method, these methods necessitate the use of long, complicated protocols and instruments at the expense of its sensitivity. In practical considerations, our method is sensitive enough to detect concentrations of sodium azide well below lethal doses in

human blood samples. Moreover, the cobinamide-based method is fast, efficient, and easy to use, which makes it very likely to be adapted into a field deployable method in the future. Lastly, we have found that 25 µM azide was stable and recoverable in blood even after three days when incubated at 4°C.

V.

Conclusion

We have developed a new method to measure azide. It is simple, practical, and could be applied to biological samples.

VI.

References

Anton, A., Dodd, J.G., and Harvey, A.E., "Spectrophotometric determination of azide with ferric perchlorate," Anal. Chem. 32:1209–1210 (1960).

Baldwin DA, Betterton EA, Pratt JM. J.Chem.Soc.Dalton Trans 1983:217–223. Bennet, M.C., Mlady, G.W., Kwon Y.H., Rose, G.M., "Chronic In Vivo Sodium Azide Infusion Induces Selective and Stable Inhibition of Cytochrome C Oxidase," *Journal of Neurochemistry,* Vol. 66, 1996, 2606-2611.

Betterton, E.A. Environmental fate of sodium azide derived from automobile airbags. Crit. Rev. Environ. Sci. Technol. 33: 423–458 (2003).

Blackledge, W., "A new facile method to measure cyanide in blood" Anal Chem. 2010 May 15; 82(10): 4216–4221

Broderick, K.E., Singh V., Kambo A., Chen J.C., Sharma V.S., Pilz R.B., Boss G.R. "Nitric Oxide Scavenging by the Cobalamin Precursor Cobinamide" The Journal of Biological Chemistry, *Vol.280,* March 11, 2005, 8678-8685.

Casiday, Rachel and Frey,Regina. "Gas Law Saves Lives: The Chemistry Behind Airbags." Washington University Chemistry. 1998. Washington University. October 2000.
<http://www.chemistry.wustl.edu/~edudev/LabTutorials/Airbags/airbags.html>

Centers for Disease Control and Prevention. (2010). *MMWR weekly: Summary of notifiable diseases*. Retrieved from
        <http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6125a1.htm>

Cerda, V., Cerda, A., Cladera, A., Oms, M.T., Mas, F., Gomez, E., Bauza, F., Miro, M., Forteza, R., Estela, J.M (2001). *Trends in Analytical Chemistry. Vol. 20:8 (2001).*

Chan, A., Balasubramanian, M., Blackledge, W., Mohammad, O.M., Alvarez, L., Boss, G.R., Bigby, T.D. "Cobinamide is superior to other treatments in a mouse model of cyanide poisoning," *Clin Toxicol,* Vol. 48, No. 7, August 2010, 709-717.

David Blair, "Al-Qa'eda Plot Would Have Killed 20,000," The Daily Telegraph (London), April 19, 2004,11; see also "Chemical Attack Said Thwarted on Jordan Security HQ, US Embassy," BBC Monitoring International Reports, April 16, 2004; "Jordan 'Was Chemical Bomb Target,'" BBC News World Edition, April 17, 2004.

Fanchiang, Y., Bratt, G.T., Hogenkamp, H.P.C., "Methyl transfer from methylcobinamide to diaquocobinamide" *proc. Natl. Acad. Sci.,* Vol. 81, May 1984, 2698-2702.

Farm Chemicals Handbook 1999. Willoughby, OH: Meister Publishing Co., 1999., p. C-355.

George, M., Nagaraja, K.S., Balusubramanian N. "Spectrophotometric Determination of Hydrazine using Bromine and Methyl Red," Indian Journal of Chemistry, *Vol46A,* October 2007, *1621-1624.*

Hazardous Substances Data Bank [Internet]. Bethesda (MD): National Library of Medicine (US); [Last Revision Date 2005, June 24; cited 2012 August]. Sodium Azide; Hazardous Substances Databank Number: 695; [about 2 screens]. Available from:
http://toxnet.nlm.nih.gov/cgibin/sis/search/f?./temp/~f7KHT6:1:FULL

Howard, J.D., Skogerboe, K.J., Case, G.A., Raisys, V.A., Lacsina, E.Q., "Death Following Accidental Sodium Azide Ingestion," *Journal of Forensic Sciences, JFSCA,* Vol. 35, No. 1, Jan. 1990, 193-196.

Kage S. , Kudo K., and Ikeda N. Determination of azide in blood and urine by gas chromatography–mass spectrometry. J. Anal.Toxicol. 24: 429–432 (2000).

Kosol, M.E., "Near Term Threats of Chemical Weapons Terrorism," *Strategic Insights,* Vol.10:6, July 2006, 1-14.

Krusyna R., Smith R.P., Kruzyna, H., "Determining sodium azide concentration in blood by ion chromatography," *J Forensic Sci*, Vol. 43:1, 1998, 200-202.

Lichstein, H.C., and Soule, M.H., "Studies of the Effect of Sodium Azide on Microbe Growth and Respiration." Ph.D dissertation, University of Michigan, 1943.

Ma, J., Ohira, S., Mishra, S.K., Puanngam, M.M., Dasgupta, P.K., Mahon, S.B., Brenner, M., Blackledge, W., Boss, G.R., "Rapid point of care analyzer for the measurement of cyanide in blood," *Anal Chem.,* Vol. 83:11, June 1, 2011, 4319-4324.

Malaki, E.A., and Koupparis, M.A., "Kinetic Study of the Determination of Hydrazines, Isoniazid and Sodium Azide by Monitoring their Reactions with 1-fluoro-2,4-dinitrobenzene, by means of a Fluoride-Selective Electrode," *Talanta,* Vol. 36:4. 1989, 431-436.

Minakata, K., Nozawa, H., Yamagishi, I., Gonmori, K., Hasegawa, K., Suzuki, M., Watanabe, K., Suzuki, O., "Determination of azide in gastric fluid and urin by flow-injection electrospray ionization tandem mass spectrometry" *Ana.I Bioanal. Chem.,* June 2012, Vol. 7, 1793-1799.

Nishikawa M., and Tsuchihashi, H., "Simultaneous GCMS Determination of Azide, Cyanide and Thiocyanate via Phasetransfer-Catalyzed Pentafluorobenzylation. *J. Health Sci*, 2000, Vol.46, 81–88.

Subramanian, N.H., Sankar Babu, V.R., Jeevan, R.G., and Radhakrishnan, G., "Matrix Elimination Ion Chromatography Method for Trace Level Azide Determination in Irbesartan Drug," J*ournal of Chromatographic Science*, August 2009, *Vol. 47*, 529-533.

Echols, R.T., James, R.R., and Aldstadt, J.H., "Determination of primary explosive azides in environmental samples by sequential injection amperometry," *Analyst,* Vol 122, 1997, 315–319.

Okumura, N., Ninomiya,M., Ohta, The chemical disaster response system in Japan. Prehosp Disaster Med 2003;18:189-92

Terpenski, E.A., "Spectrophotometric determination of sodium azide," *Analyst* Vol. 110, 1985, 1403-1405.

Tim Hacker. Arizona woman on trial in husband's death. USAtoday.com. December 1, 2004. Available at http://www.usatoday.com/news/nation/2004-12

Tsuge, K., Kataoka M., and Seto Y. "Rapid determination of cyanide and azide in beverages by microdiffusion spectrophotometric method," *J. Anal. Toxicol*. Vol.25, June 2001, 228–236.

Christova-Bagdassarian, V., Atanassova, M., "Spectrophotometric Determination of Sodium Azide in Workplace Air," *Journal of the University of Chemical Technology and Metallurgy*, Vol. 42, August 2007, 311-312