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Development of a new method to measure hydrogen sulfide using the vitamin B12 precursor cobinamide

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Development of a New Method to Measure Hydrogen Sulfide Using the Vitamin B12 Precursor Cobinamide

A Thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

Lauren Lucille Jann

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Professor Tracy L. Johnson, Co-Chair
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Professor Kim F. Albizati

2012
The Thesis of Lauren Lucille Jann is approved and it is accepted in quality and form for the publication on microfilm and electronically

Co-Chair

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University of California, San Diego

2012
DEDICATION

This thesis is dedicated to my parents, Art and Louise, for their encouragement, love, and support.
I love you.
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ABSTRACT OF THE THESIS

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by

Lauren Lucille Jann

Master of Science in Biology

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Professor Gerard R. Boss, Chair
Professor Tracy L. Johnson, Co-Chair

Hydrogen sulfide is a deadly gas for which no point-of-care device for measuring biological samples exists. Current methods of measuring sulfide include high performance liquid chromatography and gas chromatography/mass spectrometry, but these methods require non-portable equipment. Another technique for measuring sulfide is Ellman’s test; although it has a high level of sensitivity and can be measured in a spectrophotometer, it has a low dynamic range, as precipitates can form at high concentrations. The results presented here use the vitamin B12 precursor, cobinamide, which goes through a spectral shift upon binding of sulfide, to measure sulfide levels. Here it is shown that cobinamide binds to sulfide with a binding affinity of $5.27 \times 10^8$
The limit of detection and limit of quantification of this assay are calculated to be 1.88 μM and 7.21 μM, respectively. Although the cobinamide assay is not as sensitive as Ellman’s test, the cobinamide assay is simpler and these limits are still sufficient since sulfide poisoning victims have blood concentrations of sulfide in the micromolar range. To simulate biological samples, Conway diffusion cells have been used. In these cells, the smallest percentage of recovery of hydrogen sulfide is 95.9%, which suggests that measurement in biological samples is possible.
INTRODUCTION

Hydrogen Sulfide

Hydrogen sulfide is a flammable, colorless gas that has the characteristic smell of rotten eggs; it is heavier than air and accumulates in low-lying areas such as basements and sewers. The human nose can begin to detect levels of hydrogen sulfide at concentrations ranging from 0.003-0.02 parts per million (ppm). However, when concentrations reach 100 ppm or if a person is continuously exposed to low-levels, olfactory fatigue can set in, where the ability to smell the gas is lost even if it is still present. If the concentration of hydrogen sulfide exceeds 500 ppm, loss of consciousness, respiratory failure, and even death can occur (Reiffenstein et al., 1992).

The gas is naturally produced when organic compounds break down under low oxygen conditions, such as in hot springs, volcanoes, and sewers, but hydrogen sulfide poisoning is more common in the petroleum, mining, and tanning industries as well as in paper mills (Saint Louis University, 2002). The main route of poisoning is by inhalation and its mechanism of action is similar to that of hydrogen cyanide. Hydrogen sulfide binds to the iron ion of cytochrome c oxidase of mitochondria, thus ceasing aerobic respiration (Cooper and Brown, 2008; Saint Louis University, 2002).

Although hydrogen sulfide is better known for its deadly toxicity, it also has a biological function; at concentrations in the nanomolar range, it acts as a signaling molecule in the inflammatory, nervous, and cardiovascular systems (Hughes et al., 2009). Additionally, it may function as a smooth muscle relaxant and can protect neural cells from oxidative stress (Kimura et al., 2005). In vivo, it is generated when the sulphydryl group is removed from L-cysteine by the enzymes cystathionine-β-synthase,
cystathionine-γ-lyase, or 3-mercapto-sulfurtransferase (Shen et al., 2011; Tangerman, 2009; Hughes et al., 2007).

In 2008, there were reports in Japan in which people mixed sulfur-based bath products with household chemicals in confined spaces to generate lethal amounts of hydrogen sulfide gas in order to commit suicide. Between March 27, 2008 and June 15, 2008, there were 220 attempts and 208 fatalities (Morii et al., 2010). When a toxicological analysis was performed, 17 cases had blood concentrations of hydrogen sulfide that ranged from 3-995 μM, with an average of 191 μM (Maebashi et al., 2011).

**Current methods to measure hydrogen sulfide**

Currently, hydrogen sulfide levels can be measured using a polarographic sensor (Doeller et al., 2005), colorimetric analysis (Hughes et al., 2009), gas chromatography/mass spectrometry (GC/MS) (Tangerman, 2009) or high performance liquid chromatography (HPLC) (Shen et al., 2011). However, there are problems with each of these methods. The polarographic sensor has a detection limit around 10 nM, which is fairly low, but calculation of the sulfide concentration is not simple since a lot of information is needed: the Henderson-Hasselbach equation in conjunction with the pKa value of H₂S at the temperature of the experiment and the pH of the solution. Additionally, the electrodes need to be conditioned as precipitates can form on them (Hughes et al., 2009).

The colorimetric analysis using methylene blue is based on the theory that high levels of hydrogen sulfide in tissues or blood; however, bodily hydrogen sulfide levels have recently been reported to be in the nanomolar range, which means that the colorimetric assay is not sensitive enough (Hughes et al., 2009; Furne et al., 2008). A
major issue with the colorimetric analysis and polarographic sensor, as well as other assays, is that they function in acidic conditions. At acidic pH levels, acid-labile sulfide (ALS) is released as \( \text{H}_2\text{S} \) from iron-sulfur proteins. Most assays do not differentiate between ALS and \( \text{H}_2\text{S} \), which could explain the discrepancies of reported sulfide concentrations (Tangeman, 2009). Although GC/MS and HPLC are reliable ways of accurately measuring \( \text{H}_2\text{S} \) concentrations, both require large, bulky instruments that cannot be used in the field.

Another established method is Ellman’s test, which uses Ellman’s reagent, 5, 5’-dithiobis-(2-nitrobenzoic acid), also known as DTNB. The sulfur in the sample breaks the disulfide bond in DTNB to produce 2-nitro-5-thiobenzoate, a molecule that is yellow in color. This color is quantified when scanned at 412 nm (Riddles et al., 1983). Although this assay is sensitive and fast, it only works at a neutral pH and cannot directly measure sulfide levels in biological samples. A method to measure hydrogen sulfide that is facile, reliable, and field-deployable is needed.

**Cobinamide**

Cobalamin (vitamin B\(_{12}\)) is composed of a cobalt atom in the center of a corrin ring that forms a strong coordinate bond with negatively charged ligands such as cyanide and nitric oxide (Hayward et al., 1965; Wolak et al., 2001). Cobinamide, the penultimate precursor in the biosynthesis of cobalamin, has two free binding sites because it lacks 5, 6-dimethylbenzimidazole moiety that is present in cobalamin (Figure 1). The loss of this group allows the cobalt atom to be in the plane of the ring, which leads to higher binding affinities of the same negatively charged ligands that bind to cobalamin (Sharma et al., 2003). Prior research showed that cobinamide binds to
cyanide with very high affinity; the first cyanide ion binds with an affinity of $10^{14} \text{ M}^{-1}$ and the second ion binds with an affinity of $10^8 \text{ M}^{-1}$, leading to an overall affinity of $10^{22} \text{ M}^{-2}$ (Hayward et al., 1965) When cyanide binds to cobinamide, there is a spectral change where the α, β, and γ peaks of the cobinamide spectrum (Supplemental figure 1) shift to the right; this shift can be measured by spectrophotometry and can quantify cyanide in a sample (Blackledge et al., 2010). As cyanide and hydrogen sulfide are both anions and have similar mechanisms of toxicity, the theory is that cobinamide might be able to measure and possibly treat hydrogen sulfide poisoning.

**Using cobinamide to measure hydrogen sulfide**

To determine if cobinamide can be used to develop an accurate, field-deployable method of measuring hydrogen sulfide, a spectrophotometer will be used to obtain spectral scans of cobinamide with differing amounts of NaSH in the ultraviolet and visible wavelengths. Analysis of these spectra would allow determination of the limit of detection and limit of quantification, which are indicative of the sensitivity of an assay. Ma and colleagues recently demonstrated that cobinamide can quickly and accurately measure blood levels of cyanide in portable, point-of-care applications (Ma et al., 2011). Demonstrating that cobinamide can measure sulfide levels will hopefully lead to a similar point-of-care device for hydrogen sulfide. Identifying an inexpensive, accurate, facile way of measuring hydrogen sulfide in the field could lead to rapid treatment of poisoning victims.
Figure 1: Structures of cobalamin and cobinamide at neutral pH. Cobinamide lacks the 5, 6-dimethylbenzimidazole ribonucleotide group (shown in blue) that is present in cobalamin.
MATERIALS AND METHODS

De-amination of cobinamide

When cobalamin is converted to cobinamide, it is produced as ammonia cobinamide. The ammonia has a binding affinity (log10K) for cobinamide of greater than or equal to 9, which might prevent the sulfide from binding (Hayward et al., 1971). Therefore, the cobinamide was de-aminated in order to obtain ligand-free cobinamide that would best bind sulfide. Solid ammonia cobinamide was acidified to pH 5 using hydrochloric acid (HCl). Cobinamide was then eluted with hydrochloric acid, at a pH of 5, over a mixed bed ion exchange column. After eluting, the cobinamide, now as aquohydroxocobinamide, was adjusted a pH of 5 using hydrochloric acid.

Spectrophotometer

Samples were measured using a Kontron Instruments UVikon 860 spectrophotometer. The scans were performed from 350-550 nm, at a sampling interval of 0.5 nm, and a speed of 120 nm/min.

Hydrogen sulfide

Hydrogen sulfide was generated by acidifying sodium hydrosulfide hydrate (NaSH) that was dissolved in sodium hydroxide.

Binding Affinity Calculations

To determine the binding affinity, increasing concentrations of NaSH, ranging from 1μM to 3 mM, were added to give a final concentration of 5 μM cobinamide. The solutions were combined at a ratio of 90% 10 mM sodium hydroxide (NaOH)-sulfide to 10% cobinamide, made up in 200 mM pH 4 sodium phosphate buffer until the
cobinamide was fully saturated with sulfide. The cobinamide and sulfide solution was placed into a quartz cuvette and scanned in the spectrophotometer.

**Limit of Detection and Limit of Quantification calculations**

Baseline solutions, containing 10% cobinamide, final concentration 5 μM, in 200 mM pH 4 sodium phosphate buffer and 90% 10 mM NaOH were measured by spectrophotometric analysis. The limit of detection (LOD) and limit of quantification (LOQ) were calculated for each wavelength using standard deviations. The limit of detection is the minimum point at which a sample can be considered above background and, for wavelengths 368.5 nm to 478 nm where the absorbance increases, is defined as baseline average plus three times the standard deviation of the blanks. In the case of the wavelengths from 478.5 nm to 550 nm, where increased sulfide causes a decrease in absorption, the LOD is defined as the baseline average minus three times the standard deviation of the blanks. The limit of quantification is the point at which a sample can be detected and quantified. The LOQ was calculated similarly to the LOD, by adding or subtracting ten times the standard deviation, instead of three times. The LOD and LOQ were converted into moles by comparing the absorbance limits to the absorbance values of standard samples, which were composed of the same percentage of NaOH and cobinamide as the baselines.

**Ellman’s test**

DTNB was dissolved in phosphate buffer and added to samples containing 90% 10 mM NaOH and 10% 200 mM sodium phosphate buffer, pH 4, to achieve a final concentration of 1 mM. The sample was briefly mixed and then spun down in a centrifuge at 13,200 rpm for 15 seconds to pellet any precipitate. The supernatant was
scanned by spectrophotometry at 412 nm to indicate the amount of sulfur present in sample.

**Conway assay**

Conway diffusion cells (Supplemental figure 2) are white polypropylene dishes with isolated inner and outer wells for measurement of gases released from biological samples. NaSH and 1M hydrochloric acid were placed on opposite sides of the inner well. 10 mM sodium hydroxide was placed in the outer well for trapping. The cells were covered with lids and sealed with Dow Corning high vacuum grease. The contents of the inner well were mixed to convert the sodium hydrosulfide hydrate to hydrogen sulfide gas. After a 1-hour incubation period, the cells were uncapped, cobinamide was added to the sodium hydroxide with sulfide, and the cobinamide-sulfide solutions were scanned in quartz cuvettes by spectrophotometer.
RESULTS

Change in cobinamide spectrum upon binding of sulfide

Incrementally decreasing amounts of NaSH were added to cobinamide solutions and scanned until no changes in the spectrum were observed. When the amount of NaSH reached 300 pmol (600 nM), the samples were indistinguishable from baseline cobinamide. As the amount of sulfide increases, the absorbance of cobinamide decreases from 350 nm to 368 nm, increases from 368.5 nm to 478 nm, and decreases again from 478.5 nm to 550 nm (Figure 2).

Figure 2: Changes in cobinamide spectra upon addition of sodium hydrosulfide. The NaSH concentrations that were measured using cobinamide (solid line) were: 0.6 μM (dotted line), 3 μM (short dashed line), 6 μM (short dash with one dot line), 30 μM (long dashed line), and 60 μM (hollow line).
Determining the affinity constant

To determine the binding affinity of hydrogen sulfide for cobinamide, 1 μM, 3 μM, 10 μM, 30 μM, 100 μM, 300 μM, 1 mM, and 3 mM NaSH were added to 5 μM cobinamide and scanned in the spectrophotometer to determine that saturation had been achieved. The largest spectrum change occurred at 429 nm. At this wavelength, the change in absorption between the samples with and without NaSH was plotted against NaSH concentration (Figure 3A). The changes in absorption and NaSH concentrations were inverted to give a Lineweaver-Burk plot of the data (Figure 3B). From the x-intercept, the binding affinity was calculated to be $5.27 \times 10^8 \text{M}^{-1}$ ($\log_{10} K = 7.28$).

![Figure 3A](chart.png)

Figure 3A: Change in absorption of cobinamide at 429 nm plotted against NaSH concentration.
Figure 3B: Lineweaver-Burk transformation of the data in Figure 2A. The binding affinity was calculated from the x-intercept of the line of best fit.

**Determining the limit of detection and limit of quantification**

Multiple baseline samples, containing only NaOH and cobinamide in buffer, were scanned in order to calculate the limit of detection and limit of quantification (Supplemental figure 3). At 429 nm, the baselines had an average absorption of 0.04611 absorbance units (AU) with a standard deviation of 0.00129. Multiplying the standard deviation by 3 and 10 and adding that to the baseline average gave a LOD absorbance value of 0.04999 AU and a LOQ absorbance value of 0.05905 AU. When the concentrations of NaSH were extrapolated from the trendline, the LOD and LOQ were found to be 1.88 μM and 7.21 μM, respectively.
Figure 4: Absorbance of cobinamide with increasing concentrations of NaSH at 429 nm showing LOD and LOQ.

**Determining the limit of detection and limit of quantification for Ellman’s test**

As a comparison cobinamide, Ellman’s reagent, DTNB, was used to measure thiol levels. The baseline samples were composed of 90% 10 mM NaOH and 10% 200 mM pH 4 sodium phosphate buffer. Upon extrapolation of the LOD and LOQ to the trendline generated by the standard concentrations of 0.3 μM, 1.5 μM, 3 μM, 6 μM, 15 μM, and 30 μM, the LOD and LOQ were determined to be 0.18 μM and 0.84 μM, respectively.
Figure 5: Absorbance of DTNB with increasing concentrations of NaSH scanned at 412 nm, showing LOD and LOQ.

**Measuring hydrogen sulfide levels in Conway cells**

Using the previous cobinamide tests as a standard, cobinamide was then tested in Conway cells. The Conway assay was performed on NaSH amounts of 300 pmol, 1.5 nmol, 3 nmol, 15 nmol, and 30 nmol. Similarly to the standards, increased amounts of sulfide increase the absorbance from 368.5 nm to 478 nm and decrease the absorbance from 478.5 nm to 550 nm (Figure 5). When compared to the previous standard amounts of NaSH that were assayed in cuvettes, the lowest recovery of NaSH occurred in the 30 nmol sample, where the recovery was 95.9%.
Figure 6: Changes in cobinamide spectra upon addition of hydrogen sulfide in Conway cells. The NaSH amounts that were transformed into gaseous $H_2S$ and then added to cobinamide (solid line) were: 0.6 nmol (dotted line), 1.5 nmol (short dashed line), 3 nmol (short dash with one dot line), 15 nmol (long dashed line), and 30 nmol (hollow line).
DISCUSSION

There are multiple assays available that can determine sulfide concentrations in samples; however, the necessity for bulky lab equipment and the inability to measure sulfide in biological samples prohibit these methods from being used in point-of-care devices. Here, the foundation has been laid for the development of an assay to measure hydrogen sulfide levels in biological samples using cobinamide.

The binding affinity of cobinamide for hydrogen sulfide has not yet been reported in the literature. The data presented here shows that cobinamide can bind sulfide with a binding affinity of $K = 5.27 \times 10^{8} \text{M}^{-1}$. If the concentrations of NaSH and cobinamide are equal and the binding affinity is around $10^{7} \text{M}^{-1}$, the cobinamide should be 90% saturated (Perrin and Sayce, 1967). If the NaSH concentration is increased to three times as high as the cobinamide concentration, the cobinamide should be 99.5% saturated. Taking these numbers into consideration, it seems reasonable that cobinamide would be able to bind and measure hydrogen sulfide levels.

When comparing cobinamide to DTNB, in terms of quantifying sulfide levels, DTNB is about ten times more sensitive; however, the cobinamide assay has advantages over Ellman’s Test: the cobinamide assay is facile, will not form a precipitate, and has a larger range of detection. Additionally, this ten-fold difference seems negligible when blood levels of hydrogen sulfide in suicide victims averaged 191 μM (Maebashi et al., 2011).

Performing the cobinamide assay in Conway cells worked well. When analyzed, the cobinamide spectrum changed as expected and the minimum recovery was 95.9%. Although this research is limited in that it has only been chemical-based thus far, it is
highly likely that this assay would work for biological samples. It has previously been shown that cobinamide has the ability to measure cyanide levels in both blood and plasma in a similar assay (Blackledge *et al.*, 2010).

There was a lot of trial and error involved in this project. Initially, NaSH standards were measured directly in cuvettes, using 5 μM cobinamide in 25 mM sodium phosphate buffer that produced beautiful spectra that had very high sensitivity. The next step was to trap the hydrogen sulfide directly in cobinamide in phosphate buffer, but this yielded no results, even when shaken and incubated at 37°C overnight. When this didn’t work, NaSH standards were measured in cobinamide that was diluted into 10 mM, 1 mM, and 100 μM NaOH. The 10 mM and 1 mM NaOH did not yield spectra, and although the 100 μM NaOH gave a spectrum, it was not as well defined and the absorption was not as great as the spectra from cobinamide in phosphate buffer. When attempting to trap the hydrogen sulfide in NaOH in Conway cells, there appeared to be full trapping in 100 mM and 10 mM NaOH, where the pH was high. To bring the pH to 7, NaOH would need to be combined with a higher concentration phosphate buffer at a percentage of 90% NaOH and 10% phosphate buffer, in order to keep the sulfide, which would be trapped in the NaOH, as concentrated as possible. The optimal solution was a mixture of 90% 10 mM NaOH and 10% 200 mM sodium phosphate buffer at a pH 4. When tested for its ability to measure NaSH, although not as sensitive as the cobinamide in phosphate buffer, the 90% NaOH, 10% phosphate buffer gave a predicted but less dynamic spectrum. When this mixture was tested in the Conway cells, the assay worked as it should and produced spectra similar to the standards done in just the cuvette.
The research presented here lays the foundation for further research to be done in order to develop a field-deployable method to measure hydrogen sulfide. The next step would be to test this method with biological samples, such as with blood that has been spiked with sodium hydrosulfide hydrate and blood from animals that have been poisoned with hydrogen sulfide. Ultimately, this could lead to an antidote for sulfide poisoning. In mice, hydroxocobalamin has been shown to be an effective antidote against hydrogen sulfide poisoning (Truong et al., 2007). As cobinamide is a precursor of cobalamin with a higher binding affinity for ligands, it seems possible that cobinamide would be able to treat H₂S poisoning just as effectively as, or better than, hydroxocobalamin.
REFERENCES


Supplemental Figure 1: Spectrum of cobinamide, with labeled $\alpha$, $\beta$, and $\gamma$ peaks

Supplemental Figure 2: Diagram of a Conway cell
Supplemental Figure 3: Spectra of baseline samples of cobinamide