UNIVERSITY OF CALIFORNIA, SAN DIEGO

Novel Methods of Measuring Nitric Oxide and Nitrite Concentrations Using Cobinamide and Cobalamin

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

in

Biology

by

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Professor Gen Sheng Feng

2012

The Thesis of Kailin Catherine Duan is approved, and it is accepted in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego
2012

DEDICATION

I dedicate my thesis to my family.

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ABSTRACT OF THE THESIS

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Professor Gerard R. Boss, Chair
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Nitric oxide (NO) is an important signaling molecule produced by isoforms of nitric oxide synthase in mammals. Methods of measuring NO must take into consideration the low concentrations (nanomolar to micromolar) at which it is found in the body. We developed a novel method of direct nitric oxide measurement by measuring the absorbance change of the binding of nitric oxide to cobinamide(II) (Cbi), a vitamin B_{12} analogue. The absorbance values of NO-Cbi change linearly at 366 nm and 469 nm as increasing amounts of NO are added. The K_a of nitric oxide binding to Cbi was found to be $5.10 \times 10^8 \ \frac{1}{M}$. The LOD and LOQ of the Cbi method performed in water were measured at 1.98 μM and 4.39 μM, respectively. Because this method was only viable in anaerobic systems, we used cobalamin(III) (Cbl) to measure nitrite concentrations. At 375 nm, the absorbance values of NO_2-Cbl increase linearly as nitrite concentration

increases. The K_a of this method was measured at $7.23 \times 10^7 \frac{1}{M}$, and the LOD and LOQ in water were measured at 0.672 μM and 3.91 μM, respectively. Compared to the Griess reagent assay, the Cbl(III) method was less sensitive. Improvements upon both the Cbi(II) method and the Cbl(III) method are required for their potential use as cost-effective, sensitive, and easy methods of determining nitric oxide or nitrite concentrations in cell culture. However, the Cbl(III) method may be a viable method of taking nitrite and nitrate measurements in biological samples.

Introduction

Nitric oxide (NO) is a small gas molecule that has many important biological functions. Ignarro, Murad, and Furchgott won the Nobel Prize in Physiology or Medicine in 1998 for discovering the signaling properties of NO in the cardiovascular system. Since then, a multitude of physiological and pathophysiological functions of NO have been elucidated. In the vasculature, NO serves as a potent vasodilator of smooth muscle cells by activating soluble guanylyl cyclase and increasing the concentration of 3,5-cyclic guanosine monophosphate (cGMP) (1). Nitrergic neurons in both the peripheral and central nervous system generate NO as a neurotransmitter, which has effects such as the modulation of neurotransmitter release and regulation of long-term potentiation in the hippocampus (2, 3). Abnormal NO levels have also been implicated in several disease states, including bacterial sepsis, cerebral thrombotic disorder, and hypertension (4). The importance of NO in regulating many different cellular functions has also given rise to research into NO-releasing compounds, such as nanoparticles and polymers, as possible therapeutic agents or tools in studying its effects *in vitro* (5).

Given NO's significance in biological systems and the use of NO-releasing compounds in medicine and research, it is important to have a reliable, quick, and sensitive method for its measurement. Physiological concentrations of NO are dependent on the three different nitric oxide synthase (NOS) isoforms. Endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutively active and generate concentrations of NO in the low nanomolar range. Inducible NOS (iNOS) is active during inflammation or infection and can generate large quantities of NO in the micromolar range. As NO's role in the body varies widely with its concentration, it is important to determine its

concentration in an efficient manner. For example, NO has anti-apoptotic functions at a concentration of 1-30 nM but at concentrations above 1 μM, it inactivates mitochondrial respiration and activates caspase (6).

Though NO is an important signaling molecule, its short half life makes it difficult to study. Under physiological conditions, NO has a half life that can range from 1-30 seconds, depending on its concentration and the presence of hemoglobin (7). Such a short half life is due to NO's high reactivity with oxygen and reactive oxygen species (ROS). NO reacts with oxygen to form NO_2^- (nitrite) which can further be oxidized to form NO_3^- (nitrate). NO can also react with superoxide to form $ONOO^-$ (peroxynitrite), a reactive nitrogen species (RNS) that reacts with nucleophiles to change structures of proteins and other molecules (8). NO's short half life poses a challenge to developing an accurate method for its measurement.

Current popular methods of measurement include the Griess assay, chemiluminescence, and microelectrodes. All of these methods have advantages and disadvantages. The Griess assay is the most widely used method of measurement of NO. It indirectly measures NO concentrations *in vitro* by converting nitrate in solution to nitrite and measuring the total amount of nitrite spectroscopically via the reaction of nitrite with sulfanilamide and *N*-1-napthylethylenediamine. Nitrite reacts with sulfanilamide to form a diazonium salt which reacts with *N*-1-napthylethylenediamine to generate an azo dye, the concentration of which can be quantified by measuring absorbance at 540 nm. Using Active Motif's Griess assay kit, we measured the limit of detection of the assay to be 120 nM in a solution of molecular biology grade water and

149 nM in EBSS with 1% FBS. The usefulness of the method is hampered by the long reaction times required and relatively expensive materials.

Chemiluminscence is a direct method of measuring NO concentrations. NO reacts with O_3 (ozone) to generate NO_2 (nitrogen dioxide), O_2, and light.

$$NO + O_3 \rightarrow NO_2^* + O_2 \qquad (1)$$

$$NO_2^* + O_3 \rightarrow NO_2 + h\nu \qquad (2)$$

The light released by the excited NO_2 molecules can be detected in the red region of the visible spectrum using a photomultiplier tube (9). Chemiluminescence can measure NO concentrations down to 30 nM. However, its sensitivity is highly dependent on maintaining NO levels before the reaction with O_3 occurs. Given NO's short half life, it is possible to lose NO and consequently underestimate its concentration. In addition, the presence of gases that absorb emissions from the excited NO_2 molecules may hamper the measurement. Finally, this method is more expensive and requires more technical expertise to perform than the Griess reagent assay.

Another direct method of measurement of NO uses electrodes. Microelectrodes specific for NO can be directly inserted into a fluid and measure in real time the amount of NO produced. This method is extremely sensitive and is able to quantify NO at very low picomolar concentrations (10). The major drawback to using microelectrodes is their cost, as the price can range up to thousands of dollars.

Given the pros and cons of currently available methods of NO measurement, we aimed to develop a cheap, quick, and sensitive method of measuring NO. Certain vitamin B_{12} analogues have high binding affinity for NO and nitrite and can be quickly and easily

used to determine NO or nitrite concentrations in solution via spectroscopy. We focused on two specific vitamin B_{12} analogues, cobalamin and cobinamide, shown in Figure 1.

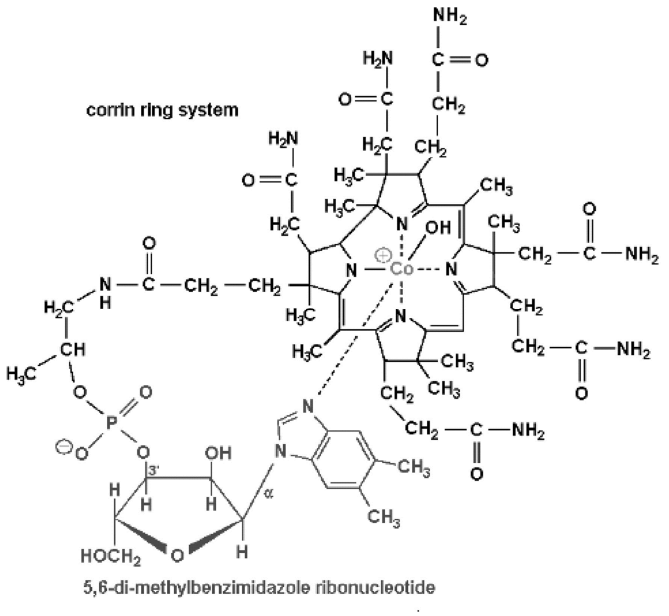

Figure 1: Cobalamin is shown bound to a hydroxyl group. Omission of the grey DBZ group yields cobinamide, a cobalamin precursor that can bind two ligands instead of only one (11).

Cobalamin (Cbl), or Vitamin B_{12}, contains a cobalt ion, either in the oxidized (III) or reduced state (II), surrounded by a corrin ring. Of the six binding sites available on the cobalt ion, four are occupied by the corrin ring, and a fifth is occupied by a dimethylbenzimidazole (DBZ) tail, leaving one site open for ligand binding. In its oxidized form, Cbl can take on nitrite as a ligand, forming nitrito-Cbl (NO_2-Cbl). The binding of nitrite shifts the absorption spectra of Cbl(III), especially at 375 nm. We measured the K_a of Cbl(III) for nitrite to be $7.23 \times 10^7 \frac{1}{\mathrm{M}}$. The sensitivity of Cbl(III) for nitrite in non-physiological and *in vitro* samples was tested by quantifying nitrite concentrations via spectroscopy. The limit of detection of Cbl(III) for nitrite in molecular biology grade water was 0.672 μM. In Earle's Balanced Salt Solution (EBSS) with 0.1%

BSA, it was found to be 3.91 μM. Attempting to measure nitrite concentrations in PAC1 cells stimulated by calcium ionophore A23187 did not produce quantifiable results.

Cobinamide (Cbi) is another vitamin B_{12} highly similar to Cbl. Cobinamide has essentially the same structure as Cbl except that it lacks Cbl's DBZ tail. The omission of the tail allows Cbi to bind to two ligands, suggesting that it may be more sensitive than Cbl when measuring the same compounds. Although Cbi also binds nitrite, its propensity to be reduced limits its effectiveness as a method of quantifying nitrite concentrations. Nitrito-Cbi (NO_2-Cbi) was found to degrade slowly over time, likely due to the oxidation of nitrite. Adding sodium ascorbate to slow the oxidation of nitrite did not work, as Cbi(III) is too readily reduced by even low levels of a reducing agent. However, Cbi binds NO very well in its reduced form. NO-Cbi can also be quantified spectroscopically and exhibits large changes in absorption from Cbi(II), especially at 366 nm and 469 nm. The sensitivity of Cbi(II) for NO was measured in a non-physiological system in deoxygenated glass-distilled H_2O. The limit of detection of Cbi(II) for NO was determined to be 1.98 μM and the K_a of Cbi(II) binding to NO was found to be $5.10 \times 10^8 \frac{1}{\mathrm{M}}$. We were unable to apply this method to an *in vivo* system, due to the oxidation of Cbi(II) to Cbi(III) upon contact with oxygen. However, if a protocol can be developed that would allow Cbi(II) to remain reduced in an *in vivo* setting, it could lead to a promising novel method of NO measurement alongside Cbl(III).

Materials and Methods

Cobinamide and Cobalamin. Hydroxyaminocobinamide (Girindus) was converted into aquohydroxycobinamine before use by the removal of the ammonia group via desalting. Hydroxyaminocobinamide was dissolved in glass-distilled H_2O and its pH was brought down to 3.5 using 6 N HCl. The resulting solution was eluted through an mixed bed resin ion exchange column with a 1000 molecular weight limit (Biorad), producing aquohydroxycobinamide. The final pH of aquohydroxycobinamide was adjusted to 4.5 using 6 N HCl. A spectral scan was done at 300-600 nm to determine the purity and concentration of the final solution, which was calculated based on the molar extinction coefficient of aquohydroxycobinamide at 349 nm (ε=28,000$\frac{1}{\mathrm{M \cdot cm}}$). The solution was stored at 4ºC.

Hydroxycobalamin acetate was obtained from Molekula. It was dissolved in glass-distilled H_2O and its pH was reduced to 4.6 using 6 N HCl. The solution was stored at 4ºC.

Reduction of Cbi was done with sodium L-ascorbate (Sigma Aldrich). Reduction of Cbi(III) to Cbi(II) was achieved by adding 1.5x sodium ascorbate to the Cbi(III) solution. In addition, 10 μM sodium ascorbate was used to keep nitrite reduced in solutions containing Cbl(III). Spectrophotometric scans were done on a Uvikon 860 spectrophotometer or a Uvikon 943 spectrophotometer. A Biotek Synergy 2 plate reader was used to read 96 well plates.

Nitric oxide and NO-Cbi. NO solutions were prepared by passing NO gas through glass-distilled H_2O. Glass-distilled H_2O was deoxygenated by passing through argon gas (Airgas) until the solution was fully saturated with argon. NO gas was passed through a

solution of 10 M KOH for purification (Praxair, 99.5% pure). It was then passed through the deoxygenated solution of glass-distilled H_2O until the solution was fully saturated with 1.946 mM NO. The NO solution was injected into rubber septa-sealed, deoxygenated cuvettes (Precision Cells) containing 25 μM Cbi(II) using Hamilton gas-tight syringes. Scans of NO-Cbi were done at 366 nm and 469 nm on a Uvikon 943 spectrophotomer (Kontron Instruments).

Nitrite and nitrito-Cbl. Sodium nitrite was dissolved in molecular biology grade H_2O (Sigma). Samples of 25 μM Cbl(III) with 10 μM sodium ascorbate were prepared in molecular biology grade H_2O. Sodium nitrite was added to the Cbl(II) samples and the resulting solutions were measured at 375 nm on the spectrophotometer or plate reader.

Griess reagent assay. Nitric oxide kit was obtained from Active Motif. The assay was performed as per the manufacturer's instructions. Briefly, nitrite standards were prepared alongside samples in a 96 well plate. Nitrate reductase and its cofactors were added and the plate was placed in darkness for 30 minutes. Griess reagents A and B were added to the samples and after 20 minutes, the 96 well plate was scanned at 540 nm on the plate reader.

PAC1 cell line stimulation. PAC1, a cell line of rat vascular smooth muscle cells, was grown in p100 plates in media containing DMEM and 10% fetal bovine serum (Cellgro). Cells were split every other day. At passage 15, the cells were incubated for 30 minutes with 2.5 mL Earle's Balanced Salt Solution (Invitrogen) with 0.1% BSA, 200 mg/L $CaCl_2$ (Sigma), 5 mM dextrose anhydrous, and 10 μM sodium ascorbate. To stimulate eNOS, 3 μM A23187 was added to the media and cells were incubated for

another 30 minutes (Sigma). Media was extracted after incubation and tested for nitrite using either the cobalamin method or nitric oxide kit.

Results

Nitric oxide measurement using Cbi(II). Increasing amounts of saturated NO (1.946 mM) were added to deoxygenated 25 μM Cbi(II) to generate a standard curve. The absorbance spectrum of Cbi(II) compared to the spectrum of NO-Cbi is shown in Figure 2. It has been previously reported that only one NO molecule binds per Cbi(II), which is consistent with our data.

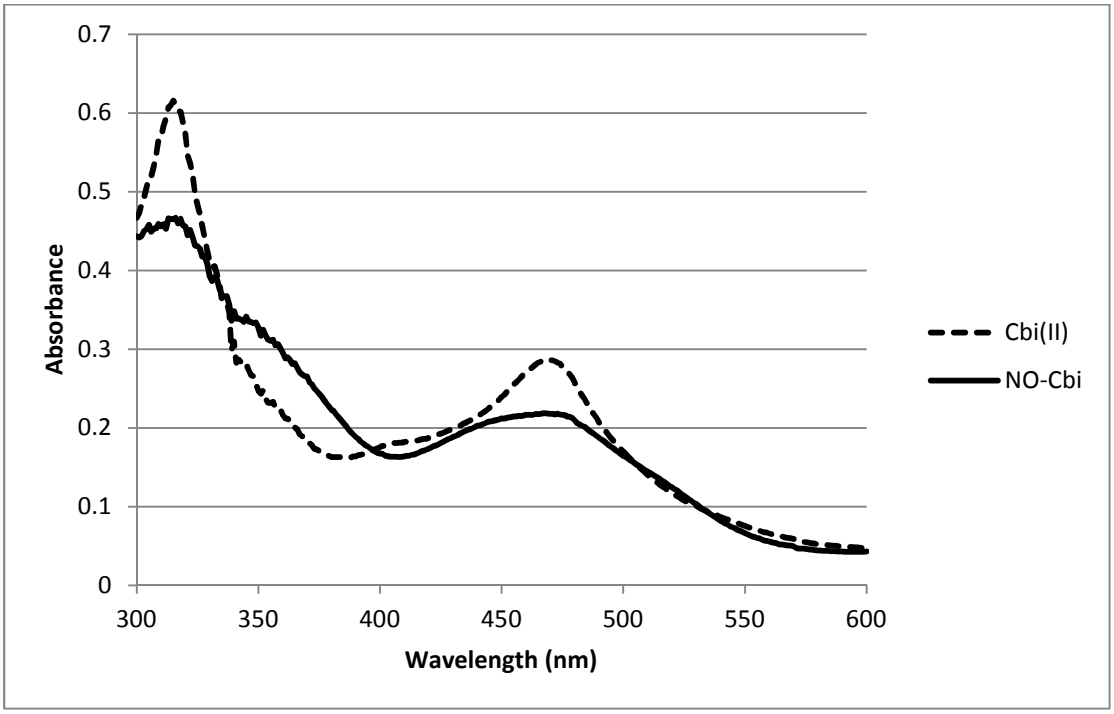

Figure 2. Absorbance spectrum of 25 μM Cbi(II) compared to saturated NO-Cbi.

The largest changes in absorbance values between baseline Cbi(II) and saturated NO-Cbi occur at 315, 366, and 469 nm. We analyzed the absorbance values at 366 nm and 469 nm for this report.

The limit of detection was determined according to the following equation, depending on whether the absorbance at the given wavelength increased or decreased.

$$\text{LOD} = \text{average} + 3 \cdot \text{standard deviation} \quad (3)$$

$$\text{LOD} = \text{average} - 3 \cdot \text{standard deviation} \quad (4)$$

The limit of quantification was determined according to the following equations, depending on whether the absorbance at the given wavelength increased or decreased.

$$\text{LOQ} = \text{average} + 10 \cdot \text{standard deviation} \quad (5)$$

$$\text{LOQ} = \text{average} - 10 \cdot \text{standard deviation} \quad (6)$$

Six baseline samples were used to determine the average and standard deviation. Absorbance values at 366 nm, 469 nm, and the ratio of the absorbance values at 366 nm to 469 nm were used to determine the limit of detection. All absorbance values increased or decreased linearly as the concentration of nitric oxide increased. Table 1 shows the LOD and LOQ of the different methods of analysis, of which the ratio method has the lowest LOQ.

Table 1. LOD and LOQ of the Cbi(II) method of detecting NO at different wavelengths and wavelength ratio. The ratios of absorbance values at 366 nm and 469 nm give the lowest LOD and LOQ.

	366 nm (μM)	**469 nm (μM)**	**366:469 (μM)**
LOD	2.50	1.11	1.98
LOQ	9.12	6.12	4.39

The K_a was determined by plotting the ratio of the change of absorbance as NO was added to Cbi(II) until the change became negligible. For a ratio of the change of absorbance at 366 nm to 469 nm, the K_a was calculated to be 5.10×10^8 1/M. The total amount of NO added to a 25 μM sample of Cbi(II) was 50 μM. Figure 3 depicts the change of absorbance as NO was added to 25 μM Cbi(II) versus [NO]. Figure 4 is the Lineweaver-Burk plot of the data from Figure 3. A best-fit line was generated from the

linearized graph that allows the calculation of K_a. K_a is defined according to the following equation, where *x* is the x-axis intercept of the graph in Figure 4.

$$K_a = \log_{10} -\frac{1}{x} \qquad (7)$$

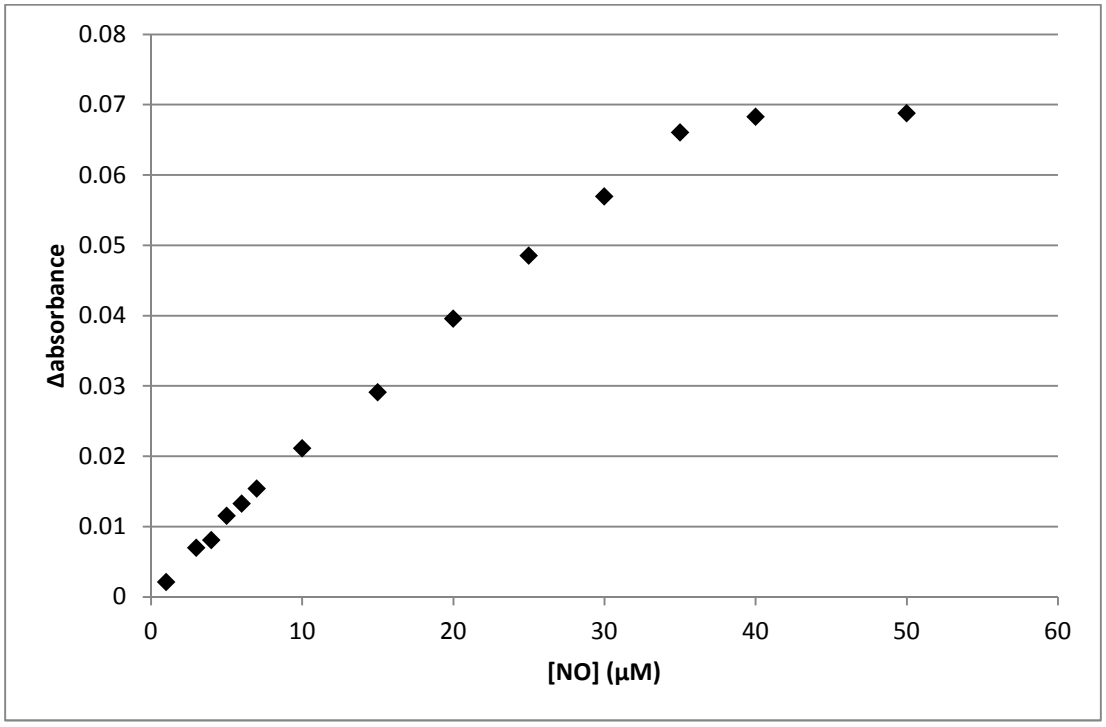

Figure 3. Change in absorbance as NO binds to Cbi(II) versus the concentration of NO added to 25 μM Cbi(II). Absorbance was measured at 469 nm.

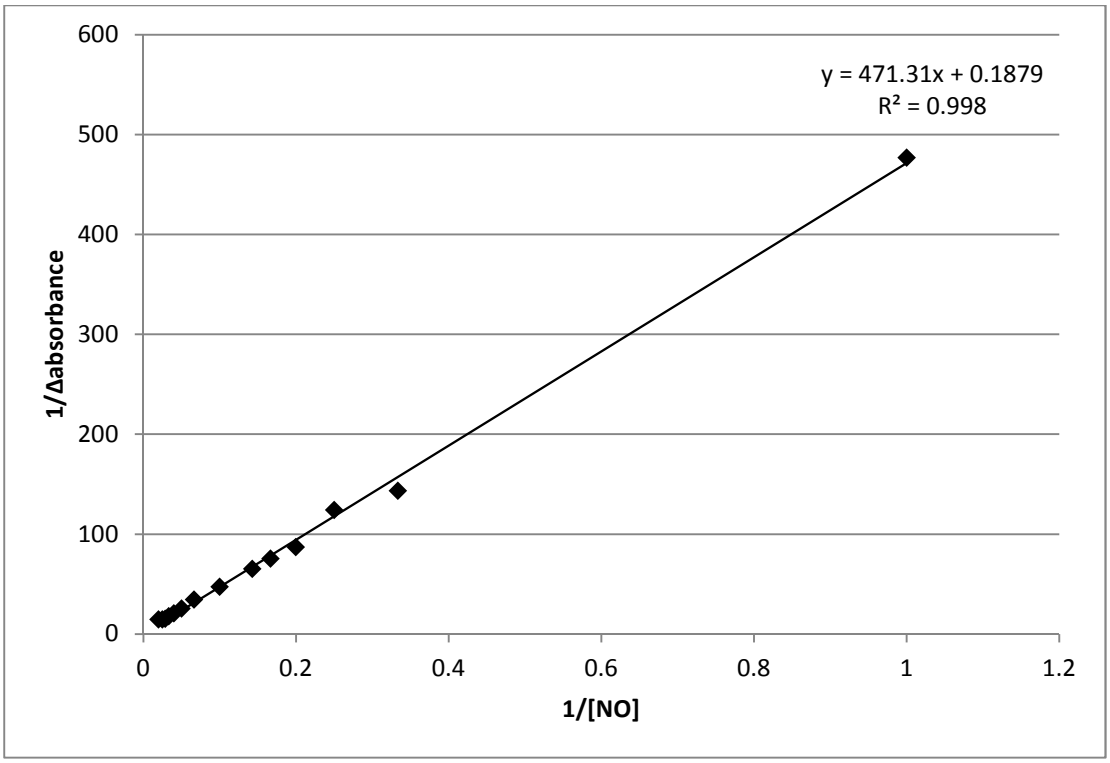

Figure 4. Lineweaver-Burk plot of the data from Figure 3. K_a was calculated using the *x*-intercept.

Nitrite measurement using Cbi(III). To measure nitrite, we first tried to use Cbi(III). One equivalent of Cbi(III) can bind to two equivalents of nitrite. Figure 5 shows the absorbance spectra of Cbi(III) and NO_2-Cbi.

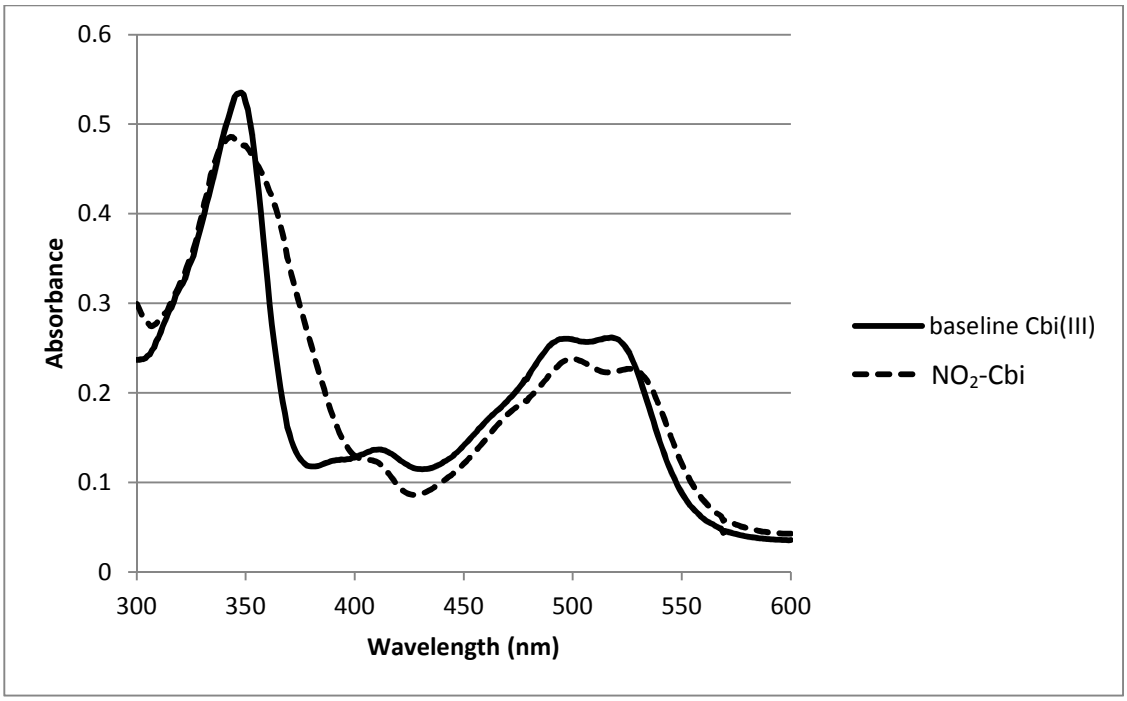

Figure 5. Absorption spectra of 25 µM baseline Cbi(III) and 25 µM NO_2-Cbi.

Absorbance values increased linearly at 375 nm as the concentration of nitrite in solution increased. Although Cbi(III) binds nitrite in solution at high affinity, we found that it is unable to remain bound to nitrite over time. To test the stability of NO_2-Cbi, 6 µM $NaNO_2$ was added to 25 µM Cbi(III). The absorbance of the sample at 375 nm was read on a plate reader over a period of 90 minutes. Figure 6 depicts the gradual decrease in absorbance for NO_2-Cbi as time passes as compared to the decrease in absorbance for only Cbi(II).

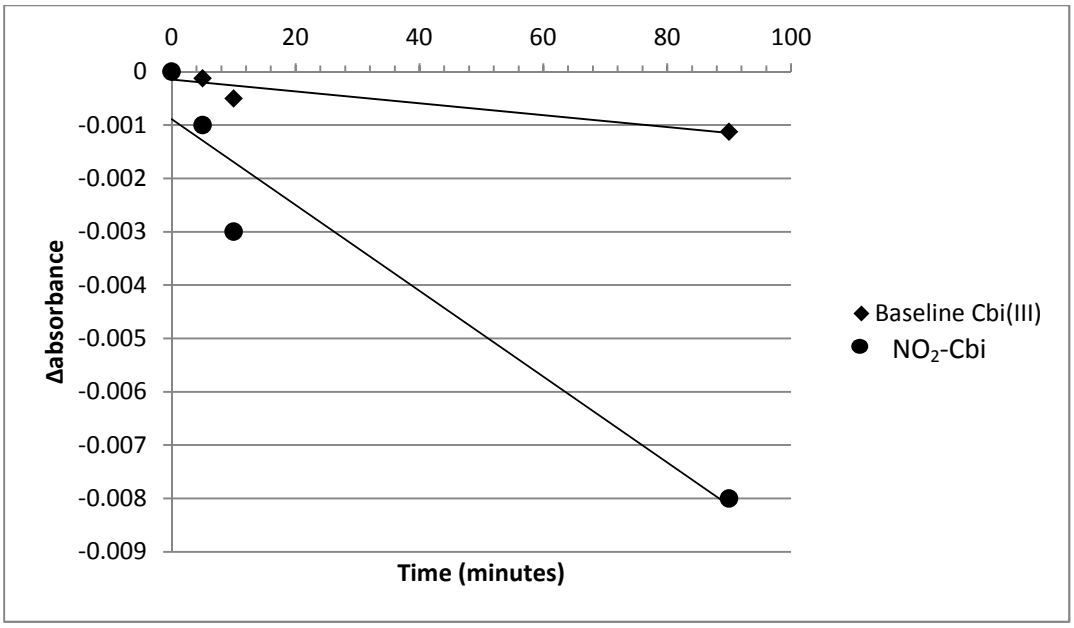

Figure 6. Change in absorbance of baseline Cbi(III) and NO_2-Cbi over a period of 90 minutes.

Because the decrease in absorbance of NO_2-Cbi is likely due to the loss of nitrite by oxidation, sodium ascorbate was added to the solution as a reducing agent. However, addition of sodium ascorbate as low as 10 μM to Cbi(III) caused reduction of Cbi(III) to Cbi(II). Cbi(II) does not bind to nitrite and can also be easily oxidized back to Cbi(III) in the presence of oxygen, rendering this method of measuring nitrite unusable due to the instability of NO_2-Cbi.

Nitrite measurement using Cbl(III). A solution of 25 μM Cbl(III) was made up in molecular biology grade water or EBSS with 200 mg/L $CaCl_2$, 10 μM sodium ascorbate, and 5 mM glucose. 0.1% FBS, 1.0% FBS, or 0.1% BSA was added to the EBSS. Figure 7 shows the absorbance spectra of Cbl(III) compared to NO_2-Cbi in water. Large absorbance changes between baseline Cbl(III) and NO_2-Cbl are present at 310 nm, 375 nm, and 469 nm. The wavelength we chose to focus on was 375 nm, due to the possibility of proteins affecting the absorbance values in the low 300 nm region.

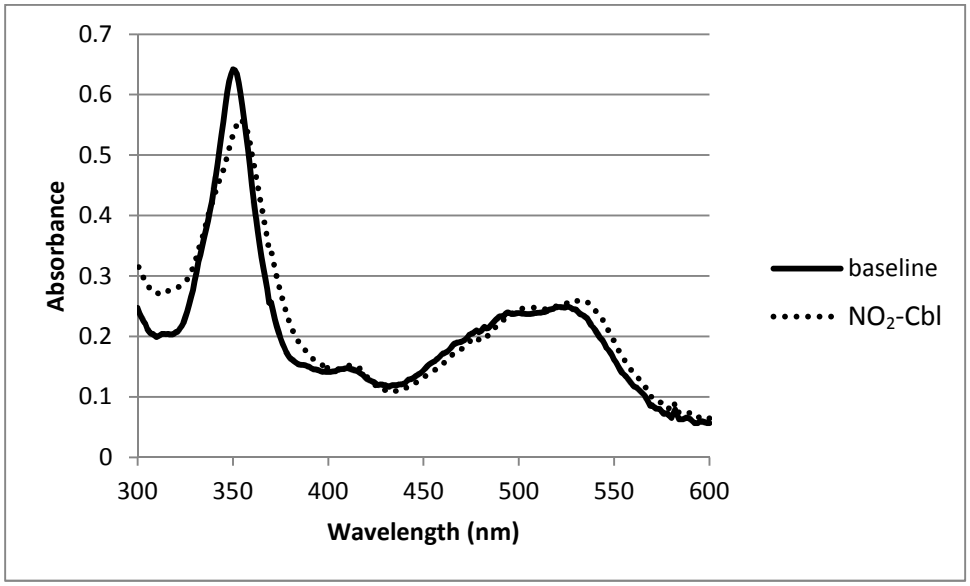

Figure 7: Absorbance spectra of 25 μM Cbl(III) compared to 25 μM NO_2-Cbl. Largest changes in absorbance values can be seen at 310 nm, 375 nm, and 469 nm.

The LOD and LOQ of the Cbl(III) method in the different solutions were determined based on equations (3) and (4); the results are summarized in Table 2. 6 baseline samples were used to determined the LOD and LOQ for each different condition and a standard curve was generated from the addition of nitrite to the Cbl(III) samples. A wavelength of 375 nm, at which the absorbance values increased linearly as the concentration of nitrite added increased, was used to measure absorbance on the plate reader. The limit of detection of Cbl(III) for nitrite in molecular biology grade water was determined to be 0.672 μM and the limit of quantification was found to be 3.91 μM.

The K_a of NO_2^- binding to Cbl(III) was found to be $7.23 \times 10^7 \frac{1}{\mathrm{M}}$ using equation 5. The binding curve and the Lineweaver-Burk plot are shown in Figures 8 and 9.

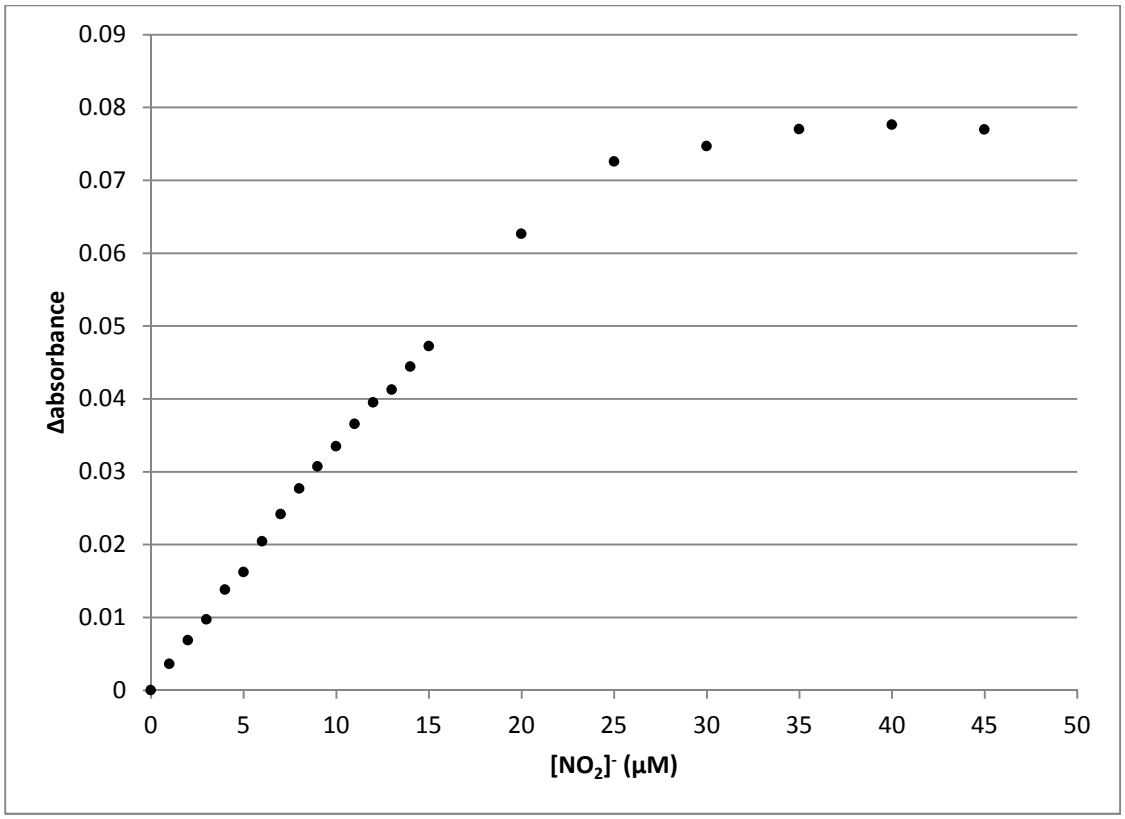

Figure 8. Change in absorbance of NO_2-Cbl as Cbl(III) is titrated with nitrite, from a concentration of 1 µM up to 45 µM.

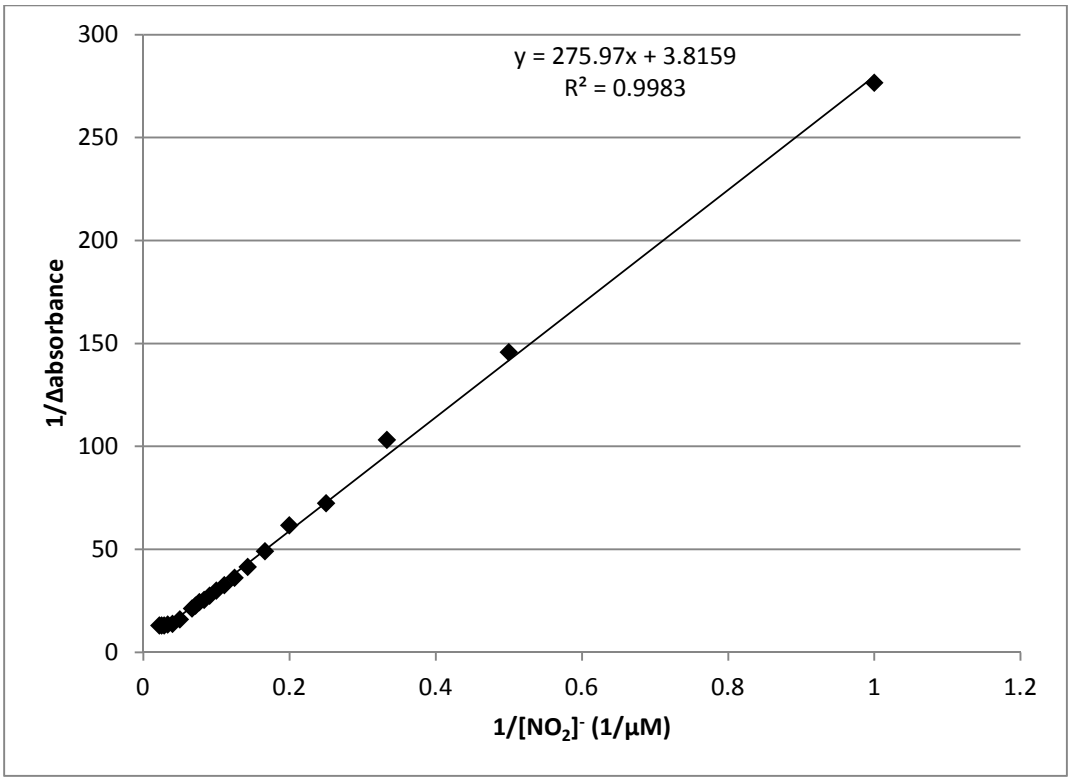

Figure 9. Lineweaver-Burk plot of the data in Figure 6. K_a was calculated using the *x*-intercept.

To test the potential use of Cbl(III) in measuring nitrite *in vitro,* we tested the method in various solutions of EBSS. However, when the Cbl(III) method was used in EBSS with 1% FBS, the limit of detection rose to 4.98 μM and the limit of quantification rose to 16.1 μM. The decrease in sensitivity can likely be attributed to the binding of Cbl(III) to proteins present in FBS. The concentration of FBS in EBSS was decreased to 0.1% to see if the sensitivity could be raised. The limit of detection decreased to 3.88 μM and the limit of quantification to 11.3 μM. However, compared to the LOD and LOQ of the Griess reagent assay, the Cbl(III) method in EBSS with FBS is not nearly as sensitive.

In order to decrease the binding of Cbl(III) to the serum proteins present in FBS, we measured nitrite in EBSS with 0.1% BSA. Under these conditions, the LOD was 2.95 μM and the LOQ was 10.6 μM, suggesting that there was decreased binding of Cbl(III)

in BSA. Nevertheless, the elevated LOD and LOQ compared to when the measurements were done in water indicates that Cbl(III) is still binding to albumin and its sensitivity is lowered in this manner.

Table 2: LOD and LOQ of Cbl(III) binding to nitrite under different conditions. 6 baseline samples were used in each condition to determine LOD and LOQ.

	Water (μM)	**EBSS + 1% FBS (μM)**	**EBSS + 0.1% FBS (μM)**	**EBSS + 0.1% BSA (μM)**
LOD	0.672	4.98	3.88	2.95
LOQ	3.91	16.1	11.3	10.6

Nitrite measurement using Griess reagent assay. The Griess reagent assay was performed in molecular biology grade water and EBSS with 200 mg/L $CaCl_2$, 10 μM sodium ascorbate, 5 mM glucose, and 0.1% FBS. In water, the Griess reagent assay had a LOD of 0.126 μM and a LOQ of 0.430 μM. In 0.1% FBS in EBSS, the Griess reagent assay had a LOD of 0.167 μM and a LOQ of 1.15 μM. The LOD and LOQ are summarized in Table 3. The difference between the LOD and LOQ for the assay in water versus EBSS and FBS is negligible, suggesting that the Griess reagent assay has high specificity for nitrite in solution and does not interact with the serum proteins.

Table 3. LOD and LOQ of the Griess reagent assay in water and EBSS with 0.1% FBS.

	Water (μM)	**EBSS + 0.1% FBS (μM)**
LOD	0.126	0.167
LOQ	0.430	1.15

Nitrite measurements in PAC1 cells. PAC1 cells were grown in p100 plates with DMEM and 10% FBS. At passage 15, they were incubated in EBSS with 200 mg/L $CaCl_2$, 10 μM sodium ascorbate, 5 mM glucose, and 0.1% BSA. 25 μM Cbl(III) was

added to media in cells that would undergo the Cbl(III) method. A23187 was added to the media to induce nitric oxide production via eNOS stimulation. Media was harvested at 30 minutes and nitrite concentrations were determined with the Cbl(III) method and the Griess reagent assay.

This experiment was repeated twice under the same conditions. The Cbl(III) method showed no consistent shift toward an absorbance spectra for nitrito-Cbl when compared to that of baseline Cbl(III) samples. In addition, when the media of cells stimulated with A23187 underwent the Griess reagent assay, absorbance values of samples from cells stimulated with A23187 were no different from baseline, indicating that no nitrite could be detected.

Discussion

Though we hoped to develop a method that would be as sensitive as the Griess reagent assay in measuring nitrites *in vitro*, our data shows that Cbl(III) binding to nitrite does not produce enough of an absorbance change to be useful in measuring nitrite in the sub-micromolar range. In addition, the use of Cbi(II) in measuring nitric oxide is also difficult to apply *in vivo* because of Cbi(II)'s propensity to oxidation in under physiological systems. However, while the Cbl(III) method may not be able to compete with the sensitivity of the Griess reagent, it may be a very useful tool in determining the total concentration of NO_x in biological samples such as plasma.

Cbi(II) was able to bind NO with a high K_a of $5.10 \times 10^8 \frac{1}{\mathrm{M}}$ in fully deoxygenated glass-distilled water. Under these conditions, the LOD and LOQ were 1.98 μM and 4.39 μM for the ratio method. Although this sensitivity is not as low as other direct methods of NO measurement such as chemiluminescence and microelectrodes, it is much cheaper and requires less technical expertise to perform. The reaction of Cbi(II) and NO has only a simple ligand-binding step and can be easily quantified with spectrophotometry. Unpublished data from our lab shows that NO-Cbi is very stable in solution under argon; very little change in the absorbance spectrum is seen even after a week in solution. In addition, Cbi has been shown to be non-toxic in cells up to 50 μM, when it starts to interfere with the function of cobalamin (11). The change in absorbance as NO binds to Cbi(II) is also linear up to the saturation of Cbi(II).

The main drawback of the Cbi(II) method is that we were unable to keep Cbi(II) reduced under physiological conditions. Even a small amount of oxygen will oxidize Cbi(II) to Cbi(III). Presence of a reducing agent even in extremely high concentrations,

such as 400x sodium ascorbate, does not completely negate the effects of oxygen. The oxidation of Cbi(II) to Cbi(III) is a gradual process and renders the sensitivity of the method null, as the spectra of Cbi(II) and Cbi(III) are markedly different. Thus, Cbi(II) is a reliable method of measuring NO in anaerobic systems but not in physiological systems. Future studies regarding the use of Cbi(II) as a method of measuring NO produced *in vivo* will require a method of keeping Cbi(II) reliably reduced in the presence of oxygen.

Although the binding affinity of Cbi(III) for nitrite is higher than that of Cbl(III), nitrite can be oxidized to nitrate in the presence of oxygen. Scans of the same sample of 6 μM NO_2-Cbi over a period of 90 minutes showed a steady shift back to the absorbance spectra of Cbi only, indicating that the nitrite in the sample was being eliminated somehow. Given that the half life of nitrite *in vivo* has been reported to be 110 seconds, it is important to have a method of measuring nitrite that can quickly bind it and remain in solution for a long time (12). To reduce the probability that nitrite would be oxidized to nitrate, we added varying amounts of sodium ascorbate to Cbi(III), but could not simultaneously prevent the oxidation of nitrite and prevent the reduction of Cbi(III) to Cbi(II), which does not bind nitrite. Thus, we turned to Cbl(III) as an alternative method of measuring nitrite. Cbl(III) is not as readily reduced with sodium ascorbate. Samples of 6 μM NO_2-Cbl were measured over the course of an hour showed no change in absorbance spectra (data not shown), indicating that nitrite remained bound to Cbl(III). Although Cbl(III) has a slightly lower binding affinity for nitrite than Cbi(III) due to the presence of the DBZ group, it remains a better option in measuring nitrite concentrations.

Compared to the Griess reagent assay, the Cbl(III) method of measuring nitrite is lacking in sensitivity. We measured the LOQ of the Griess reagent assay to be 0.430 μM

in molecular biology grade water, which is similar to previously reported sensitivities of around 0.5 μM (13). The LOQ of the Cbl(III) method in water was measured to be 3.91 μM, higher than that of the Griess reagent assay. However, when compared to the Griess reagent assay, the Cbl(III) method requires much less time and preparation to use. Cbl(III) binding to nitrite occurs within seconds and requires no additional reactions upon the addition of nitrite to a sample of Cbl(III). Thus, in non-physiological systems that don't require sensitivity in the nanomolar range, the Cbl(III) method may have an advantage over the popular Griess reagent assay.

However, when we compare the Griess reagent assay to the Cbl(III) method in media that would be typical for use in *in vivo* studies, the relative sensitivity of Cbl(III) for nitrite drops considerably. Cbl(III) binds other ligands than nitrite, including serum proteins, which limits its use in measuring nitrite levels *in vivo*. The media in which we chose to test the two methods was EBSS with added calcium chloride, sodium ascorbate, glucose, and FBS or BSA. The Griess reagent assay maintained its sensitivity in this solution and its LOD and LOQ did not change too much compared to when measured in water. However, we were unable to maintain a satisfactory LOD and LOQ for Cbl(III) under these conditions because of the unavoidable binding of Cbl(III) to the serum proteins. Lowering the concentration of FBS or BSA did not improve the sensitivity of Cbl(III) by much and further decrease in the amount of FBS or BSA would make the media non-physiological and inhospitable for cell culture. Therefore, the use of the Cbl(III) method for measuring nitrite over the use of the Griess reagent assay seems to be justified only in studies where other potential ligands or binding agents of Cbl(III) are not

present; that is, the Griess reagent is likely a better choice to indirectly measure nitric oxide in cells.

In addition, the use of Cbl(III) to measure nitrite may be a viable method of quantifying nitrite and nitrate concentrations in biological samples. Whereas the concentration of nitric oxide produced by cells is quite low, the total amount of nitrite and nitrate in the blood is much higher due to the longer half-life of both compounds compared to NO. In studies of patients with sepsis and septic shock, concentrations of NO_x in plasma was measured at 29.6 μM for the control group, and 94.5 μM for patients experiencing septic shock (14). At such high levels of NO_x, the decreased sensitivity of the Cbl(III) method compared to the Griess reagent assay is a moot point. The Cbl(III) method's ease of use could outweigh its decreased sensitivity in this case. Measurement of plasma nitrite levels of patients experiencing septic shock could easily be with a quick assay using the Cbl(III) method. In addition, if one could reduce the nitrate in plasma to nitrite in an efficient manner, the total amount of NO_x could be measured using the Cbl(III) method. Thus, whereas the sensitivity of the Cbl(III) method may be an issue when measuring nitrite in cells, the Cbl(III) method may be a viable alternative for the Griess reagent assay for biological samples, which tend to contain much higher levels of nitrite and nitrate.

When calcium ionophore A23187 was added to plates with PAC1 cells to stimulate eNOS production of NO, no nitrite could be detected by the Griess reagent assay and the Cbl(III) method. The complete lack of any detected nitrite by the more sensitive Griess reagent assay suggests that either the cells were not producing nitrite because they were not stimulated properly or that the amount of nitrite produced was

below the LOD for both methods of detection. The former is more likely to have occurred, given that nitric oxide produced by stimulated cells should be detectable. Future studies should determine how the stimulation of the cells can be improved or if the cell line used was deficient in eNOS expression.

In conclusion, the relative affordability and ease of use of the Cbi(II) method for nitric oxide measurement may be able to offset its lowered sensitivity in non-physiological measurements. However, unless there is a method to keep Cbi(II) reduced in a physiological system, Cbi(II) may not be able to measure nitric oxide concentrations *in vivo*. The Cbl(III) method is unable to rival the sensitivity of the Griess reagent assay due to Cbl(III)'s nondiscriminatory binding to various ligands present in media. Although this makes the Cbl(III) method difficult to use in cell culture, it could be valuable in measuring nitrite and nitrate concentrations in biological samples, where sensitivity is not as big of an issue.

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