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Journal
Analytical chemistry, 82(10)

ISSN
0003-2700

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Publication Date
2010-05-01

DOI
10.1021/ac100519z

Published in final edited form as:
Anal Chem. 2010 May 15; 82(10): 4216–4221. doi:10.1021/ac100519z.

A New Facile Method to Measure Cyanide in Blood

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Abstract

Cyanide, a well-known toxic substance that could be used as a weapon of mass destruction, is likely responsible for a substantial percentage of smoke inhalation deaths. The vitamin B_{12} precursor cobinamide binds cyanide with high affinity, changing color and, correspondingly, its spectrophotometric spectrum in the ultraviolet/visible light range. Based on these spectral changes, we developed a new facile method to measure cyanide in blood using cobinamide. The limit of detection was 0.25 nmol, while the limit of quantitation was ~ 0.5 nmol. The method was reliable, requires minimal equipment, and correlated well with a previously established method. Moreover, we adapted it for rapid qualitative assessment of cyanide concentration, which could be used in the field to identify cyanide-poisoned subjects for immediate treatment.

Introduction

Cyanide is a rapidly acting poison. It may be responsible for many of the 5,000–10,000 deaths due to smoke inhalation in the United States annually as it is released from the combustion of plastics, wool, and other nitrogen-containing materials [1–3]. Large amounts of cyanide are used in a variety of industries, and, since it is absorbed by multiple routes including inhalation, ingestion, and skin contact, it could be used as a terrorist weapon. Certainly, cyanide's use in murders, suicides, wars, and attempted genocide warrants concern [2].

Cyanide inactivates mitochondrial cytochrome c oxidase by binding to the enzyme's heme a_3-Cu_B binuclear center; this inhibits oxidative phosphorylation, and ATP production [4;5]. Furthermore, reactive oxygen species are increased from the ensuing backlog of the mitochondrial electron transport chain [6]. Rapid toxicity to the central nervous system and heart is likely responsible for cyanide's swift action.

A variety of methods exist for measuring cyanide in biological fluids including spectrophotometry, gas chromatography, fluorometry, gas chromatography-mass spectrometry, high performance liquid chromatography (HPLC), and HPLC-mass spectrometry [7–17]. Spectrophotometric assays can analyze multiple samples relatively quickly, but may lack sensitivity and specificity, while gas chromatography, mass spectrometry, and HPLC require expensive equipment and allow only limited sample throughput. None of these methods are amenable for use in the field.

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Cobinamide (Figure 1), a precursor in cobalamin biosynthesis, lacks cobalamin's dimethylbenzimidazole moiety. This adds a second free binding site, and ends the dimethylbenzimidazole group's steric hindrance on the *trans* site, leading to an overall cyanide binding affinity of 10^{22} M^{-2}(first cyanide molecule binds with an affinity of 10^{14} M^{-1}, and second one with an affinity of 10^{8} M^{-1}) [18]. We have developed a facile method to measure cyanide based on spectral changes that occur upon cyanide binding to cobinamide. We found that changes in single wavelengths, wavelength ratios, and wavelength range models accurately measured cyanide in rabbit blood samples. Furthermore, the qualitative color change was used to rapidly identify clinically relevant cyanide concentrations in blood.

Experimental Section

Cobinamide Synthesis

Cobinamide was synthesized from hydroxocobalamin acetate (Wockhardt, LTD, Mumbai, India) by base hydrolysis using cerium (III) hydroxide[19], produced by adding sodium hydroxide to cerium nitrate; in this reaction, the Ce^{+3} ion serves a catalytic function20. The cobinamide product was purified on a carboxymethyl cellulose cation exchange column (CM52, Whatman), and concentrated and de-salted on a reversed-phase C-18 column. Final concentration was performed by flash evaporation and lyophilization; the cobinamide product is highly stable and can be stored for months at 4°C. Purity of cobinamide preparations was evaluated by HPLC by converting all of the cobinamide to dicyanocobinamide, and analyzing the sample on a C-18 reversed-phase column eluted with a gradient from 20 mM potassium phosphate, pH 4.6 containing 0.2 mM KCN (solvent A) to 60% methanol/water (solvent B): one minute to 40% B, 11 min to 50% B, and 1 min to 100%B (flow rate 1 ml/min). The dicyanocobinamide eluted at 16 min and was detected by spectral absorption at 366 nm[21]. The concentration of cobinamide solutions was determined spectrophotometrically in 0.1 M HCl (as diaquocobinamide) using an extinction coefficient of 2.8×10^{4} /M/cm at 348 nm [22]. At neutral pH, cobinamide exists as aquohydroxocobinamide, and at a pH of > 11, it exists as dihydroxocobinamide [23].

Measurement of Cyanide Using a Standard Spectrophotometric Method

Cyanide was measured following the formation of *o*-nitrophenylhydroxylamine anion using *p*-nitrobenzaldehyde and *o*-dinitrobenzene as described by Guibault and Kramer 24 and modified by Gewitz et al.[25] (this method is subsequently referred to as the NBA/DNB method). Briefly, potassium cyanide (Sigma-Aldrich) was dissolved in 0.1 M NaOH, and placed in tubes sealed with Kontes center well-appended stoppers with the wells containing 250 µl of collection fluid (0.1 M NaOH). Trichloroacetic acid (10%, 250 µl) was injected through the stoppers' septa into the samples, and the tubes were shaken at 37°C for 60–75 min and then allowed to cool to room temperature. The released HCN was trapped in the collecting solution (pKa of HCN is 9.3). Freshly prepared reagent-grade *p*-nitrobenzaldehyde and *o*-dinitrobezene in 2-methoxyethanol (NBA/DNB method) were added to the collection fluid followed by measurement of absorbance at 560 nm 10 min later.

Measurement of Cyanide Using Cobinamide

On binding increasing amounts of cyanide, progressive changes occur in cobinamide's absorbance spectrum between 300 and 600 nm until cobinamide is converted to the fully saturated dicyano form (Figure 2). These spectral changes allowed us to develop a facile method for measuring cyanide by adding cobinamide to the center wells of the method described above. Cobinamide concentrations from 5 to 100 µM could be used to allow for a wide dynamic range of cyanide concentrations. At the end of the 37°C incubation, the 10 µM cobinamide-NaOH solution was analyzed spectrophotometrically over the range of 300–600 nm at 0.5 nm intervals using a Uvikon (Kontron 964) spectrophotometer.

Data Collection and MATLAB Program for Measuring Cyanide Using Cobinamide

A method was developed to analyze wavelength ranges of interest as follows. Anchoring spectra for cyanide-free dihydroxocobinamide (referred to as "A") and fully cyanide-bound dicyanocobinamide (referred to as "B") were obtained (Figure 2). Given an experimental spectrum, C(λ), a model spectrum, M(λ), was defined as the linear combination of spectra A (λ) and B(λ) for the wavelength range of interest:

$$M(\lambda)=aA(\lambda)+bB(\lambda) \qquad \text{(Equation 1)}$$

The coefficients a and b, sum to one. The coefficient b was solved whereby the sum of the squared differences of M(λ) from C(λ) was minimized for the wavelength range of interest. That is, the following expression was minimized:

$$\sum_{\lambda}(C(\lambda) - M(\lambda))^2$$

The following analytical solution for b (shown below in Equation 2) was utilized as part of a MATLAB program, whereby analysis of wavelength/absorbance datasets could be performed. For discrete wavelengths represented by index k, Equation 1 becomes the following:

$$M_k=(1 - b)A_k+bB_k=A_k+b(B_k - A_k)$$
$$\varepsilon=\sum_{k}(C_k - A_k - b[B_k - A_k])^2$$

Minimizing with respect to b:

$$\partial\varepsilon/\partial b=\sum_{k}(C_k - A_k - b[B_k - A_k])(B_k - A_k) \equiv 0$$
$$\Longleftrightarrow \sum_{k}(C_k - A_k)(B_k - A_k) - b\sum_{k}(B_k - A_k)(B_k - A_k)=0$$

$$\Longleftrightarrow b=\frac{\sum_{k}(C_k - A_k)(B_k - A_k)}{\sum_{k}(B_k - A_k)^2} \qquad \text{(Equation 2)}$$

Equation 2's solution of b provides the model-based absorbance "equivalent" for the range of interest, which, when plotted versus standard concentrations, may be used for concentration estimation in the same manner as for single wavelengths or wavelength ratios. Various single wavelengths, wavelength ratios, and wavelength ranges were evaluated. A MATLAB program was generated which simultaneously provided standard curves and cyanide measurements based on up to three range models, three wavelength ratios, and three single wavelength analyses.

Measurement of Cyanide in Rabbit Blood

Extra rabbit blood was obtained from ongoing studies conducted at the University of California, Irvine, according to NIH Guidelines for the Care and Use of Laboratory Animals, and approved by the Institutional Animal Care and Use Committee [26]. Briefly, New Zealand white rabbits

weighing ~ 4 kg were anesthetized and administered 10 mg sodium cyanide dissolved in 60 ml 0.9% NaCl intravenously by pump over 60 min followed by experimental treatments. Serial venous blood samples were obtained at baseline, at time of treatment, and at multiple times thereafter until 90 min following treatment. The blood was immediately cooled to 4° C, centrifuged, and the red blood cells (RBCs) were diluted 10× in ice-cold water resulting in osmotic pressure-driven lysis. Concentration-gradient driven collection of vapor-phase HCN following acidification of the samples with 10% trichloroacetic acid was performed in the same manner as described for both the NBA/DNB and cobinamide-based methods [27].

Qualitative Assay of Cyanide

A qualitative assay for cyanide was developed based on the color change that occurs when cobinamide binds cyanide. Cyanide solutions or lysed New Zealand white rabbit RBCs ranging in volume from 1000–1500 μl were placed in a Conway microdiffusion cell with a 1 mm radius piece of glass fiber paper saturated with 3 μl of 80 μM cobinamide in 0.1 M NaOH placed in the center of the interior chamber. Trichlororacetic acid (10%, 1 ml) was mixed with the fluid in the exterior chamber after sealing the cell, and color change of the filter paper was read 5 min later.

Results

Measurement of Cyanide in Standard Solutions

The concentration of cyanide in 250 μl standard solutions was measured over a range from 0.5 to 16 μM using both the cobinamide-based method and the NBA/DNB method (Figure 3). For the cobinamide-based method, seven analyses of the data are presented, i.e., two range models (300–600 nm and 490–590 nm, panel a), three ratios (366/505, 562/505, and 580/505, panel b), and two single wavelengths (366 and 580 nm, panel c). The 490–590 nm range analysis provided the best accuracy and precision over the range of 2–16 μM cyanide; this is based on having the lowest mean standard deviation (SD) of residua and highest mean correlation coefficient (r^2) with coefficients of variation and percent deviations from target < 10% (Table 1). Cyanide concentrations of 2–16 μM were chosen to allow comparison with the NBA/DNB method, which had a relatively narrow dynamic range, and could reliably be used only in this range (Table 1).

For both the 490–590 nm and 300–600 nm range analysis, the lower limit of quantitation was 2 μM (0.5 nmol), using a coefficient of variation and percent deviation from target value of < 20%. By the same criteria, the three ratio analyses and NBA/DNB method were satisfactory at ≥ 4 μM (1 nmol) cyanide, while the cobinamide single wavelength analyses were useable at ≥ 8 μM (2 nmol) cyanide (Table 1).

For the range and ratio analyses, the limit of detection was found to be < 1 μM (0.25 nmol) cyanide using the upper limit of noise as the mean plus three times the standard deviation of blank samples. The linear dynamic range of the assay is estimated to range from one-half to three times the cobinamide concentration for the ratio analyses; adjustment of the cobinamide concentration from 5–100 μM allows a linear dynamic range from < 2.5 to > 300 μM cyanide.

Measurement of Cyanide in Rabbit Blood

The concentration of cyanide in rabbit blood was measured by both the cobinamide-based method and the NBA/DNB method. Data from three experiments are plotted in each curve of Figure 4 revealing the correlation of these values. The r^2 of the 490–590 nm range analysis in comparison to the NBA/DNB method was 0.9 (Figure 4, panel a), whereas it was 0.85 for the 580/505 ratio (Figure 4, panel b), and 0.7 for the 580 nm single wavelength analysis (Figure

4, panel c). Thus, the cobinamide-based method when applied to biological samples shows good correlation with an established method.

Qualitative Assay of Cyanide

We performed a qualitative assay of cyanide based on the color change of cobinamide-impregnated paper on exposure to vapor-phase cyanide in a Conway microdiffusion chamber. The assay was as effective in rabbit blood as in cyanide standards, and could detect as little as 15 nmol of cyanide, corresponding to a cyanide concentration of ~30 μM in 0.5 ml of whole blood (Figure 5).

Discussion

Multiple methods exist for measuring cyanide in biological fluids. Each method has advantages and disadvantages, and no particular method is used widely and is generally accepted as the gold standard. Thus, improved methods for measuring cyanide in biological fluids are needed, particularly methods that provide for a rapid turn around in the clinical setting.

We have developed a new method to measure cyanide, based on the extremely high affinity of cobinamide for cyanide and the spectral changes that occur when cyanide binds to cobinamide. The assay can be used in both a quantitative mode using a spectrophotometer, and in a qualitative mode by visually observing the color change in cobinamide-impregnated paper. Both methods have a high throughput capacity. The slowest part of the quantitative assay is trapping cyanide in the alkaline cobinamide solution, which requires about 30 min using a Conway microdiffusion cell.

In addition to a high throughput capacity, other advantages of the cobinamide-based method are ease of use, stability of cobinamide, and application across a wide, adjustable dynamic range, depending on the cobinamide concentration used. In contrast to other colorimetric methods such as the NBA/DNB method, which requires two aromatic compounds and an unstable ether, the cobinamide-based method uses non-toxic reagents having less environmental concerns. For the NBA/DNB method, the dynamic range varied based on differing lots of *o*-dinitrobenzene and on the freshness of the 2-methoxyethanol.

Low cobinamide concentrations were used to validate the new method with respect to a standard method. However, higher concentrations of cobinamide would likely be used in a clinical laboratory, since cyanide concentrations in blood of ~ 40 μM are considered potentially toxic [10], and a clinical screening modality in the setting of mass casualty should minimize false positives and maximize true positives. While clearly dependent on blood volumes and dilution factors used, cobinamide solutions from 5–100 μM can be used to achieve this degree of sensitivity. The qualitative assay could detect a cyanide concentration > 30 μM in clinical blood samples in 5 min at room temperature, and this might be very useful to rapidly identify cyanide-poisoned patients for early treatment. Moreover, it could be used easily in the field and made quantitative by using a hand-held spectrophotometer or light-emitting diodes.

The major disadvantage of this newly-developed method is the lack of readily available cobinamide. However, it is easy to make from hydroxocobalamin, which is available from multiple suppliers. Dicyanocobinamide is commercially available, but due to cobinamide's extremely high binding affinity for cyanide, we have found it virtually impossible to remove both cyanide ions quantitatively. Hence the reason it is necessary to make cobinamide from hydroxocobalamin.

While this manuscript was being prepared, Mannel-Croise and Zelder published a paper showing that aquocyanocobinamide could be used to measure cyanide [28]. Because one cyanide

molecule is already bound to cobinamide, the dynamic range of the assay is considerably less. Moreover, they applied the method to neat cyanide standards only, and did not test it on biological samples.

Conclusions

We have developed a new method to measure cyanide. It is simple, and could be applied to biological samples. When used in a qualitative mode, it can provide a quick diagnosis of cyanide poisoning, which could be life-saving since cyanide is a rapidly acting toxicant.

Acknowledgments

This work was supported by NINDS grant U01 058030 as part of the NIH CounterACT program. W.B. was supported by an institutional NIH training grant, T32 DK 069263.

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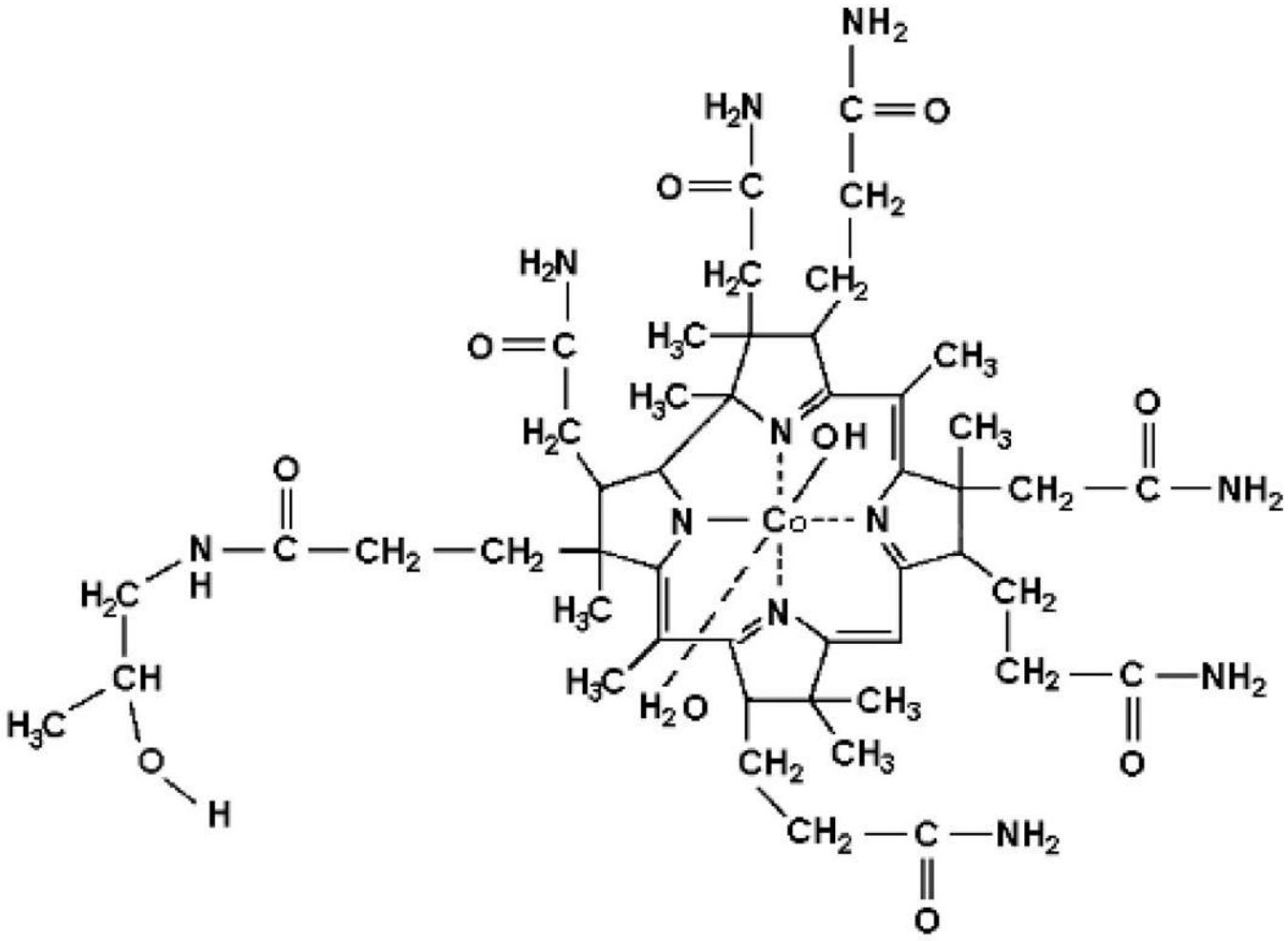

Figure 1. Structure of Cobinamide

Cobinamide is shown in its aquohydroxo form, which is present at neutral pH. Cyanide binds to the cobalt atom, replacing the water and hydroxyl groups.

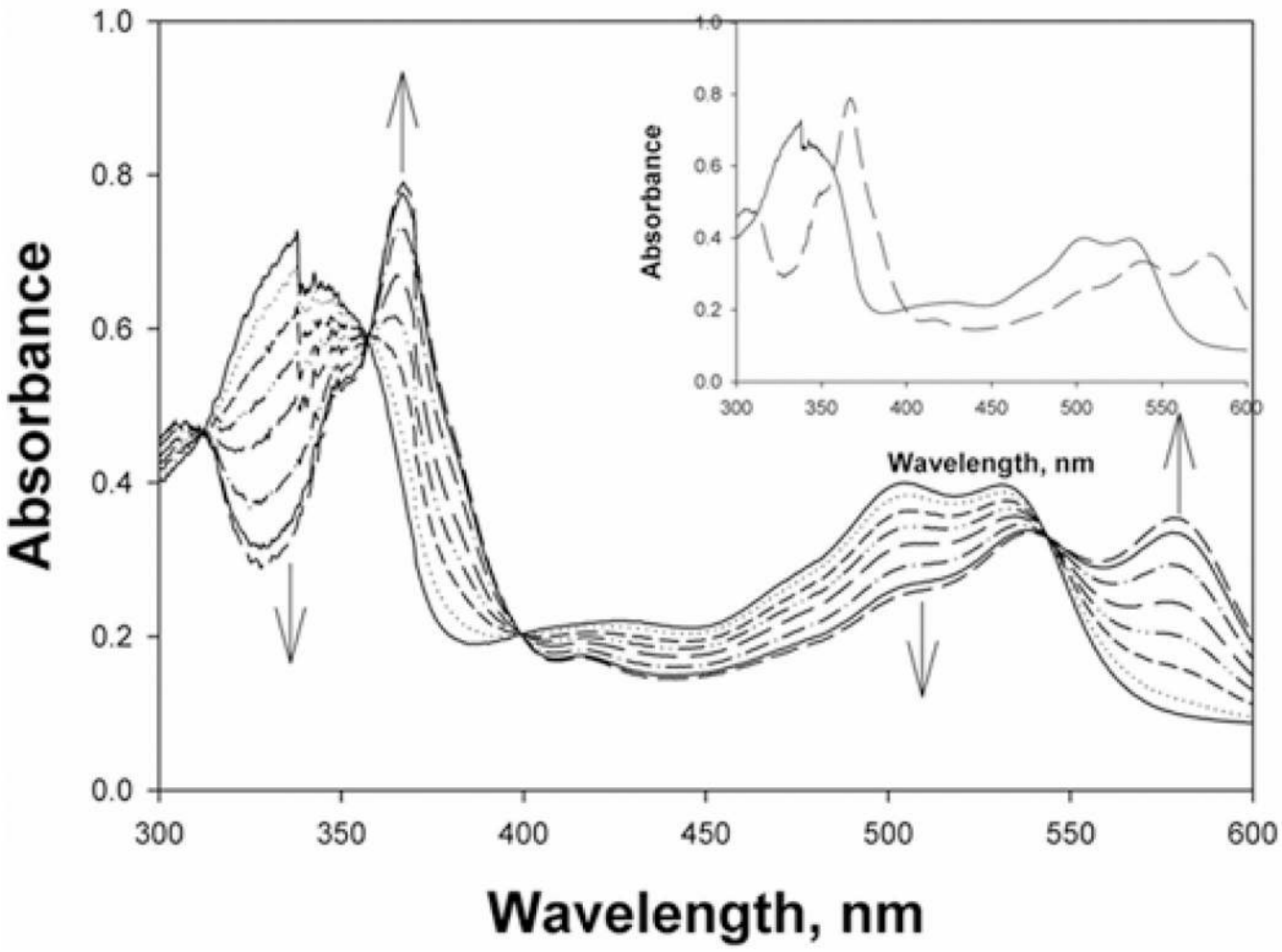

Figure 2. Spectral Changes of Dihydroxocobinamide on Binding Cyanide

The ultraviolet/visible wavelength spectra of cobinamide (solid line) in 0.1 M NaOH is shown during transition to complexed dicyanocobinamide (dashed line). Serial addition of KCN to 25 μM cobinamide gradually changes the spectrum: shown are cyanide concentrations of 10 μM (dotted line), 20 μM (line with small dashes), 30 μM (line with small dashes and two dots), 40 μM (line with large dashes), 60 μM (line with small dashes and one dot), 80 μM (solid line) and 100 μM (line with large dashes); arrows indicate direction of change toward dicyanocobinamide. Inset shows dihydroxocobinamide (solid line) and dicyanocobinamide (dashed line).

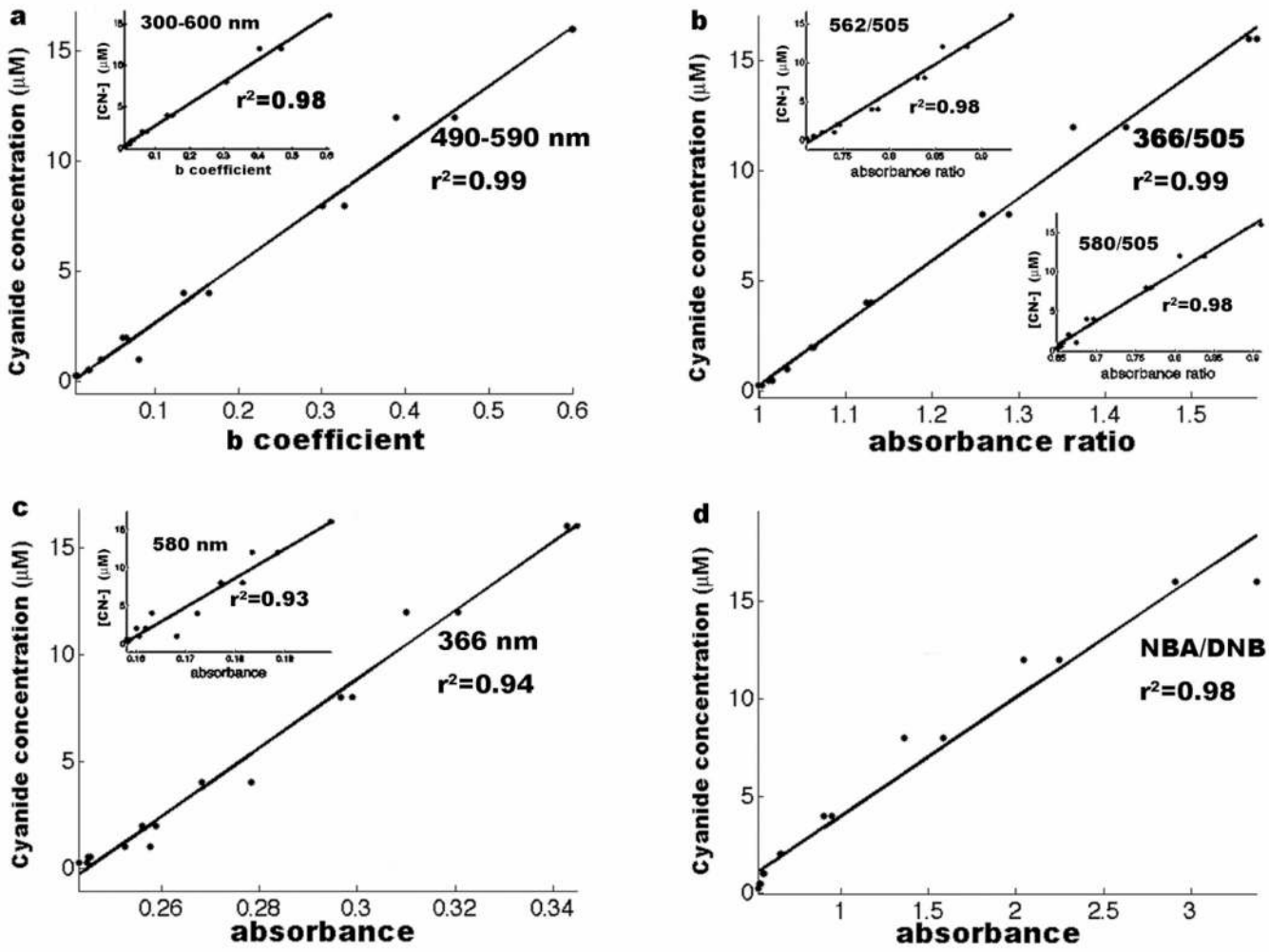

Figure 3. Measurement of Cyanide

The concentration of cyanide in standard solutions varying from 0.5 to 16 μM was measured using the cobinamide-based method (panels a–c) and the NBA/DNB method (panel d). The data from the cobinamide-based method were analyzed in three different ways: by two wavelength ranges (300–600 nm and 490–590 nm, panel a); by three wavelength ratios (366/505, 562/505 and 580/505, panel b); and by two single wavelengths (366 and 580 nm, panel c). Lines were generated by standard linear regression. The b coefficient for the wavelength range analyses (panel a) is defined in Methods.

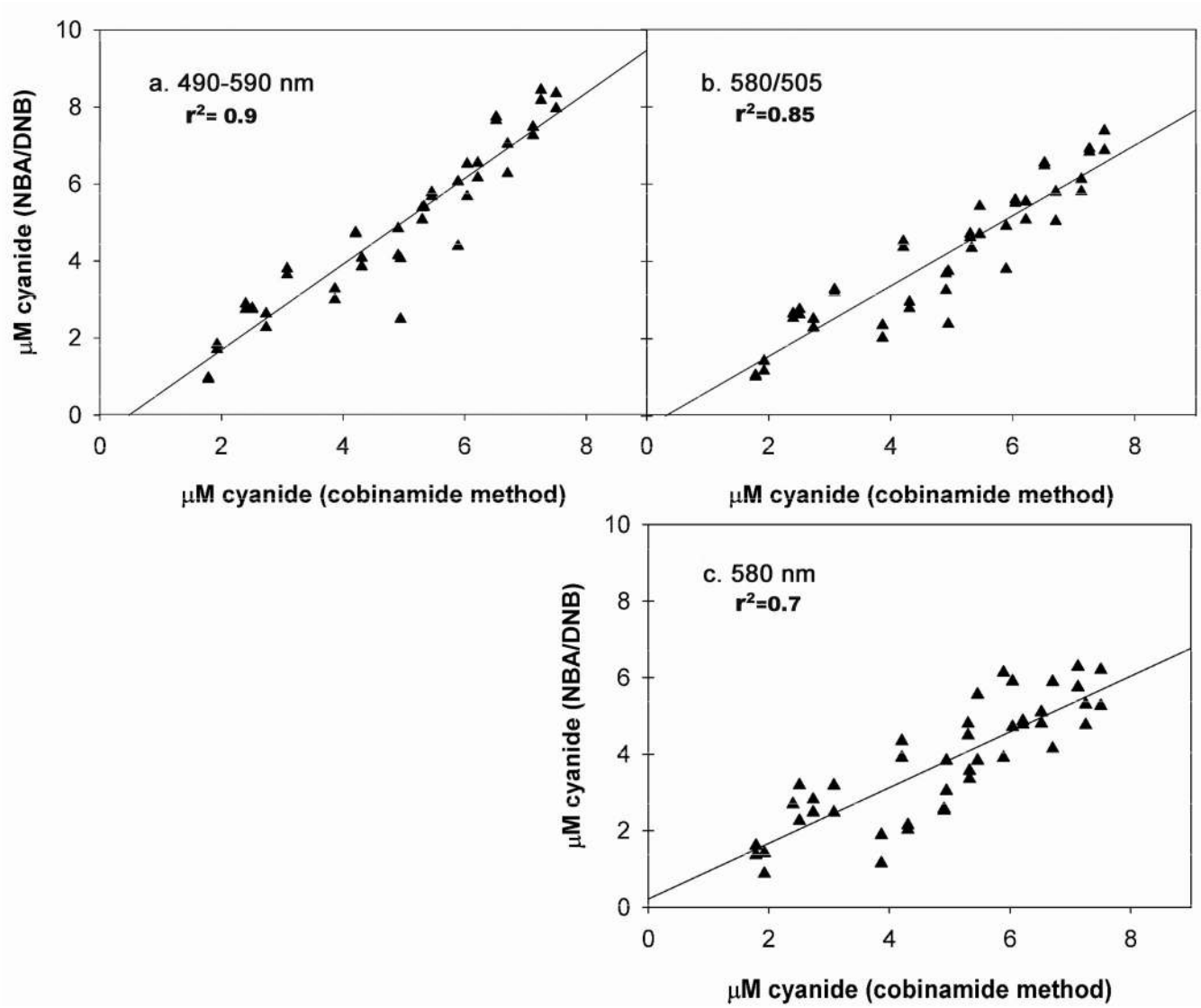

Figure 4. Comparison of Cobinamide-Based Method to NBA/DNB Method for Measuring Cyanide in Rabbit RBCs

Blood was obtained from rabbits that had been exposed to cyanide, and the RBCs were separated from the plasma. Concentrations of cyanide in the RBCs measured by the cobinamide-based method (x-axis) and by the NBA/DNB method (y-axis) are plotted. For the cobinamide-based method, three analyses of the data are shown: Panel a, 490–590 nm range analysis, panel b, 580/505 ratio analysis, and panel c, 580 nm single wavelength analysis.

Figure 5. Qualitative Cyanide Assay

A cobinamide-saturated glass fiber filter was placed in the center well of a Conway microdiffusion cell containing 1 ml of rabbit RBCs and 1 ml of 10% trichloroacetic acid in the outer well. The cell was capped and the acid and RBCs were mixed. The filters were removed after 5 min. 1) Left filter is from a rabbit RBC sample containing no cyanide. 2) Right filter is from a rabbit RBC sample containing 15 μM cyanide, as measured by the quantitative cobinamide method.

Table 1

Assessment of Accuracy and Precision of Measuring Cyanide Concentrations by a Cobinamide-Based Method and the NBA/DNB Method

The concentration of cyanide in standard solutions was measured by the newly-described cobinamide-based method and the NBA/DNB method as described in Methods. The correlation coefficient (r^2), the standard deviation (SD) of the residua, and accuracy estimates are shown as means from ≥ 3 experiments. The precision and accuracy estimates are represented by coefficients of variation and mean percent deviation from target, respectively, in the range of 2–16 μM cyanide.

			Cyanide Concentration			
Analysis	**r^2**	**SD of residua**	**2 μM**	**4 μM**	**8 μM**	**16 μM**
Wavelength range			Coefficient of variation / % deviation from target			
300–600 nm	0.98	1.9	19 / 16	10 / 8.7	15 / 9.7	
490–590 nm	0.99	1.8	9.5 / 8.3	6.3 / 5.0	7.6 / 5.8	5.8 / 3.3
Wavelength ratio						
366/505	0.99	1.4	22 / 18	12 / 8.6	4.3 / 3.5	
562/505	0.98	2.5	34 / 27	19 / 19	7.8 / 8.6	2.5 / 4.8
580/505	0.98	2.2	40 / 28	18 / 18	7.4 / 5.8	3.5 / 3.2
Single wavelength						
366 nm	0.94	3.4	54 / 56	34 / 25	11 / 11	
580 nm	0.93	4.7	58 / 47	38 / 27	20 / 16	13 / 10
NBA/DNB method	0.98	2.6	24 / 45	11 / 9.8	9.8 / 9.9	5.4 / 3.8

Anal Chem. Author manuscript; available in PMC 2011 May 15.