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Rapid Point of Care Analyzer for the Measurement of Cyanide in Blood

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Abstract

A simple, sensitive optical analyzer for the rapid determination of cyanide in blood in point of care applications is described. HCN is liberated by the addition of 20% H_3PO_4 and is absorbed by a paper filter impregnated with borate-buffered (pH 9.0) hydroxoaquocobinamide Hereinafter called cobinamide). Cobinamide on the filter changes color from orange (λ_{max} = 510 nm) to violet (λ_{max} = 583 nm) upon reaction with cyanide. This color change is monitored in the transmission mode by a light emitting diode (LED) with a 583 nm emission maximum and a photodiode detector. The observed rate of color change increases 10x when the cobinamide solution for filter impregnation is prepared in borate-buffer rather than in water. The use of a second LED emitting at 653 nm and alternate pulsing of the LEDs improve the limit of detection by 4x to ~ 0.5 μM for a 1 mL blood

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Supporting Information Available Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

sample. Blood cyanide levels of imminent concern (≥ 10 μM) can be accurately measured in ~ 2 min. The response is proportional to the mass of cyanide in the sample – smaller sample volumes can be successfully used with proportionate change in the concentration LODs. Bubbling air through the blood-acid mixture was found effective for mixing of the acid with the sample and the liberation of HCN. A small amount of ethanol added to the top of the blood was found to be the most effective means to prevent frothing during aeration. The relative standard deviation (RSD) for repetitive determination of blood samples containing 9 μM CN was 1.09% (n=5). The technique was compared blind with a standard microdiffusion-spectrophotometric method used for the determination of cyanide in rabbit blood. The results showed good correlation (slope 1.05, r^2 0.9257); independent calibration standards were used.

INTRODUCTION

Hydrogen cyanide (HCN) and its common alkali metal salts (NaCN, KCN, etc.) are well known for their high toxicity;[1] tales of cyanide being used as a poison are legend.[2] Cyanide is industrially important; although for the most part HCN is no longer used to make acrylonitrile, it is still used in large amounts to manufacture methyl methacrylate and adiponitrile (and thence, Nylon 66). In addition, alkali cyanides are used in electroplating, gold and silver extraction, tanning, and metallurgy. In the US alone, hundreds of kilotons of cyanide are manufactured annually.[3,4] Cases of cyanide poisoning from improperly prepared cyanoglucoside-rich foods like cassava are common in the African continent and chronic exposure leads to *Konzo*, a neuropathic disease.[5]

HCN can be inhaled or absorbed across the skin, or a cyanide salt can be ingested to manifest toxicity. While there may be chemicals more toxic than HCN, the clandestine production of significant quantities of cyanide is easy and will be nearly impossible to prevent. Hence, there is concern about its use as a terrorist weapon.[6,7] In 1995, the Japanese Aum Shinrikyo cult released HCN in Tokyo subway toilets. An Al-Qaeda plot to release HCN in the New York subway system was more recently revealed.[8,9] However, a less dramatic but far more common cause of fatal cyanide poisoning is smoke inhalation in fires.[10-13] Fuel-rich fires produce both CO and HCN. Acrylonitrile in carpet fibers and other nitrogenous polymers produce HCN. Tobacco smoke contains sufficient HCN to distinguish smokers from non-smokers based on their blood cyanide levels.[14,15]

Cyanide binds reversibly to the iron containing heme group of cytochrome a3 and stops the mitochondrial electron chain. Decreased energy production and cellular redox changes cause metabolic acidosis. Like O_2, HCN binds to the heme Fe(II) in reduced cytochrome a3. However, in the presence of O_2, the heme is oxidized readily to Fe(III), driven by a much greater binding constant.[16] Blood cyanide (BCN) levels are generally measured in whole blood; ≥98% of the cyanide is in the red cells bound to methemoglobin.[17] BCN concentrations differ widely based on smoking status and cyanide exposure. In healthy unexposed adults, the background concentration is sub-μM, toxic or fatal blood concentrations are generally considered to be >40 μM. Concentrations in survivors can exceed 200 μM; values exceeding 400 μM have been recorded in fatalities.[15,18,19,20]

If antidotes are promptly administered in adequate dosage, the effects of cyanide poisoning can be reversed. Until recently, hydroxycobalamin was considered to be the best antidote. However, it has recently been proposed that cobinamide, which binds cyanide with even higher affinity, may prove to be superior.[21-26] Cobinamide reverses the effects of cyanide more rapidly and more completely than hydroxycobalamin; this has been demonstrated in experimental animals in real time by diffuse optical spectroscopy.[27] Rapid quantification of BCN will be of great value not only in clinical examination and forensic investigations[28] but

it will be especially useful in the field as a point of care (POC) method to detect cyanide exposure and decide on antidote dosage.

We recently reviewed the current literature on the general determination of cyanide[29], and noted that some of the corrinoid based methods[30,31,32] have demonstrated applicability to the measurement of cyanide in real biological samples. Earlier Lindsay et al.[15] had reviewed the measurement of cyanide in blood. For blood, cyanide must first be released from methemoglobin: Cavett flasks to mini-stills to membrane-based and various microdiffusion apparati (the Conway Microdiffusion Cell being the most popular) have been used.[33] Commonly, either the sample preparation/analyte release or the actual measurement step is slow.[34] In terms of measurement techniques, recent approaches like isotope dilution headspace GC-MS[35] or derivatization LC-MS/MS[36] are highly sensitive and accurate but impractical in the field. For Forensic investigations, it is to be noted that cyanide half-life can be <2 h.[37] Presently, no rapid POC methods exist to measure BCN.

We demonstrate here a practical instrument that uses 0.2-1 mL blood in a disposable microcentrifuge tube, to which H_3PO_4 is added. Air is bubbled in the solution to purge the HCN through a cobinamide impregnated filter located between a light emitting diode (LED) based light source and a photodetector (both located in a specially designed cap for the vial). The color change of cobinamide is monitored as HCN reacts with the filter. An LOD of 0.5 μM cyanide with a 1 mL sample is attainable in 5 min; higher concentrations can be detected faster.

EXPERIMENTAL SECTION

CAUTION

Cyanide is extremely toxic and hazardous. HCN is easily evolved. Care must be taken to avoid skin contact and inhalation/ingestion. The entire experimental setup, standard sample preparation and filter treatment were conducted in a flow-monitored well-ventilated hood. For operator protection and handling of the waste, the waste was collected in alkaline hypochlorite (5% bleach solution containing added alkali) before disposal.[32,38, 39] Comparable measures should be taken if similar experiments are performed.

Reagents

All chemicals used were reagent grade or better. 18.2 MΩ·cm Milli-Q water (www.millipore.com) was used throughout. Pure cobinamide was produced by base hydrolysis of cobalamin (www.sial.com) following Blackledge *et al.*[30] The stock cyanide solution was prepared by dissolving KCN in 1 mM NaOH, calibrated by a standard titrimetric method,[40] and stored refrigerated. The cyanide working solution was prepared in 1 mM NaOH solution daily. Blood samples were spiked with cyanide immediately before use. The 0.1 M borate buffer solution was prepared by dissolving sodium tetraborate ($Na_2B_4O_7{\cdot}10H_2O$, www.emdchemicals.com) in Milli-Q water and adjusted to 9.00 with 2 M NaOH or HCl solution using an ALTEX Φ71 pH meter (Beckman), calibrated immediately before measurement by bracketing NIST-traceable pH 7 and 10 standard buffers.

Filter Pre-treatment Procedure

Type 09-801AA paper filters (www.fishersci.com) were cut to ~7 × 7 mm square, and immersed in 1 mM cobinamide prepared in pH 9.00 borate buffer solution for 5 min. They were removed and allowed to dry in clean air until completely dry. The prepared filters were kept in sealed plastic bags in the dark at room temperature in a closed glass jar; a few NaOH pellets were put in the jar to absorb any CO_2. They were stable in this condition for weeks.

Experimental Arrangements

The analyzer is schematically shown in Figure 1. Several different analyzer designs were experimented with (Figures S1-S9 in the Supporting Information (SI) provides detailed description and photographs). All relied on single-or multi-wavelength transmission photometry of reagent-impregnated filters in the sample vial cap. In a typical configuration, a 583 nm LED source (Hewlett-Packard HLMP 3850A, viewing angle 24°) was driven at a current of 10 mA. A 10 Ω dropping resistor was used with a supply voltage of 2.1 V; previous studies have shown that driving an LED with low value serial resistors provides considerable immunity against temperature induced light intensity changes.[41] A light to voltage converter (monolithic integrated photodiode-operational amplifier combination) TSL257 (www.taosinc.com) was used as detector. The detector output data were acquired with a 14-bit resolution (USB-1408FS, www.measurementcomputing.com) using a 1 s time constant RC filter. In a later modification, instead of the LED, the common leg of a bifurcated fiber optic (FO, http://www.dolan-jenner.com) was the light source. The two free distal ends of the FO were coupled to a 583 and a 653 nm LED (signal and reference),[42,43] respectively driven at 30 and 31 mA. The two LEDs were alternately turned on and off at 0.33 Hz (1.5 s on-period, each) controlled via an N-channel logic level MOSFET switch (IRLI530N, www.irf.com) (the schematic appears in Figure S10 in the SI). Data acquisition and LED on-off control was governed by routines written in Labview 8.5 (www.ni.com). For full-spectrum experiments, the light source was a white LED (NSPW500BS, www.nichia.com, driven at 25 mA, a 10 Ω dropping resistor, V_{supply} 3.8 V, usable light output spans 400-700 nm)and a fiber optic brought the transmitted light to a miniature CCD spectrophotometer (USB 2000, www.oceanoptics.com).

Measurement Procedure—Calibration experiments were conducted with bovine blood (defibrinated, P/N R1000250, www.vwrsp.com). One millilitre aliquots of blood (pre-spiked with known amounts of cyanide) was pipetted into a screw-top microvial (www.axygen.com P/N ST-200, capacity 2 mL). Ethanol (100 μL) was then added as de-foamer. The vial was capped with the analyzer cap (this has built in o-ring seal to seal on top of the vial). 0.1 mL 20% (v/v) H_3PO_4 was added from the top of the tube by a 1 mL syringe. Air was then bubbled through the solution to mix the acid and the blood, using a 30-50 cc disposable syringe and delivering ~ 20 cc with reasonable evenness over 1 min. Experience showed that this method worked as well (if not better, as the operator has visual feedback of any foaming in the container) as an electrically operated miniature air pump. The aeration is begun simultaneously with the acid addition and time zero on the depicted data indicates this temporal point. Data is acquired at 1 Hz. Filter transmittance increases as the filter equilibrates with moisture and during the first few seconds the detector voltage generally increases regardless of the cyanide content. Even at ~40 μM BCN, the absorbance signal does not actually start increasing until $t \simeq 15$ s. For processing the data, the detector voltage at t = 10 s, is therefore taken to be the initial transmittance, I_0. The detector signal I_t was recorded up to 300 s and the absorbance A_t was computed as $\log I_0/I_t$. In the two wavelength measurement technique, 653 nm intensity is considered the reference; for any particular 583 nm read, the immediately preceding 653 nm light intensity read is taken as I_0.

RESULTS AND DISCUSSION

Choice of Chemistry

Cobinamide is not only an antidote for cyanide, it undergoes a very significant spectral change as it binds cyanide. Notably, the absorbance at ~580 nm increases upon binding cyanide while ~650 nm can be used as a reference wavelength: absorbance here is negligible and is unaffected by cyanide binding.[38] Cobinamide has already been used for monitoring cyanide in aqueous solution or in blood after microdiffusion.[30,31] Because of its excellent

sensitivity and rapid reaction rate, we wished to explore this chemistry rather than the more classically established pyridine based methods for the colorimetric measurement of cyanide.[44] The structural formulae of cobinamide (strict nomenclature: what we use is hydroxoaquocobinamide) and other corrin-based chemosensors are given in Figure S11 in the Supporting Information and are discussed in more detail elsewhere.[29]

Choice of Filter Substrate

We tested a number of different filter material to find the most suitable substrate. The filter will not only need to be impregnated with cobinamide, it will need to remove the HCN passing through it by reaction. While this removal is likely to be more complete with a thicker filter, light throughput, vital for a sensitive optical method, will decrease with filter thickness. Octadecyl silica microparticle impregnated PTFE filters (Empore, 3M) were eliminated because of solvent manipulations needed for impregnation and poor light transmission. Other synthetic polymer filters, e.g., Versapor AA (acrylic copolymer, Pall-Gelman) were also found unsuitable. Of paper filters (Grade 1 qualitative cellulose filter, porosity 11 μm; Grade 2 qualitative cellulose filter, porosity 8 μm, Grade 5 qualitative cellulose filter, porosity 2.5 μm (all from Whatman)), and P5 Grade medium porosity cellulose filters (5-10 μm retention, Fisher), the last performed the best in terms of attainable sensitivity and was henceforth used.

Effect of Cobinamide Concentration used for Filter Impregnation—Cobinamide solutions (0.2, 0.5, 1.0 and 2.5 mM in water) were used to impregnate filters. Aqueous cyanide (0-1 mM), used in lieu of cyanide-spiked blood, were used to construct a calibration curve for each cobinamide impregnation concentration. In our device, the light source side is sealed; the purge gas must pass through the filter and then around the photodetector/FO. We anticipated that the capture efficiency/sensitivity will increase with increasing amounts of cobinamide on the filter. In practice, the slope of the calibration curve did increase as [cobinamide] increased from 0.2 to 0.5 mM, but there was no significant further increase (see Figure S12 in Supporting Information). Attainment of a plateau at [cobinamide] ≥ 0.5 mM suggests quantitative capture of HCN. There is a slight but statistically insignificant decrease at the highest cobinamide loading; if real, it is likely an artifact. The overall transmittance is low and stray light effects increase at high absorbance as the LED source is not truly monochromatic; such effects have been discussed previously.[45,46]

Mixing Efficiency and Defoaming of Blood—It is essential that the sample and the added acid mix rapidly. Some active mixing means is necessary, especially as blood samples are viscous and the viscosity increases on acid addition. In initial experiments with aqueous samples, magnetic stirring was found to increase dA/dt > 6x but the release rate was still slow. We realized that HCN has a high intrinsic solubility;[38] without active purging, HCN release, driven only by the diffusive capture by the filter, will be very slow. While using a magnetic stirrer in the field is not impossible, it adds another active element. It occurred to us that both mixing and purging could be accomplished by air bubbles that can be readily delivered by thumb-size air pumps incorporated into the electronics/ readout enclosure. While as little as 4-5 mL/min air was effective, indigenous surfactants in blood induce frothing and can contaminate the optical components.

Several avenues were explored to minimize the foam: reducing the sample volume to 0.5 mL leaves a larger headspace and allows the foam to form and dissipate. The LOD is sufficiently good that two-fold loss of sensitivity will be acceptable in most emergency POC applications. The use of an even larger vial and a wire-mesh screen midway to interrupt the foam was also tested. The results were not satisfactory. Silicone based defoaming agents (Antifoam A/B/C, www.sial.com) added in the recommended amount had little or no effect

in this system. Interestingly, addition of 100 μL ethanol atop the blood stopped frothing for the first 1~2 min; by this time, mixing was complete. The calibration slopes in water vs. blood as matrix (using ethanol as a defoaming agent) were 205.5±3.8 and 210.4±6.4 mAU/mM CN^-, respectively, for a 1 mL sample aliquot, indicating no statistical difference.

Nature and Amount of Acid as Cyanide Release agent from Blood—We experimented with Fe complexing agents like EDTA and fluoride in neutral to modestly acid media and failed to observe rapid cyanide release from cyanide-spiked blood. Strong acids in an adequate amount do affect this release and the attendant high ionic strength also decreased the intrinsic solubility of unionized HCN. One hundred μL volumes of 11% and 50%, v/v H_2SO_4 and 20% and 50% v/v H_3PO_4 were evaluated. The average sensitivity was similar with all of the above because all lead to a sufficient final acidity to completely release the BCN. However, the reproducibility (n=5) differed greatly. Sulfuric acid, especially at the higher concentration, resulted in a large amount of solids that led to poor mixing and very poor reproducibility. With 50% H_3PO_4, the mixture remains liquid even after acid addition. There was no significant difference in performance between 20% and 50% H_3PO_4; the former was henceforth used.

Performance

Originally cobinamide for impregnation was prepared without any buffer. The *p*Ka of cyanide at room temperature is ~9.2;[38] and having a base-buffered matrix should improve capture efficiency and cyanide reactivity. However, preparing cobinamide in buffers approaching a pH ≥10 caused slow conversion to dihydroxocobinamide; this does not react with cyanide as rapidly on the filter. But impregnating filters with cobinamide buffered at a more modestly alkaline pH (pH 9.00, borate) dramatically increased the response by an order of magnitude relative to an unbuffered solution (2.095±0.068 vs. 0.2103±0.0064 AU/mM CN) due to improved reaction kinetics. Figure 2 shows the spectral changes of cobinamide impregnated filters which have been subjected to blood samples with 0-162 μM BCN, measured according to the stated protocol, using the white LED source - CCD spectrometer detector.

Figure 3 shows the response of the single wavelength analyzer to 0-36 μM cyanide using 1 mL sample aliquots. The relative standard deviation (RSD) was 1.09% (n=5) for blood sample spiked with 9 μM BCN. The limits of detection (LODs), based on three times the standard deviation of the blank divided by the calibration slope, was 2 μM for single wavelength detection. This improved to 0.5 μM for dual wavelength detection, which compensates much better for variations in moisture induced transparency. Because these LODs are more than adequate for emergency POC situations, we also explored if smaller sample volumes, e.g., 0.2 and 0.5 mL can be used. Not surprisingly, the response was independent of the volume of the sample and was directly related to the mass of cyanide in the sample. The inset in Figure 3 shows data where each point represents the same mass of cyanide contained in a 0.2, 0.5 or a 1 mL sample aliquot with the error bar indicating the standard deviation. The S/N =3 mass LOD based on these data is 26 ng cyanide or 1 nmol CN.

Intercomparison with Real Samples

A tripartite collaborative experiment was conducted. At the University of California Irvine Medical Center and Beckman Laser Institute, the efficacy of cobinamide as a cyanide antidote is being tested with rabbits as experimental animals. In a typical experiment, 10 mg of NaCN is infused over one hour. Normally this would be a lethal dose but cobinamide is administered as an antidote afterwards. Approximately 1- mL blood samples were taken periodically through the infusion process and split into two parts and shipped refrigerated to

the authors' respective laboratories at the University of California at San Diego (UCSD) and University of Texas at Arlington (UTA). At UCSD the samples were analyzed by a microdiffusion procedure[30] using Conway diffusion cells and a spectrophotometric method using *p*-nitrobenzaldehyde and *o*-dinitrobenzene. This involves the formation of the *o*-nitrophenylhydroxylamine anion; it was originally described by Guilbault and Kramer[47] and modified later by Gewitz et al.[48] Hereinafter it is referred to as the NBA/DNB method. Blind analyses at UTA were conducted using the presently described system. The amount of sample available permitted only a single determination at each location.

Figure 4 shows the results. Except for one sample, the overall correlation for cyanide is excellent, especially considering independent calibration standards and blind analyses.

In summary, we describe a simple and sensitive optical analyzer that relies on light transmission measurement of a cobinamide impregnated filter for fast determination of BCN (~120 s for BCN levels of concern, ≥ 10 μM) that can be deployed in emergency POC situations. The use of inexpensive light source and detector components potentially permit a disposable cap (see e.g., Figure S13 in the Supporting Information) and sample vial that will be umbilically connected to a palmtop controller/reader.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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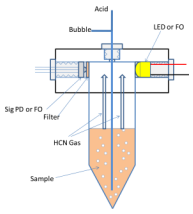

Figure 1.
Schematic diagram of the detection system. FO: fiber optic; PD: photodiode.

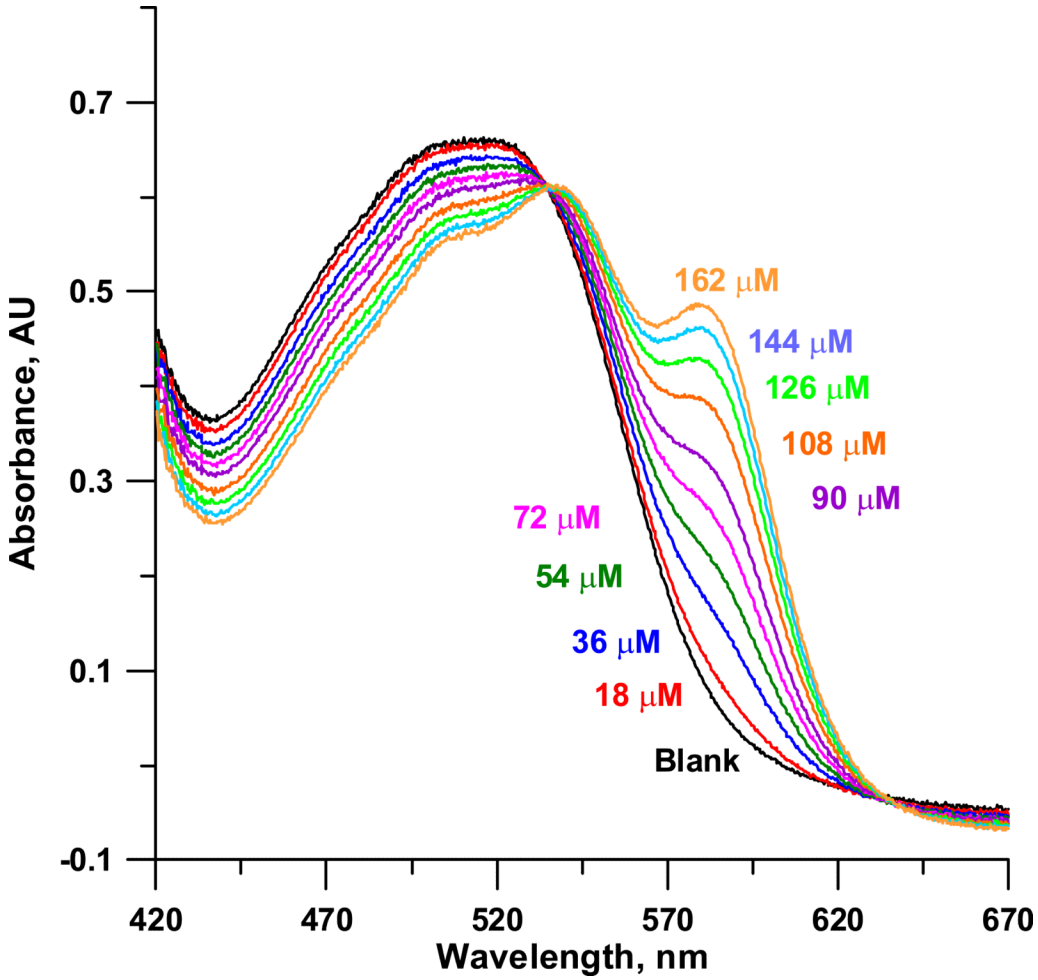

Figure 2.
Spectrum change of cobinamide impregnated filter with different cyanide concentrations.

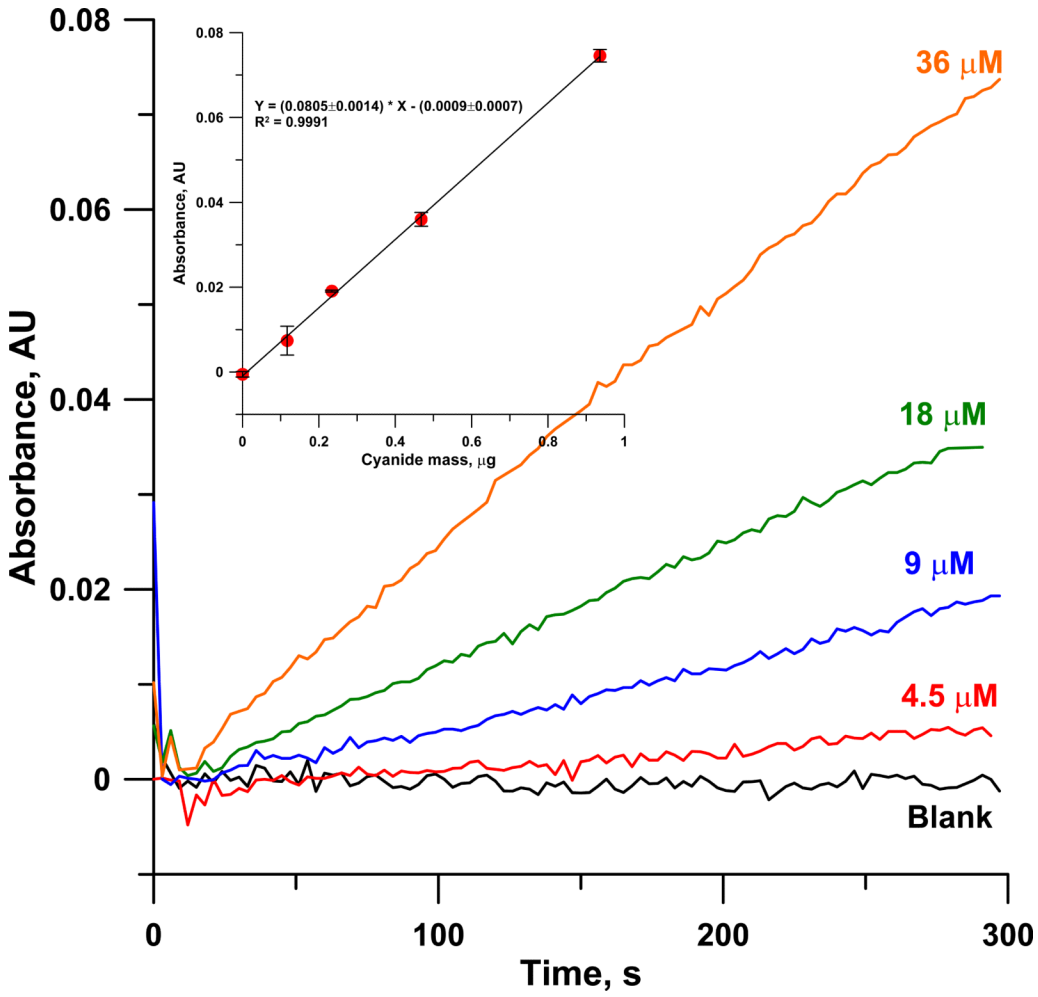

Figure 3.

System response. The inset shows the calibration curve with abscissa units in terms of cyanide mass; three different using different sample volumes (0.2, 0.5, 1 mL), were used for each point.

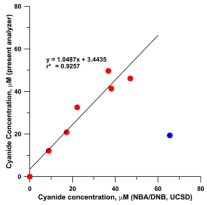

Figure 4.
UCSD NBA/DNB results vs. cyanide analyzer. See text for details.