Published in final edited form as:

Anal Methods. 2015 ; 7(13): 5707–5711. doi:10.1039/C4AY00190G.

Comment on “Rapid visual detection of blood cyanide” by C. Männel-Croisé and F. Zelder, *Analytical Methods*, 2012, 4, 2632

Akinde F. Kadjoⁱ, Purnendu K Dasguptaⁱ, and Gerry R Bossⁱⁱ

ⁱⁱDepartment of Chemistry and Biochemistry, University of Texas at Arlington, Arlington TX 76019-1065, USA

ⁱDepartment of Medicine, University of California, San Diego, La Jolla, CA 92093-0652, USA

Abstract

Cyanide poisoning from Inhaled HCN is all too common in victims of smoke inhalation in fires. While the toxic effects arise primarily from its inhibitory effects on *cytochrome c oxidase*, the majority of the cyanide binds to methemoglobin (metHb) in the blood. It can be considered as the detoxification mechanism: one of the antidotes used earlier was nitrite which primarily works by converting hemoglobin to metHb (normally present to the extent of ~1% of the total hemoglobin). Vitamin B12 (hydroxocobalamin) and related analogs have long been known to have high affinity for cyanide and has been used as antidotes – the binding of cyanide to many compounds in this general family also results in a significant change in color that can be used for analytical purposes. Männel Croisé and Zelder (*Anal. Methods*, 2012, 4, 2632) have advocated direct addition of a related compound to blood samples and isolating the colored measurand on a solid phase extraction cartridge. While they demonstrated attractive rapid measurement of cyanide in spiked blood samples, we believe that this is not a practically usable procedure regardless of the exact chromogenic reagent used. Cyanide bound to metHb dissociates too slowly for a 1 min reaction to work as suggested – we believe for reasons unknown (eg., metHb levels in their blood samples unusually low), cyanide added to their blood samples did not (have time to) bind to metHb and these samples may not resemble real situations where significant amount of the cyanide will be bound to metHb.

Männel Croisé and Zelder published an attractive method for rapid visual detection of blood cyanide in this journal.¹ There has been considerable valuable work done by these authors on the use of corrinoids for the colorimetric detection of cyanide.^{2–6} These compounds are related to Vitamin B-12 (hydroxocobalamin) and the great affinity of B-12 to bind cyanide to form cyanocobalamin is also well known. Cyanide binding also typically results in a considerable change in the visible absorption spectrum that can be analytically exploited. Referring to Figure 1, aquahydroxocobinamide ($R = -NH_2$, $X = -OH$, $OH(H_2O)Cbi^+$) binds up to two moles of cyanide per mole and with a greater affinity than metHb and hence functions as an attractive antidote.⁷ Judicious dosage of this or any antidote requires a knowledge of the total blood cyanide levels and for this reason rapid determination of blood cyanide is needed in emergency situations. Aquacyanocobinamide (Figure 1, $CN(H_2O)Cbi$

+) and other related structures (the compound used in Ref. 1 has $R = -OCH_3$, $X = -CN$, hereinafter called compound **1**, as in the original paper), have emerged in recent years as excellent colorimetric reagents for measuring cyanide, the hydroxo group is replaced with a second cyano group, a change in color also results.. Independently, Boss et al. have been interested in aquahydroxocobinamide ($OH(H_2O)Cbi^+$) as an antidote for cyanide poisoning,^{8-9,10,11,12,13,14} and Boss et al. independently¹⁵⁻¹⁷ or in collaboration with Dasgupta et al.¹⁸⁻²⁰ have used cobinamide based reagents (both $OH(H_2O)Cbi^+$ and $CN(H_2O)Cbi^+$) for the determination of cyanide. Spectrally $CN(H_2O)Cbi^+$ is very similar to $OH(H_2O)Cbi^+$ while $(CN)_2Cbi$ is very different. It was thus somewhat of a mystery as to how traces of cyanide can be detected with an excess of $OH(H_2O)Cbi^+$ as $CN(H_2O)Cbi^+$ will presumably be formed. We jointly solved the puzzle:²¹ the reason is kinetic and not thermodynamic: $CN(H_2O)Cbi^+$ is much more rapidly attacked by CN^- to form $(CN)_2Cbi$; any $CN(H_2O)Cbi^+$ formed from $OH(H_2O)Cbi^+$ is rapidly converted to $(CN)_2Cbi$ in preference to more $CN(H_2O)Cbi^+$ being formed from $OH(H_2O)Cbi^+$. The preferred reagent will therefore be $CN(H_2O)Cbi^+$ as Männel Croise and Zelder have previously used; it leads to approximately twice the sensitivity as the amount of $(CN)_2Cbi$ formed is twice as much when one starts from $CN(H_2O)Cbi^+$ rather than $OH(H_2O)Cbi^+$.¹⁹

In the paper of present concern,¹ Männel Croisé and Zelder propose measuring cyanide in (spiked) blood samples by (a) adjusting the pH to 9.6 with CHES buffer, adding (b) **1** to the blood sample (c) allowing 60 s for replacement of $-OH$ by $-CN$ to form **1-CN**, (d) putting the mixture through a C-18 based solid phase extraction cartridge where the **1-CN** is retained, (e) washing the cartridge with 3 mL of water to remove any adherent blood, (f) quantitating the cyanide visually by the extent of the red-violet coloration at the top of the column, or removing the top 2 mm of the sorbent layer and examining it by diffuse reflectance spectroscopy, or for the most accurate results, eluting the $(CN)_2Cbi$ with 400 μL of methanol, making up to 500 μL and measuring the product by solution phase absorbance measurements at 583 nm. In most other previous efforts in blood determination involving cobinamide or derivatives,^{15,17,19,20} HCN had to be first released from the cyanide bound to blood by strongly acidifying the sample, prior to capturing the gaseous HCN and measuring the resulting cyanide. In fact, it was pointed out¹ that a paramount advantage of the proposed approach, regardless of the technique or reagent used, is that this release of HCN along with a matrix isolation step (e.g., microdiffusion/microdistillation), common to all other approaches, is not necessary.

While we agree that a direct determination of cyanide without the need for microdistillation etc. is indeed attractive, there are several aspects of the proposed approach that are of concern to us. While it may seem late to offer a critique in 2015 to a paper published in 2012, we deemed repeated experimental verification is needed to offer written criticism of the work of an esteemed colleague.

Previous work on blood cyanide determination without prior liberation of HCN

The sole exception (to previous approaches using microdistillation) cited by Männel Croisé and Zelder¹ is the work of Lacroix et al.²² These authors spike the blood sample with an

internal standard containing $^{13}\text{C}^{15}\text{N}$ and after deproteinization and centrifugation add Taurine and naphthalenedialdehyde (NDA) and allow it to react for 10 min at 4 °C to form the 1-cyano-2-alkyl-benz[f]isoindole derivative and quantitate the same by LC-isotope dilution MS/MS. The authors achieve a limit of detection of 10 ng CN/mL (0.4 μM). In comparison, cobinamide based colorimetric methods have been shown to provide an LOD as good as 8 nM; indeed even with portable analyzers for cobinamide-based blood cyanide, LODs were equal to or better than the Lacroix et al approach despite the fact that these authors used relatively large volume sample injections (100 μL on 2.1 mm ϕ columns) and the well-known high response of benz[f]isoindoles in ESI-MS. A likely possibility that one can surmise is that very little of the derivative is actually formed under the conditions used by Lacroix et al., albeit there may not be major quantitation errors because of the use of isotope ratio measurements. The use of NDA and Taurine to measure blood cyanide can be traced back to the work of Sano et al.²³ and Chinaka et al.²⁴ Both these authors state that ~83 % of the added cyanide is recovered after 30 min of reaction time at room temperature. If the formation proceeded at a first order rate, the putative first order rate constant would be $9.8 \times 10^{-4} \text{ s}^{-1}$.

It is also not correct that cobinamide based approaches have been carried out only with prior HCN release. One of us coauthored efforts to measure cyanide in which cyanide was spiked to whole blood, aquahydroxocobinamide was then added, allowed to react for 5 min and then the plasma, containing $(\text{CN})_2\text{Cbi}$, was separated for spectrophotometric measurement¹⁰ or visual estimation.¹⁷ These approaches were abandoned because we subsequently realized that how much of the cyanide actually binds to the blood may depend on the time allowed for the cyanide to react with blood and conversely how much of the bound cyanide can be extracted by cobinamide depends on the time allowed for the cobinamide to react with the spiked sample.

Intrinsic concerns. Reagent amount and thermodynamic limitations

The final concentration of reagent **1**, which reacts with CN^- to form **1-CN** in the proposed method is 42 μM prior to reaction with any cyanide present. The blood sample (0.5 mL) taken is spiked with up to 100 μM CN^- ; with a final volume of 1 mL, in the absence of any reaction the cyanide concentration will be 50 μM , in addition to endogenous cyanide already present in any blood sample (this is typically in the low single digit μM level²⁰). Regardless of the value of the equilibrium constant of the reaction

Obviously there is not enough reagent present to react with all the cyanide present at the high end of the spike level and suggesting a straight line response through the entire 0–100 μM range (Figure S3 in their Supporting Information) is misleading. The equilibrium constant for reaction 1 is given by Männel Croisé and Zelder¹ to be $1.7 \times 10^6 \text{ M}^{-1}$, based on their own as well as other prior work. While this may seem a large enough binding constant to provide for quantitative binding, at the levels involved, it is not so. Even with only 1 μM total cyanide (at which point the reagent is certainly present in large excess), it is easily calculated that 98.6% of the cyanide will react and the response will of course get steadily

lower with increasing cyanide levels. Even in the absence of any competing ligands (notably metHb in blood), the reaction will not result in quantitative formation of $(\text{CN})_2\text{Cbi}$ and by $\sim 30 \mu\text{M}$ total CN^- in final solution, the difference with the initial response slope will become apparent (Figure 2). If in fact a competing ligand such as MetHb is present, the reaction will be substantially less complete.

Methemoglobin (metHb) is the primary cyanide binding agent in blood.^{25,26} The normal range of metHb is 0.5–2% of the total hemoglobin, often taken to be 1% of the total hemoglobin²⁷ the normal range for which is 120–175 g/L.²⁸ Although metHb typically is in the form of a tetramer in solution, it is customary to express the molarity in terms of molarity of the iron;²⁹ we assume here an average concentration of 1.5 g/L or 90 μM assuming an MW of 16,700 for the monomer. It is to be understood that metHb concentrations may be quite different in the animal blood samples with which Männel Croisé and Zelder as well as we experimented (in addition, there is some evidence that metHb concentration may increase some during storage³⁰ - these authors report a mean MetHb concentration of 1.6% in 312 banked fresh blood samples); however, ultimately the interest is in determining cyanide in human blood. After the addition of an equal volume of buffer, reagent etc., the metHb concentration will be 45 μM . The binding constant of MetHb with CN^- is given in two sources, one due to Klapper and Uchida³¹ suggests that there are two binding sites with respective association constants of 0.45×10^6 and $3.5 \times 10^6 \text{ M}^{-1}$. Figure 2 also shows traces K&U-1 and K&U-2, that respectively represents these two binding constants. Obviously, the formation of **1-CN** is expected to be much less if the complexation by metHb is taken into account.

In fact there is an earlier study by Anusiem et al. that looks at cyanide binding to both metHbA and metHbC.³² Because it provides an extensive data set (it reports the binding constants both as a function of temperature and pH, these data are used more often (see e.g., ref. 33). The data does not extend to pH 9.6. By extrapolating the data given for pH 7.0 to 7.8 for metHbA (Table 13, reference 32), and that given for pH 7.6 to 7.8 for metHbC (Table 14, reference 32), we estimate the respective binding constants to be 4.5×10^7 and $3.0 \times 10^6 \text{ M}^{-1}$ at 20 °C. Two further traces, Anusiem-a and Anusiem-c are shown also in Figure 1 that respectively assume these binding constants and an average metHb concentration of 45 μM in the final solution being measured, to complete the picture. It should become clear that at the level of reagent added, based on available thermodynamic data in the literature, it is extraordinarily unlikely that a quantitative linear response in the range of blood cyanide concentrations explored in this paper will be expected *a priori*, especially with the amount of reagent used.

Kinetic Considerations

An even more troubling aspect is kinetics. The issue here is that regardless of what chromogenic reagent is used to extract the cyanide bound to met-Hb in blood, the dissociation of cyanomethemoglobin to provide cyanide to the extractant is likely to be too slow to provide a method that reacts in a minute. Klapper and Uchida²⁹ provide a second order rate constant of $180 \text{ M}^{-1} \text{ s}^{-1}$ (k_f) for the forward reaction

The rate constant k_r for the reverse reaction, the dissociation, that must take place before **1** can form **1-CN**, is readily calculated from the overall binding constant, equal to k_f/k_r . For the range of binding constants discussed above (4.5×10^{-5} to $4.5 \times 10^{-7} \text{ M}^{-1}$) k_r is calculated to be in the range of 4×10^{-4} to $4 \times 10^{-6} \text{ s}^{-1}$, corresponding to half-lives of ~30 min to 2 days; if any of the above literature data are to be trusted, it is simply not possible for the cyanide bound to methHb to significantly react with **1** in 1 min, much less do so quantitatively.

One plausible explanation is that due to whatever reason, the cyanide spiked into the blood samples in the experiments of Männel Croisé and Zelder did not have enough time to bind to the methHb. To test this hypothesis, we experimented with both bovine and porcine blood in an identical fashion to that described in the paper¹ with the sole difference being that we used $\text{CN}(\text{H}_2\text{O}) \text{Cbi}^+$ as the cyanide extracting chromogenic reagent and waited 0.5, 5, 10, 20, 30, and 60 min between spiking cyanide to the extent of 50 μM and adding $\text{CN}(\text{H}_2\text{O}) \text{Cbi}^+$ to the extent of 42 μM . The results were interpreted in terms of a calibration curve done in the 3.8% citrated water in place of blood, with the C18ec cartridge eluted by methanol and spectrophotometric measurement at 583 nm. The results are shown in Figure 3, along with a best fit line describing the disappearance of the recoverable cyanide with time to some final equilibrium value as a first order process. The best fit rate constants are 5.5×10^{-4} and $1.8 \times 10^{-3} \text{ s}^{-1}$, respectively.

In a real situation with a human how much of the cyanide intake is already complexed with methHb will depend on the exact exposure scenario and duration but there is little doubt that a significant if not a major fraction can already be bound to methHb and this cannot be measured by the proposed method.

Acknowledgement

This work was funded in part by Jenkins Garrett Professorship funds at UTA and by the CounterACT Program, Office of the Director, National Institutes of Health and the National Institute of Neurological Disorders and Stroke, Grant # U01NS058030

References

1. Männel-Croisé C, Zelder F. *Anal. Methods*. 2012; 4:2632–2634.
2. Zelder F. *Inorg. Chem*. 2008; 47:1264–1266. [PubMed: 18205304]
3. Männel-Croisé C, Zelder F. *Inorg. Chem*. 2009; 48:1272–1274. [PubMed: 19161297]
4. Männel-Croisé C, Probst B, Zelder F. *Anal. Chem*. 2009; 81:9493–9498. [PubMed: 19842647]
5. Männel-Croisé C, Meister C, Zelder F. *Inorg. Chem*. 2010; 49:10220–10222. [PubMed: 20964392]
6. Männel-Croisé C, Zelder F. *ACS Appl. Mater. Interfaces*. 2012; 4:725–729. [PubMed: 22211318]
7. Chan A, Balasubramanian M, Blackledge W, Mohammad OM, Alvarez L, Boss GR, Bigby TD. *Clin. Toxicol*. 2010; 48:709–717.
8. Broderick KE, Potluri P, Zhuang S, Scheffler IE, Sharma VS, Pilz RB, Boss GR. *Exp. Biol. Med*. 2006; 231:641–649.
9. Broderick KE, Balasubramanian M, Chan A, Potluri P, Feala J, Belke DD, McCulloch A, Sharma VS, Pilz RB, Bigby TD, Boss GR. *Exp. Biol. Med*. 2007; 232:789–798.

10. Kreuter KA, Lee J, Mahon SB, Kim JG, Mukai D, Mohammad O, Blackledge W, Boss GR, Tromberg BJ, Brenner M. *Chest*. 2008; 134 (4_MeetingAbstracts):p124001.
11. Chan A, Balasubramanian M, Blackledge W, Mohammad OM, Alvarez L, Boss GR, Bigby TD. *Clin. Toxicol.* 2010; 48:709–717.
12. Lee J, Mohammad O, Brenner M, Mahon SB, Sharma VS, Kim J, Mukai D, Blackledge W, Boss GR, Goodman S, Kreuter KA, Ahdout R. *J. Biomed. Opt.* 2010; 15(1):017001. [PubMed: 20210475]
13. Brenner M, Kim JG, Mahon SB, Lee J, Kreuter KA, Blackledge W, Mukai D, Patterson S, Mohammad O, Sharma VS, Boss GR. *Ann Emerg. Med.* 2010; 33:352–363. [PubMed: 20045579]
14. Chan A, Crankshaw DL, Monteil A, Patterson SE, Nagasawa HT, Briggs JE, Kozocas JA, Mahon SB, Brenner M, Pilz RB, Bigby TD, Boss GR. *Clin. Toxicol.* 2011; 49:366–373.
15. Blackledge WC, Blackledge CW, Griesel A, Mahon SB, Brenner M, Pilz RB, Boss GR. *Anal. Chem.* 2010; 82:4216–4221. [PubMed: 20420400]
16. Swezey R, Shinn W, Green C, Drover DR, Hammer GB, Schulman SR, Zajicek A, Jett DA, Boss GR. *J. Anal. Toxicol.* 2013; 37:382–385. [PubMed: 23653045]
17. Boehringer H, Tong W, Chung R, Boss G, O'Farrell B. *Proc. SPIE*. 2012; 8317 831710Z-1.
18. Ma J, Dasgupta PK, Blackledge W, Boss GR. *Anal. Chem.* 2010; 82:6244–6250. [PubMed: 20560532]
19. Ma J, Ohira S-I, Mishra SK, Puanngam M, Dasgupta PK, Mahon SB, Brenner M, Boss GR. *Anal. Chem.* 2011; 83:4319–4324. [PubMed: 21553921]
20. Dasgupta PK, Mahon SB, Ma J, Brenner M, Wang J-H, Boss GR. *Anal. Chim. Acta.* 2013; 768:129–135. [PubMed: 23473259]
21. Ma J, Dasgupta PK, Zelder FH, Boss GR. *Anal. Chim. Acta.* 2012; 736:78–84. [PubMed: 22769008]
22. Lacroix C, Sausseureau E, Boulanger F, Goull JP. *J. Anal. Toxicol.* 2011; 35:143–147. [PubMed: 21439149]
23. Sano A, Kakimoto N, Takitani S. *J. Chromatogr.* 1992; 582:131–135. [PubMed: 1491032]
24. Chinaka S, Takayama N, Michigami Y, Ueda K. *J. Chromatogr. B.* 1998; 713:353–359.
25. Lundquist P, Rosling H, Sorbo B. *Clin. Chem.* 1985; 31:591–595. [PubMed: 3978792]
26. Vessey CJ, Cole PV, Simpson PJ. *Br J Anaesth.* 1976; 48:651–660. [PubMed: 797397]
27. Williams, SR. <http://toxicology.ucsd.edu/art%202%20methemoglobin.pdf>
28. http://www.drstandley.com/labvalues_hematology.shtml
29. Uchida H, Berliner LJ, Klapper MH. *J. Biol. Chem.* 1970; 245:4606–4611. [PubMed: 4318478]
30. Uchida I, Tashiro C, Koo YH, Mashimo T, Yoshiya I. *J. Clin. Anesth.* 1990; 2:86–90. [PubMed: 2346656]
31. Klapper MH, Uchida H. *J. Biol. Chem.* 1971; 246:6849–6854. [PubMed: 5126226]
32. Anusiem AC, Beetlestone JG, Irvine DH. *J. Chem. Soc. A.* 1968:960–969.
33. Blumenthal DC, Kassner RJ. *J. Biol. Chem.* 1980; 255:5859–5863. [PubMed: 6247350]

Figure 1.

(a) Aquahydroxoquacobinamide R = -NH₂, X = -OH; (b) Aquacyanocobinamide R = -NH₂, X = -CN; (c) extractant used in ref. 1: R = -OCH₃, X = -CN, in ref. 1 and herein called **1**.

Figure 2.

Computed equilibrium 1-CN concentrations vs. total cyanide concentration initially present, 42 μM total **1** added. Solid black line: in the absence of competing agent; Dashed red line: hypothetical case where the initial linear slope observed at very low added cyanide levels is maintained throughout. Other traces represent computations that take into account competition of methHb present (45 μM) using different sets of methHb-CN binding constants from Klapper and Uchida³¹ (K&U-1 and K&U-2) and by Anusiem et al.³² for methHbA and methHbC (Anusiem-a and Anusiem-c). The computations are based on thermodynamic data, i.e., infinite reaction time is presumed.

Figure 3. Measured concentration of $(CN)_2Cbi$ vs. the incubation time of CN^- in Bovine and Porcine blood.