New methods for the detection of cyanide based on displacement of the glutathione ligand of glutathionylcobalamin by cyanide

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ABSTRACT

Glutathionylcobalamin (GSCbl) is a vitamin B\textsubscript{12} derivative that contains glutathione as the upper axial ligand to cobalt via a Co–S bond. In the present study, we discovered that cyanide reacted with GSCbl, generating cyanocobalamin (CNCbl) and reduced glutathione (GSH) via dicyanocobalamin (diCNCbl) intermediate. This reaction was induced specifically by the nucleophilic attack of cyanide anion displacing the glutathione ligand of GSCbl. Based on the reaction of GSCbl with cyanide, we developed new methods for the detection of cyanide. The reaction intermediate, violet-coloured diCNCbl, could be applied for naked eye detection of cyanide and the detection limit was estimated to be as low as 520 $\mu$g L$^{-1}$ (20 $\mu$M) at pH = 10.0. The reaction product, CNCbl, could be applied for a spectrophotometric quantitative determination of cyanide with a detection limit of 26 $\mu$g L$^{-1}$ (1.0 $\mu$M) at pH = 9.0 and a linear range of 26–520 $\mu$g L$^{-1}$ (1.0–50 $\mu$M). In addition, the other reaction product, GSH, could be applied for a fluorometric quantitative determination of cyanide with a detection limit of 31 $\mu$g L$^{-1}$ (1.2 $\mu$M) at pH = 9.0 and a linear range of 31–520 $\mu$g L$^{-1}$ (1.2–20 $\mu$M). These new GSCbl-based methods are simple, highly specific and sensitive with great applicability for the detection of cyanide in biological and non-biological samples.

1. Introduction

Cyanide is acutely toxic to humans via all routes of administration, with a very steep and rate-dependent dose–response curve. The toxicity of cyanide involves inactivation of cytochrome oxidase and inhibition of cellular respiration resulting in cellular anoxia \cite{1,2}. Cyanide poisoning in humans most often occurs via combustion products of synthetic materials due to fire accidents. Cyanide also occurs naturally as cyanogenic glycosides in certain plants, including fruits and vegetables, which can release cyanide upon acid hydrolysis \cite{3}. Additionally, hydrogen cyanide is ubiquitous in nature and released into the atmosphere from biomass burning and the combustion of synthetic materials. Despite of its toxicity, cyanide is synthesised and used for industrial applications, such...
as the manufacture of paper, textiles, dyes and plastics, as well as in metallurgy for electroplating, metal cleaning and extracting gold.

There are many international, national and local regulations and guidelines regarding cyanide in air, water and other media, and the maximum cyanide concentration in drinking water has been set at 0.07 mg L$^{-1}$ by the World Health Organization and European Union [4,5]. Various methods have been developed to detect and quantify cyanide in biological and non-biological samples [6]. Official methods of cyanide determination include titration, spectrometry [7], potentiometry with cyanide-selective electrodes [8] and amperometry [9]. Most widely used methods for cyanide determination are organic-based sensor such as picric acid [10,11] or a combination of chloramine-T and isonicotinic acid/barbituric acid [12]. However these reagents are unstable in aqueous solution with toxicity or explosiveness. Current cyanide determination methods satisfy the requirements for sensitivity and accuracy [13,14,15,16], but these are often complex and require time-consuming sample preparation with bulky laboratory systems.

Vitamin B$_{12}$ (cyanocobalamin, CNCbl) an organometallic complex containing cobalt in the centre of the corrin ring (Scheme 1) was shown to be an attractive cyanide chemosensor [17]. Cyanide reacts with CNCbl replacing the 5,6-dimethylbenzimidazole lower axial ligand ($\alpha$-DMB ligand) and generating dicyanocoblamain (diCNCbl), which allowed ready visual detection of millimolar levels of cyanide in water. Since then, CNCbl and related cobalamins have been extensively studied for sensitive and practical applications in the detection of cyanide [6,18]. Cobinamides, cobalamin derivatives without the $\alpha$-DMB ligand, are promising cyanide chemosensors that enable detection of cyanide at levels as low as 10 $\mu$M with the naked eye [19,20]. Cobinamide-based methods were further improved by using various spectrophotometric instruments, and the detection limit was decreased to 1.0 $\mu$M cyanide, which could be applied for the detection of biological and non-biological cyanide [21,22,23].

Glutathionylcobalamin (GSCbl) is a vitamin B$_{12}$ derivative that contains glutathione as the upper axial ligand to cobalt via a Co–S bond (Scheme 1). In the present study, we discovered that cyanide reacted with GSCbl, generating CNCbl and reduced glutathione (GSH) via the formation of diCNCbl intermediate (Scheme 2). This reaction was induced by the nucleophilic attack of cyanide anion (CN$^-$). Based on the reaction of GSCbl with cyanide, diCNCbl, CNCbl and GSH were applied for the detection of cyanide by the naked eye, spectrophotometric quantitation and fluorometric quantitation, respectively. All of these methods are highly specific for CN$^-$ without competition against other anions prevalent in biological and non-biological samples. The detection limits of GSCbl-based methods for the detection of cyanide were 520 $\mu$g L$^{-1}$ (20 $\mu$M) for the naked eye, 26 $\mu$g L$^{-1}$ (1.0 $\mu$M) for the spectrophotometric assay and 31 $\mu$g L$^{-1}$ (1.2 $\mu$M) for the fluorometric assay. The sensitivities of the spectrophotometric assay and fluorometric assay were sufficient to detect the maximum concentration of cyanide in drinking water (0.07 mg L$^{-1}$ $\approx$ 2.7 $\mu$M) set by the World Health Organization and European Union. These results provide new GSCbl-based methods for the detection of cyanide, which are straightforward or simple, highly specific and sensitive, and could be applied for the detection of biological and non-biological cyanide.
Scheme 1. Structural formula of cobalamin (left) and the schematic representation of GSCbl (lower right). R = cyanide in CNCo, and glutathione (upper right) in GSCbl.
2. Experimental

2.1. Materials

Chemicals were purchased from Sigma, unless otherwise indicated. GSCbl was synthesised in the reaction of aquacobalamin (OH₂Cbl) with reduced glutathione (GSH) by following a patented method (US patent number 7,030,105) with extensive washing to remove free GSH as previously described [24]. Concentrations of GSCbl were determined based on ε₅₃₄ nm = 7.97 mM⁻¹ cm⁻¹ [25]. Potassium cyanide (KCN) solution was freshly prepared in 20 mM NaOH and neutralised before use and a cyanide standard from Sigma used for quality control of determination methods. Chloro-2,4-dinitrobenzene (CDNB) and monochlorobimane (MCB) were freshly dissolved in methanol for use. Stock solutions (500 mM) of Cl⁻, F⁻, Br⁻, I⁻, SCN⁻, S²⁻, SO₃²⁻, S₂O₃²⁻, HSO₃⁻, NO₃⁻, HCO₃⁻, PO₄³⁻ or SO₄²⁻ were prepared by direct dissolution of proper amounts of sodium salts in distilled and deionised water. All chemicals and solvents were of analytical grade.

2.2. Reaction of GSCbl with cyanide and spectrophotometric determination of cyanide

Reactions contained 20–50 μM GSCbl in 50 mM Tris/HCl pH 7.5 or indicated buffers and were initiated by the addition of indicated concentrations of KCN. Following incubation in the dark at room temperature, absorption spectra were recorded using a Cary 100 UV-Vis spectrophotometer (Varion). Absorption spectra of the reaction were compared with absorption spectra of the indicated authentic cobalamins in the same buffer. The absorption spectrum of diCNCbl was obtained from the reaction of 20 μM CNCbl with 10 mM KCN in 50 mM Tris/HCl pH 7.5. Influence of pH on the generation of diCNCbl and CNCbl was examined in the range of pH = 4.0–12.0 using buffers of 50 mM Na citrate pH 4.0–5.0, 50 mM MES pH 5.5–6.5, 50 mM Tris/HCl pH 7.0–8.5, 50 mM CHES 9.0–10.0, 50 mM NaHCO₃ pH 10.5–11.0 and 50 mM Na₂HPO₄ pH 11.5–12.0. The formation of diCNCbl and CNCbl was followed at 580 and 360 nm, respectively. Maximum levels of diCNCbl and CNCbl were measured by taking maximum values of ΔA₅₈₀ nm (A₅₈₀ nm at 10 min – A₅₈₀ nm at 0 min) and maximum values of ΔA₃₆₁ nm (A₃₆₁ nm at 120 min – A₃₆₁ nm at 0 min).

![Scheme 2](image-url)

Scheme 2. Mechanism of the GSCbl-based cyanide detection. CN⁻ triggers the generation of CNCbl (right) and GSH via diCNCbl intermediate (middle).
The generation of CNCbl was titrated with 50 μM GSCbl and increasing concentrations of KCN (0–200 μM = 0–5200 μg CN⁻ L⁻¹) in 50 mM Tris/HCl pH 9.0. Absorption spectra were recorded after reactions were completed by incubation for 2 h in the dark at room temperature. The molar ratio of CNCbl and CN was estimated from the plot of ΔA361 nm (Δε361 nm = 14.2 mM⁻¹cm⁻¹) versus KCN concentrations by simple linear regression analysis. The detection limit was calculated according to IUPAC recommendation [26]: lower limit of detection = 3SD/√n, SD is the standard deviation of the blank measurements (n ≥ 7), s is the slope of the titration curve.

2.3. HPLC analysis

Cobalamin product was identified by HPLC analysis as previously described [27]. A reaction mixture was prepared with 50 μM GSCbl and 200 μM KCN (5200 μg CN⁻ L⁻¹) in 50 mM Tris/HCl pH 7.5. After incubation of the reaction mixture for 2 h in the dark at room temperature, the mixture was loaded on an Inerstil ODS-3 V C₁₈ reversed phase column (250 × 4.6 mm, 5 μm, GL Sciences). The column was then eluted with a gradient ranging from 0% to 40% acetonitrile in 0.1% TFA over 40 min at a flow rate of 1 mL min⁻¹ while monitoring the eluent at 254 nm. Under these conditions, standard cobalamins OH₂Cbl, CNCbl and GSCbl were eluted at retention times of 17.6 min, 20.9 min and 22.6 min, respectively. The retention time of the cobalamin product from the reaction of GSCbl with KCN was compared with the retention times of standard cobalamins.

Glutathione product was identified and quantified by HPLC analysis as previously described [24,27]. Briefly, reaction mixtures were prepared with 50 μM GSCbl and 0–200 μM KCN (0–5200 μg CN⁻ L⁻¹) in 50 mM Tris/HCl pH 7.5. After incubation of the reaction mixtures for 2 h in the dark at room temperature, amino groups of glutathione were derivatised with 2,3-dinitrofluorobenzene following the reaction of free thiols with monooiodoacetic acid and injected on a Bondclone NH₂ column (300 mm × 3.9 mm, 10 μm, Phenomenex) equilibrated with solvent A of 4:1 (v/v) methanol/water. The column was eluted using solvent B (the mixture of 400 mL of solvent A with a 100 mL solution of 272 g sodium acetate trihydrate, 122 mL water and 373 mL glacial acetic acid) under the following conditions: from 0–5 min, isocratic 30% solvent B; from 5–30 min, linear gradient from 30–100% solvent B. Elution peaks of GSH were monitored by measuring the absorption at 355 nm. The retention time of the glutathione product from the reaction of GSCbl with KCN was compared with the retention times of standard GSH and GSSG. Concentrations of the GSH product were determined by comparing integrated peak areas with the standard curve obtained using commercial GSH compound. The stoichiometry of GSH:CN⁻ was estimated from the plot of GSH concentrations versus KCN concentrations by a simple linear regression analysis.

2.4. Preparation of glutathione S-transferase

Glutathione S-transferase (GST) from Schistosoma japonicum was prepared by over expression of the encoding gene in an expression vector pGEX-4T3 (GE Healthcare). Pre-culture of E. coli BL21 (DE3) (Novagen) harbouring the plasmid pGEX-4T3 was grown in LB medium/ampicillin (100 μg/mL) at 37°C overnight. The main culture of 1 L LB/
ampicillin (100 μg/mL) was inoculated with 1% pre-culture and incubated at 37°C to reach A600 nm ≈ 0.8. Gene expression was induced by the addition of 50 μM isopropyl β-D-thiogalactopyranoside (IPTG, Qiagen). After 5 h of incubation at 37°C, cells were harvested and lysed for protein purification. GST was purified by an affinity column chromatography using a GSH sepharose 4b affinity column (5 mL column volume, GE Healthcare) following manufacturer’s instruction. Purified GST was extensively dialysed in PBS to remove GSH contaminated during affinity purification. Protein concentrations were determined by Bradford assay [28] and GST activity was determined spectrophotometrically using CDNB (Δε340 nm = 9.6 mM−1 cm−1) [29].

2.5. Fluorometric determination of cyanide

Reaction mixtures were prepared with 20 μM GSCbl and 0–20 μM KCN (0–520 μg CN− L−1) in 50 mM Tris/HCl pH 9.0, and then incubated for 2 h in the dark at room temperature. The pH of reaction mixtures was adjusted to 7.5 by the addition HCl, and then the fluorescent reagent MCB (100 μM) and 1U/mL GST were added. After 20 min of incubation at room temperature, the fluorescence intensity was measured using a Synergy HT microplate reader (BioTek) using an excitation wavelength at 360 ± 40 nm and an emission wavelength of 460 ± 40 nm. GSH concentrations were determined by comparison with a standard curve obtained with commercial GSH in the presence of 20 μM GSCbl as described above. The detection limit was calculated according to IUPAC recommendation [26]: lower limit of detection = 3SDb/s, SDb is the standard deviation of the blank measurements (n ≥ 7), s is the slope of the standard curve.

3. Results

3.1. Displacement of the glutathione ligand of GSCbl by cyanide, generating CNCbl via diCNCbl intermediate

The addition of excess cyanide to GSCbl induced immediate and significant changes in the absorption spectrum, showing the development of the absorption peaks at 368 nm, 541 nm and 580 nm (Figure 1(a,c)) those are characteristic of diCNCbl (Figure 1(d)). Further incubation showed additional slow changes in the absorption spectrum of diCNCbl, with absorption decreases at 368 nm, 541 nm and 580 nm, and concomitant absorption increases at 361 nm, 518 nm and 550 nm. In the slow reaction phase, isosbestic points were observed at 364 nm, 399 nm and 560 nm (Figure 1(b,c)). The generated absorption spectrum was characteristic of cyanocobalamin (CNCbl) (Figure 1(d)). Similar absorption spectral changes were also observed for the reaction of GSCbl with low concentrations of cyanide at a ratio of [GSCbl]/[cyanide] = 2.0 (Figure 2). Absorption peaks at 368 nm and 580 nm were developed in the fast reaction phase, which was characteristic of the generation of diCNCbl, although to a lower extent than that obtained with excess cyanide (Figure 2(a)). In the later slow reaction phase, absorption peaks at 368 nm and 580 nm decreased with concomitant absorption increases at 361 nm and 550 nm (Figure 2(c)), which was characteristic of the conversion of diCNCbl into CNCbl. These results indicate
that cyanide reacts with GSCbl and displaces the glutathione ligand generating CNCbl via diCNCbl intermediate.

3.2. Induction of reaction by the nucleophilic attack of cyanide anion

The influence of pH on the reaction of GSCbl with cyanide was examined in the range of pH = 4.0–12.0 by incubation of fixed concentrations of GSCbl and cyanide at the ratio of [GSCbl]/[cyanide] = 0.8. The generation of the reaction intermediate diCNCbl and the reaction product CNCbl was followed by measuring the A580 nm and A361 nm, respectively (Figure 3 (a,b)). The absorption at 580 nm increased for 10 min and then decreased with further incubation (Figure 3(a)), indicating the rapid generation of diCNCbl and its disappearance.
The maximum levels of diCNCbl at 10 min were increased by increasing pH, which was plotted in a sigmoidal-shaped curve (Figure 3(c)). It was evident that the optimum range for the generation of diCNCbl was pH > 9.0. This result indicates that the reaction of GSCbl with cyanide is induced by the nucleophilic attack of cyanide anion (the pK$_a$ of HCN/CN$^-$ = 9.04 [30]). The absorption at 361 nm increased following incubation and slowly reached equilibrium, indicating the saturation of CNCbl generation. Levels of CNCbl at 2 h were increased by increasing pH, which also produced a plot in a sigmoidal-shaped curve (Figure 3(d)). The generation of CNCbl appeared to be optimised in the range of pH ≥ 8.0. However, above pH = 8.5, small interference in the absorption at 361 nm occurred, which was due to the enhanced stability of diCNCbl at high pH and its incomplete conversion into CNCbl.
3.3. Confirmation of reaction products, CNCbl and GSH

Products from the reaction of GSCbl with cyanide were identified by HPLC analysis, as described in the materials and methods (Section 2.3). As expected in the absorption spectrum for the reaction of GSCbl with cyanide (Figure 1(c,d)), the cobalamin product was identified as CNCbl being eluted at the same retention time of 20.9 min (Figure 4(a)). The other reaction product was assumed to be glutathione released from GSCbl by cyanide displacement. HPLC analysis revealed that the glutathione product was in the reduced form, GSH being eluted at the same retention time of 17.5 min (Figure 4(b)). In addition, a quantitative analysis showed a linearly proportional relationship between GSH and CN$^-$ (Figure 4(c)). A regression analysis yielded a slope of 0.76 ± 0.04 ($n \geq 3$, $r^2 = 0.9954$) that corresponds to the molar ratio of [GSH]:[CN$^-$] $\approx 1.0:1.3$. 

Figure 3. Influence of pH on the generation of diCNCbl and CNCbl. The generation of diCNCbl (a) and CNCbl (b) in the reaction of 50 μM GSCbl with 40 μM KCN in buffers of pH = 4.0–12.0. Arrows indicate the increases in the generation of diCNCbl at 10 min and CNCbl at 120 min by increasing pH of the reaction buffer. Maximum levels of diCNCbl and CNCbl were measured, as described in the materials and methods, and plotted against pH (c and d, respectively).
3.4. Anion specificity for displacement of the glutathione ligand of GSCbl

To examine the anion specificity for the reaction of GSCbl with CN$^-$, various anions Cl$^-$, F$^-$, Br$^-$, I$^-$, SCN$^-$, S$^{2-}$, SO$_3^{2-}$, S$_2$O$_5^{2-}$, HSO$_3^−$, NO$_3^−$, HCO$_3^−$, PO$_4^{3-}$ or SO$_4^{2-}$ in excess (100 equiv.) were incubated with GSCbl in 50 mM Tris/HCl pH 7.5. The addition of CN$^-$ to GSCbl immediately developed the absorption spectrum of diCNCbl ($\lambda_{max} = 368$ nm, 541 nm and 580 nm) (Figure 5(a)). Two anions, SO$_3^{2-}$ and HSO$_3^−$, appeared to react with GSCbl developing an absorption spectrum of an unidentified cobalamin derivative (see Supplementary figures). Except for SO$_3^{2-}$ and HSO$_3^−$, all other tested anions did not induced a significant change in the absorption spectrum of GSCbl and the characteristic
absorption bands ($\lambda_{\text{max}} = 288 \text{ nm}, 334 \text{ nm}, 428 \text{ nm} \text{ and } 534 \text{ nm}$) remained unaffected, indicating no glutathione ligand displacement occurred (Figure 5(a)). Moreover, the addition of $\text{CN}^-$ to GSCbl in the presence of other additional anions, including $\text{SO}_3^{2-}$ and $\text{HSO}_3^-$, at 100-fold excess over $\text{CN}^-$ showed the same absorption spectral changes of diCNCbl generation. These results indicate that displacement of the glutathione ligand of GSCbl is specific for $\text{CN}^-$ without competition against any of other tested anions.

3.5. Naked eye detection of cyanide using diCNCbl

The reaction of GSCbl with cyanide generated the intermediate diCNCbl, which was accompanied by a large bathochromic shift ($\Delta \lambda_{\text{max}} = 45 \text{ nm}$) (Figure 1(a)) that could be used for the detection of cyanide by the naked eye. The addition of excess $\text{CN}^-$ to GSCbl generated diCNCbl, resulting in an immediate colour change from red to violet that was not detected with other anions ($5 \text{ mM each of } \text{Cl}^-, \text{F}^-, \text{Br}^-, \text{I}^-, \text{SCN}^-, \text{NO}_3^-, \text{HCO}_3^-, \text{PO}_4^{3-}, \text{S}^{2-}, \text{SO}_3^{2-}, \text{S}_2\text{O}_3^{2-}, \text{HSO}_3^- \text{ or } \text{SO}_4^{2-}$) (Figure 5(b)). Moreover, solutions of GSCbl with any of tested anions, including $\text{SO}_3^{2-}$ and $\text{HSO}_3^-$, at 200-fold excess over $\text{CN}^-$ turned to violet after the addition of $\text{CN}^-$ (Figure 5(c)), consistently indicating the $\text{CN}^-$-specific displacement of glutathione ligand of GSCbl. Under optimised conditions (20 $\mu$M GSCbl in the buffer pH = 9.0–12.0 and 10 min incubation), GSCbl could be applied for the naked eye detection of cyanide with an apparent detection limit of 20 $\mu$M (520 $\mu$g L$^{-1}$) (Figure 5(d)).

3.6. Quantitation of cyanide by spectrometric determination of CNCbl

The generation of CNCbl in the reaction of GSCbl with increasing concentrations of cyanide was measured by UV-Vis spectroscopy. Absorption spectra for the reaction product showed the increase of CNCbl in response to increasing the concentration of
cyanide (Figure 6(a)). The plot of A361 nm versus concentrations of cyanide revealed a linear proportional relationship between CNCbl and CN\(^{-}\) in the cyanide concentration range of 0–50 μM (26–520 μg L\(^{-1}\)) (Figure 6(b)). A regression analysis yielded a slope of 0.83 ± 0.09 (n ≥ 6, \(r^2 = 0.9922\)) that corresponds to the molar ratio of [CNCbl]:[CN\(^{-}\)] ≈ 1.0:1.2 (Figure 6(b) inset). This spectrometric titration could be applied for cyanide quantitation with a detection limit of 1.0 μM (26 μg L\(^{-1}\)).

3.7. Quantitation of cyanide by fluorometric determination of GSH

GSH released from GSCbl by cyanide displacement was determined to be linearly proportional, therefore, it could be used for cyanide quantitation by HPLC analysis (Figure 4(b)). However, for simplicity with high sensitivity, we applied a thiol-specific fluorescence reagent, MCB, for GSH determination. GSCbl was incubated with different concentrations of cyanide and GSH released from GSCbl was conjugated with MCB by catalysis of GST. The fluorescence intensities measured for the determination of GSH were linearly proportional to CN\(^{-}\) in the cyanide concentration range of 0–20 μM (Figure 7). A regression analysis yielded a slope of 0.66 ± 0.05 (n ≥ 6, \(r^2 = 0.9941\)) that corresponds to the molar ratio of [GSH]:[CN\(^{-}\)] ≈ 1.0:1.5 (Figure 6(b) inset). This fluorometric determination of GSH could be applied for cyanide quantitation with a detection limit of 1.2 μM (31 μg L\(^{-1}\)).

3.8. Application in water samples

The applicability of GSCbl-based methods for the detection of cyanide in real samples was examined by the addition of cyanide to tap water and pond water. Concentrations

Figure 6. Spectrometric titration of CNCbl for quantitation of cyanide. Absorption spectra (a) were obtained for the reaction of 50 μM GSCbl with 0–200 μM KCN (0–5200 μg CN\(^{-}\) L\(^{-1}\)) in 50 mM Tris/HCl pH 9.0 after 2 h of incubation. The arrow indicates the increase in A361 nm by increasing concentrations of KCN. (b) Plot of ΔA361 nm versus the concentration of KCN shows a linear proportional generation of CNCbl (inset). Solid lines are for regression analyses of data.
of cyanide in water samples were determined as described in the materials and methods, comparing with standard curves obtained in distilled water. The naked eye method consistently yielded a detection limit of 20 μM (520 μg L⁻¹) cyanide in water samples (Figure 8). Results obtained by spectrophotometric assay and fluorometric assay were in good agreement with the amounts of cyanide added in water samples (Table 1).

4. Discussion

GSCbl is a physiologically relevant thiolatocobalamin and a precursor in the biosynthesis of 5′-adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl), the enzyme cofactors of methylmalonyl-CoA mutase and methionine synthase, respectively [31,32]. Moreover, GSCbl has attracted considerable interest due to its therapeutic application for diseases involving oxidative stress [33,34]. In the present study, we developed new methods for the detection of cyanide based on displacement of the β-GS ligand of GSCbl by cyanide (Scheme 2). The reaction of GSCbl with cyanide generated CNCbl and GSH via an intermediate diCNCbl. The influence of pH on the generation of diCNCbl showed that the reaction of GSCbl with cyanide is initiated by the nucleophilic attack of CN⁻ (Figure 3 (a,c)). The generation of the reaction intermediate diCNCbl requires the displacement of both the α-DMB ligand and the β-glutathione ligand of GSCbl. It is known that the generation of diCNCbl from CNCbl by cyanide displacement of the α-DMB ligand requires excess cyanide over CNCbl [17]. However, diCNCbl was generated in the reaction of GSCbl with excess cyanide, but also with low concentrations of cyanide.
Therefore, the generation of diCNCbl from GSCbl is likely through sequential cyanide displacement of the $\alpha$-DMB ligand followed by the $\beta$-GS ligand. The reaction of MeCbl with cyanide showed displacement of the $\alpha$-DMB ligand, but no displacement of the $\beta$-methyl ligand. An intermediate, ($\beta$-GS)($\alpha$-CN)Cbl, may be formed prior to the formation of diCNCbl, however the Co–S bond of GSCbl in the base-off state, in which the $\alpha$-DMB ligand is removed and replaced by exogenous ligand, was assumed to be too unstable to be detected. Therefore, ($\beta$-GS)($\alpha$-CN)Cbl would be highly transient, if it forms, and be immediately converted into diCNCbl by cyanide displacement of the $\beta$-GS ligand. The reaction intermediate diCNCbl was slowly converted into CNCbl by dissociation of the $\alpha$-CN ligand in the reaction with low concentrations of cyanide. The generation of CNCbl and GSH from GSCbl were linearly

**Figure 8.** Detection of cyanide in water samples by the naked eye. Indicated $\mu$M concentrations of cyanide were added to tap water (a) and pond water (b) samples. Colours of solutions were obtained as described in Figure 5(d). Violet-coloured vials with $\geq$ 20 $\mu$M cyanide (520 $\mu$g L$^{-1}$) are indicated in boxes.

**Table 1.** Determination of cyanide in water samples by spectrophotometric assay and fluorometric assay.

| Cyanide added, $\mu$M ($\mu$g L$^{-1}$) | Tap water | Pond water |
|----------------------------------------|-----------|------------|
|                                        | Spectrometric determination, $\mu$M | Fluorometric determination, $\mu$M | Spectrometric determination, $\mu$M | Fluorometric determination, $\mu$M |
| 2.0 (52)                               | 2.0 ± 0.1 | 2.1 ± 0.3 | 1.7 ± 0.1 | 2.0 ± 0.3 |
| 5.0 (130)                              | 5.1 ± 0.1 | 5.1 ± 0.4 | 3.7 ± 0.5 | 5.0 ± 0.4 |
| 10 (260)                               | 11.5 ± 0.4 | 10.1 ± 0.8 | 9.1 ± 0.3 | 8.9 ± 0.4 |

Determined values are means ± SD ($n \geq 6$).
proportional to concentrations of cyanide, indicating that the reaction of GSCbl with cyanide is almost irreversible and has a high equilibrium constant for the formation of products. This aspect is an advantage to apply GSCbl for the detection of cyanide with a high sensitivity.

The reaction intermediate diCNCbl exhibits violet colour that is ready to be distinguished from red-coloured GSCbl by the naked eye. Based on the colour difference, we developed a straightforward method for the detection of cyanide. The GSCbl-based naked eye method had an apparent detection limit of 20 μM (520 μgL⁻¹) cyanide (Figure 5(d)), which is 30-fold more sensitive than the previously developed CNCbl-based method [17] and close to the detection limit of 10 μM (260 μgL⁻¹) obtained using a cobinamide derivative optimised for the detection of cyanide [20,23] (Table 2). Moreover, the generation of diCNCbl from GSCbl is highly specific for CN⁻ without competition against other relevant anions prevalent in biological and non-biological samples. Two anions SO₃²⁻ and HSO₃⁻ react with GSCbl generating an unidentified cobalamin derivative (see Supplementary figures), which might disturb the GSCbl-based detection of cyanide. However the naked eye detection of cyanide was not significantly disturbed in the presence of excess SO₃²⁻ and HSO₃⁻ over CN⁻ (Figure 5(b)). In addition, UV-Vis spectroscopy showed that CN⁻ converted the unidentified cobalamin derivative generated by SO₃²⁻ and HSO₃⁻ to CNCbl via diCNCbl intermediate (see Supplementary figures). This result indicates that the interference of SO₃²⁻ and HSO₃⁻ would be insignificant, at least, in the naked eye detection of cyanide, although further studies would be required to reveal the specificity of the GSCbl-based detection of cyanide against SO₃²⁻ and HSO₃⁻.

We also developed a GSCbl-based spectrometric quantitative method for cyanide detection. The generation of CNCbl in the reaction of GSCbl with cyanide could be sensitively determined using the difference in absorption at 361 nm (Δε361 nm = 14.2 mM⁻¹cm⁻¹) (Figure 6). The GSCbl-based spectrometric detection of cyanide was simple and the sensitivity of the method (detection limit of 1.0 μM (26 μgL⁻¹)) was 10-fold higher than that of GSCbl-based naked eye detection of cyanide (Table 2). Another product GSH generated in the reaction of GSCbl with cyanide could also be applied for the detection of cyanide. GSH can be determined using various methods, such as colorimetric assays, fluorometric assays and HPLC analysis. We adopted a fluorometric assay using a specific fluorescent reagent, MCB, which provides a high sensitivity for GSH determination and is conjugated specifically with GSH by the catalysis of GST. The

### Table 2. Comparison of GSCbl-based methods with other cobalamin derivatives-based methods for cyanide detection.

| Reagent                  | Method       | Lower detection limit, μM (μgL⁻¹) | Range of detection, μM (μgL⁻¹) | Reference |
|--------------------------|--------------|-----------------------------------|-------------------------------|-----------|
| Glutathionylcobalamin    | Visual       | 20 (520)                          | ≥ 20 (≥520)                   | This work |
|                          | UV-Vis       | 1.0 (26)                          | 1.0–50 (26–1,300)             | This work |
|                          | Fluorometric | 1.2 (31)                          | 1.2–20 (31–520)               | This work |
| Cyanocobalamin           | Visual       | 600 (15,600)                      | ≥ 600 (≥15,600)               | [17]      |
|                          | UV-Vis       | 250 (6,500)                       | 250–1,000 (6,500–26,000)      | [17]      |
| Cobyricin acid           | Visual       | 10 (260)                          | 2–45 (52–1,170)               | [20,23]   |
| Cobinamide               | Visual       | 30 (780)                          | ≥30 (≥780)                    | [21]      |
|                          | UV-Vis       | 1.0 (26)                          | 2.5–300 (65–7,800)            | [21]      |
| Cobyricin acid ester     | UV-Vis       | 0.8 (20)                          | 1.5–46 (40–1,200)             | [22]      |
GSCbl-based fluorometric assay yielded a detection limit of 1.2 μM cyanide that is highly sensitive and four-fold lower than the maximum cyanide concentration in drinking water (0.07 mg L\(^{-1}\) corresponding to ~2.7 μM) set by the World Health Organization and European Union [4,5]. We showed that GSCbl-based methods could be used to detect cyanide in real water samples with high accuracy and sensitivity (Table 1).

In conclusion, we discovered that cyanide reacts with GSCbl, generating CNCbl and GSH via diCNCbl intermediate. Based on this reaction, we developed various new methods for the detection of cyanide (Scheme 2). The reaction intermediate diCNCbl could be applied for the naked eye detection of cyanide with a detection limit of 20 μM (520 μg L\(^{-1}\)). The reaction products CNCbl and GSH could be applied for the quantitative determination of cyanide with detection limits of 1.0 μM (26 μg L\(^{-1}\)) and 1.2 μM (31 μg L\(^{-1}\)), respectively. These GSCbl-based methods are straightforward or simple, highly specific and sensitive, therefore these methods have great applicability for the detection of cyanide in biological and non-biological samples.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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References

[1] D.M. Beasley and W.I. Glass, Occup. Med. (Lond.). 48, 427 (1998). doi:10.1093/occmed/48.7.427.
[2] K.J. Van Buuren, P. Nicholis and B.F. Van Gelder, Biochim. Biophys. Acta 256, 258 (1972). doi:10.1016/0005-2728(72)90057-6.
[3] R.M. Gleadow and B.L. Moller, Annu. Rev. Plant. Biol. 65, 155 (2014). doi:10.1146/annurev-arplant-050213-040027.
[4] WHO, Guidelines for Drinking-Water Quality, Vol. 1 Recommendations (World Health Organization, Geneva, 2008)
[5] EU Council Directive on the Quality of Water Intended for Human Consumption, 98/83/EC (The Council of the European Union, Brussels, 1998).
[6] J. Ma and P.K. Dasgupta, Anal. Chim. Acta 673, 117 (2010). doi:10.1016/j.aca.2010.05.042.
[7] US Environmental Protection Agency, Method 9014 (United States Environmental Protection Agency, Cincinnati, OH, 2014)
[8] US Environmental Protection Agency, Method 9213 (United States Environmental Protection Agency, Cincinnati, OH, 1996)
[9] US Environmental Protection Agency, Method OIA-1677 (United States Environmental Protection Agency, Cincinnati, OH, 2004)
[10] J.H. Bradbury, Food Chem. 113, 1329 (2009). doi:10.1016/j.foodchem.2008.08.081.
[11] M. Bradbury, S. Egan, J. Bradbury and J. Sci, Food Agric. 79, 593 (1999). doi:10.1002/(SICI)1097-0010(19990315)79:4<593::AID-JSFA222>3.0.CO;2-2.
[12] S.A. Essers, M. Bosveld, V. Grift, R.M. Der and A.G. Voragen, J. Sci. Food Agric. 63, 287 (1993). doi:10.1002/jsfa.2740630305.
[13] F.J. Huo, J. Kang, C.X. Yin, J.B. Chao and Y.B. Zhang, Sensor Actuat. B Chem. 215, 93 (2015). doi:10.1016/j.snb.2015.03.047.
[14] Y.K. Yue, F.J. Huo, C.X. Yin, J.B. Chao and Y.B. Zhang, Sensor Actuat. B Chem. 212, 451 (2015). doi:10.1016/j.snb.2015.02.074.
[15] H. Khajehshari and M.M. Bordbar, Sensor Actuat. B Chem. 209, 1015 (2015). doi:10.1016/j.snb.2014.10.053.
[16] F.H. Zelder, Inorg. Chem. 47, 1264 (2008). doi:10.1021/ic702368b.
[17] F. Zelder and L. Tivana, Org. Biomol. Chem. 13, 14 (2015). doi:10.1039/C4OB01889C.
[18] J. Ma, P.K. Dasgupta, F.H. Zelder and G.R. Boss, Anal. Chim. Acta 736, 78 (2012). doi:10.1016/j.aca.2012.05.028.
[19] C. Mannel-Croise and F. Zelder, Inorg. Chem. 48, 1272 (2009). doi:10.1021/ic900053h.
[20] W.C. Blackledge, C.W. Blackledge, A. Griesel, S.B. Mahon, M. Brenner, R.B. Pilz and G.R. Boss, Anal. Chem. 82, 4216 (2010). doi:10.1021/ac100519z.
[21] S.S. Hassan, M.S. Hamza and A.E. Kelany, Talanta 71, 1088 (2007). doi:10.1016/j.talanta.2006.06.010.
[22] C. Mannel-Croise, B. Probst and F. Zelder, Anal. Chem. 81, 9493 (2009). doi:10.1021/ac901977u.
[23] J. Jeong, J. Park and J. Kim, Biochem. Biophys. Res. Commun. 443, 173 (2014). doi:10.1016/j.bbrc.2013.11.075.
[24] R.K. Suto, N.E. Brasch, O.P. Anderson and R.G. Finke, Inorg. Chem. 40, 2686 (2001). doi:10.1021/ic001365n.
[25] H.M.N.H. Irving and H. Freiser, in Compendium of Analytical Nomenclature: definitive Rules 1977, (Pergamon Press, Oxford, NY, 1978), 1st ed.
[26] L. Hannibal, C. Gherasim, D.W. Jacobsen and R. Banerjee, J. Biol. Chem. 284, 33418 (2009). doi:10.1074/jbc.M109.057877.
[27] M.M. Bradford, Anal. Biochem. 72, 248 (1976). doi:10.1016/0003-2697(76)90527-3.
[28] D.B. Smith, M.R. Rubira, R.J. Simpson, K.M. Davern, W.U. Tiu, P.G. Board and G.F. Mitchell, Mol. Biochem. Parasitol. 27, 249 (1988). doi:10.1016/0166-6851(88)90044-8.
[29] K.L. Brown and S. Peck, Inorg. Chem. 26, 4143 (1987). doi:10.1021/ic00272a001.
[30] L. Hannibal, A. Axhemi, A.V. Glushchenko, E.S. Moreira, N.E. Brasch and D.W. Jacobsen, Clin. Chem. Lab. Med. 46, 1739 (2008). doi:10.1515/CCLM.2008.356.
[31] E. Pezacka, R. Green and D.W. Jacobsen, Biochem. Biophys. Res. Commun. 169, 443 (1990). doi:10.1006.bbrc.1990.0351.
[32] C.S. Birch, N.E. Brasch, A. McCaddon and J.H. Williams, Free Radic. Biol. Med. 47, 184 (2009). doi:10.1016/j.freeradbiomed.2009.04.023.
[33] A. McCaddon, B. Regland, P. Hudson and G. Davies, Neurology 58, 1395 (2002). doi:10.1212/WNL.58.9.1395.
[34] J.B. Samuel, G.C. Andrew, R. Van Eldik and N.E. Brasch, Inorganica Chim. Acta 348, 221 (2003). doi:10.1016/S0020-1693(02)01475-5.
[35] K.S. Conrad and T.C. Brunold, Inorg. Chem. 50, 8755 (2011). doi:10.1021/ic200428r.
[36] L. Hannibal, C.A. Smith and D.W. Jacobsen, Inorg. Chem. 49, 9921 (2010). doi:10.1021/ic101173b.
[37] L. Xia, A.G. Cregan, L.A. Berben and N.E. Brasch, Inorg. Chem. 43, 6848 (2004). doi:10.1021/ic040022c.