Journal of Hazardous Materials, 28 (1991) 241-249
Elsevier Science Publishers B.V., Amsterdam

The determination of cyanide in water and biological tissues by methemoglobin

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(Received October 12, 1990; accepted in revised form February 14, 1991)

Abstract

The authors developed a simple and sensitive method for the determination of cyanide using spectrophotometry to measure changes caused by cyanide in the absorption spectra of methemoglobin. Met-hemes in the hemoglobin molecules react with cyanide rapidly to form a cyanomethemoglobin complex, even at low concentrations of cyanide, in a stoichiometric manner. After adding cyanide, the absorption spectra of methemoglobin changed with a typical decrease in absorbance at 630 nm, and an increase in absorbance at 540 nm. These changes were closely correlated with the concentrations of cyanide ion. From the results, it was shown that 0.2 μg/ml of cyanide ion in water and 0.4 μg/ml in biological tissues can be detected by this method. The method may be useful for the convenient and sensitive detection of cyanide in waste water and biological tissues and fluids.

1. Introduction

Since cyanide is strongly poisonous to biological systems, the handling and disposal of this compound is strictly controlled in gilding factories and plants producing integrated circuits, where it is commonly used in large amounts. A convenient and sensitive method for detecting and determining cyanide levels is therefore required in these industrial areas. However, conventional methods for measuring cyanide concentrations are somewhat complicated and time-consuming [1,2]. An improved method for measuring cyanide concentrations in biological tissues and fluids is also required in certain areas of clinical, forensic and veterinary medicine [3].

On the other hand, cyanide is commonly used for measuring hemoglobin concentrations, because it specifically and tightly binds to methemoglobin [4]. This fact prompted us to investigate whether cyanide concentrations might be estimated by measuring changes in the absorption spectra of methemoglobin, due to the binding of cyanide, and whether the changes in absorbance might

be correlated with cyanide concentrations. We observed that the absorption spectra of methemoglobin change according to the increase in cyanide, even at low concentrations. This paper deals with a simple and sensitive procedure for detecting cyanide in water and biological tissues, by using methemoglobin. The method may be useful for conveniently measuring cyanide ion in wastewater.

2. Materials and methods

2.1 Preparation of erythrocyte suspension

Heparinized human bloods were freshly obtained after receiving informed consent from the donors, and were centrifuged at $3{,}000 \times g$ for 10 min. Then, erythrocytes were collected after removing plasma and buffy coats. The cells were washed with chilled isotonic saline twice, by centrifuging at $3{,}000 \times g$ for 10 min, and were suspended in isotonic saline to make a hematocrit value of 5%. The pH of the erythrocyte suspension was adjusted to 7.4 with 0.1 *N* NaOH in isotonic saline.

2.2 Preparation of methemoglobin

Human erythrocytes were lysed with five volumes of distilled water. The lysates were centrifuged at $10{,}000 \times g$ for 20 min to remove ghosts. The supernatant, including hemoglobin, was oxidized by adding a half volume of ferricyanide solutions (20 m*M*) at room temperature. After 15 min, the solutions were passed through a column of Sephadex G-25 (fine, 3×30 cm) previously equilibrated with 200 m*M* potassium phosphate buffer (pH 7.0). By this procedure methemoglobin and ferricyanide were separated from each other. The methemoglobin solution was diluted with 200 m*M* phosphate buffer (pH 7.0) to appropriate concentrations.

3. Reagents

Potassium cyanide and potassium ferricyanide were purchased from Wako (Tokyo) and Sephadex G-25 (fine) from Pharmacia-LKB (Uppsala).

4. Experimental procedures

4.1 Time course of reaction of methemoglobin with cyanide

A solution of 55 μM methemoglobin in heme (2 ml) was placed in a quartz cuvette, and 20 μl of 6 m*M* cyanide solution was added. After mixing, the final cyanide concentration was 59.4 μM. Changes in the absorption spectra of methemoglobin were measured at intervals for up to 5 min between 450 nm and 650 nm by a spectrophotometer (Union SM-401, Union Giken, Osaka).

4.2 Reaction of methemoglobin with cyanide at different concentrations of cyanide

Solutions (20 μl) containing different concentrations of cyanide (final concentrations; 3.3 μM, 5 μM, 12.5 μM, 20 μM, 25 μM, 33.3 μM, 50 μM, 100 μM and 200 μM) were added to 2 ml 55 μM methemoglobin in heme in glass tubes. These reaction mixtures were allowed to stand at room temperature for 30 min, and their absorption spectra were measured between 450 nm and 650 nm. As a control, the absorption spectrum of methemoglobin with 20 μl of distilled water was measured.

4.3 Reaction of cyanide with different concentrations of methemoglobin

Varying volumes of 1.1 mM methemoglobin in heme (20 μl, 30 μl, 40 μl and 50 μl) were added to 2 ml 13.5 μM cyanide solutions. The solutions contained, respectively, 10.9 μM, 16.3 μM, 21.6 μM and 26.8 μM methemoglobin. The mixtures were allowed to stand at room temperature for 30 min and their absorption spectra were measured between 530 nm and 650 nm. The absorption spectra of methemoglobin itself, or those of cyano–methemoglobin, were measured after the addition of each volume of methemoglobin solution to 2 ml distilled water, or to 2 ml distilled water with excess amounts of cyanide, respectively.

4.4 Detection of cyanide in erythrocyte suspension by methemoglobin

The experiments were performed in Conway dishes [5], which contain an outer and inner chamber separated by an annular wall. Into these 1 ml of human erythrocyte suspension (hematocrit, 5%) was placed in the outer chamber, and 2.2 ml 120 μM methemoglobin in heme were placed in the inner chamber. Then, 40 μl cyanide solution with concentrations from 1.34 mM to 21.5 mM were added in the outer chamber, and mixed well for 5 min. Then 2 ml of 10% sulfuric acid was added in the outer chamber, a ground glass cover coated with silicone grease was placed over the dish and the evolution of cyanide allowed to continue for 2 h at room temperature. After 2 h, methemoglobin solutions were collected from the inner chamber and absorption spectra measured as outlined above.

4.5 Determination of methemoglobin concentration

The concentration of methemoglobin used for experiments was determined by using cyanide [6]. In this case, the value 11 was used as millimolar extinction of cyan–methemoglobin at 540 nm.

5. Results

Figure 1 shows the changes in absorption spectra with time during cyan–methemoglobin formation at room temperature. The absorbances at 630 nm

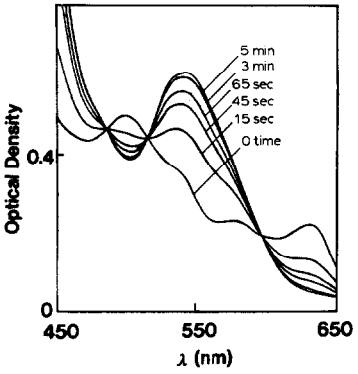

Fig. 1. Changes in absorption spectra of methemoglobin after addition of cyanide. The changes in absorption spectra of 55 μM methemoglobin in heme were measured between 450 nm and 650 nm at intervals up to 5 min after the addition of cyanide (final concentrations; 59.4 μM).

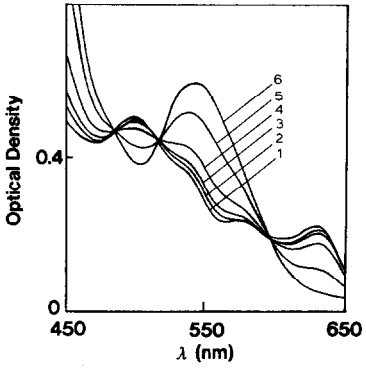

Fig. 2. Measurement of absorption spectra of methemoglobin with different concentrations of cyanide. The absorption spectra of 55 μM methemoglobin in heme with different concentrations of cyanide (3.3 μM to 200 μM), between 450 nm and 650 nm 30 min after adding cyanide to the methemoglobin solutions. The numbers 2 to 10 in the figure show the absorption spectra of methemoglobin with 3.3, 5, 12.5, 20, 25, 33.3, 50, 100 and 200 μM cyanide, respectively. Number 1 in the figure shows the absorption spectrum of methemoglobin without cyanide.

decreased, and those at 540 nm increased after the addition of cyanide (final concentrations of 59.4 μM) to 55 μM methemoglobin in heme. The changes in absorption spectra were not observed after 10 min at this cyanide concentration, showing that ferric heme in methemoglobin was saturated with cyanide.

Figure 2 shows the absorption spectra of 55 μM methemoglobin in heme with different concentrations of cyanide (final concentrations; 3.3 μM, 5 μM, 12.5 μM, 20 μM, 25 μM, 33.3 μM, 50 μM, 10μM and 200 μM). As cyanide concentrations increased, the absorbances decreased at 630 nm, and increased at 540 nm.

Figure 3 is derived from Fig. 2, in which the differences in absorbances at

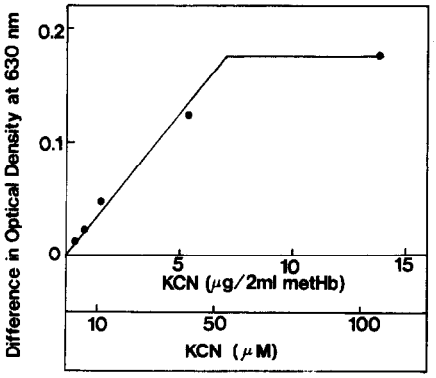

Fig. 3. Changes of differences in optical density at 630 nm of methemoglobin solutions with and without cyanide plotted against cyanide concentrations. The differences in optical density at 630 nm between methemoglobin with cyanide and methemoglobin without cyanide (these values were obtained from Fig. 2) were plotted against cyanide concentrations. The cyanide concentrations are expressed by μg/2 ml methemoglobin solutions, and by molar concentrations for convenience.

630 nm between methemoglobin without cyanide and methemoglobin with different concentrations of cyanide were measured and plotted against the changes in cyanide concentrations. Above 55 μM cyanide, no change in optical density occurred, suggesting that 55 μM of cyanide is possibly bound to an equivalent amount of ferric heme in methemoglobin. This result suggests that cyanide binds to ferric heme in methemoglobin in a 1:1 manner. Below 55 μM cyanide, the difference in absorbance at 630 nm between methemoglobin without cyanide and methemoglobin with cyanide was dependent on the concentrations of cyanide added. Therefore, it is possible to estimate unknown concentrations of cyanide solutions by measuring the differences in absorbances at 630 nm caused by adding the cyanide solution to methemoglobin and calculating the following equation:

$$\text{Methemoglobin } (\mu M \text{ in heme}) \times \frac{\text{OD without CN} - \text{OD with CN}}{\text{OD without CN} - \text{OD with excess CN}}$$

where OD without CN, OD with CN, and OD with excess CN are the optical densities of methemoglobin solutions without cyanide, the optical densities of methemoglobin solutions with unknown concentrations of cyanide, and the optical densities of methemoglobin solutions with excess amounts of cyanide, respectively. Methemoglobin concentrations in the above equation are easily calculated by measuring the absorbances at 540 nm after addition of excess amounts of cyanide according to the conventional method [6].

Since cyanide may be determined by adding a small volume of high concentrations of methemoglobin solutions to cyanide solutions, we measured the changes in absorption spectra due to the presence of different concentrations

of methemoglobin under such conditions (Figs. 4A–C). Figures 4(A)–(C) show the changes in absorption spectra for different concentrations of methemoglobin adding 20, 30, 40 and 50 μl 1.1 mM methemoglobin to 2 ml 13.5 μM cyanide solutions. As the methemoglobin concentrations decrease, the absorption spectra of methemoglobin with cyanide (b, in each figure) approaches those of cyan–methemoglobin (c, in each figure).

Figure 4(D) shows changes in the ratio (a−b)/(a−c) as a percentage of methemoglobin concentrations, where a, b and c show the optical density at 630 nm of methemoglobin, the optical density of methemoglobin in 13.5 μM cyanide, and the optical density of cyano–methemoglobin, respectively. The ratio decreased linearly with an increase in methemoglobin concentrations

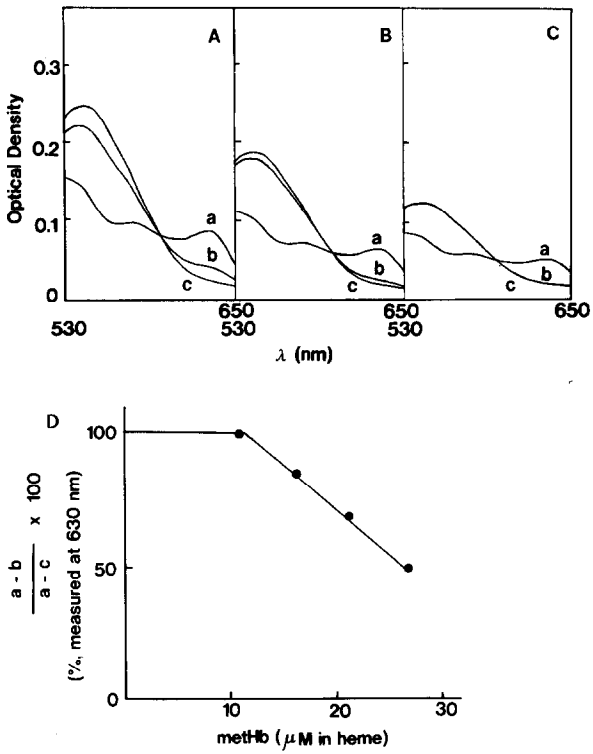

Fig. 4. Effects of cyanide on absorption spectra of methemoglobin at different concentrations of methemoglobin. Various quantities of 1.1 mM methemoglobin in heme (20, 30, 40 and 50 μl) were added to: (a) 2.0 ml distilled water without cyanide, (b) 2 ml of 13.5 μM cyanide solutions, and (c) 2 ml distilled water with excess amounts of cyanide. After mixing well, the samples were allowed to stand for 30 min at room temperature and the absorption spectra recorded between 530 nm and 650 nm. (A) 40 μl methemoglobin (final concentration, 21.6 μM in heme); (B) 30 μl methemoglobin (final concentration, 16.3 μM in heme); (C) 20 μl methemoglobin (final concentration, 10.9 μM in heme); (D) this was derived from Figs. 4(A)–(C). The a, b and c in the vertical axis show the optical density at 630 nm of methemoglobin without cyanide, methemoglobin with 13.5 μM cyanide, and methemoglobin with excess amounts of cyanide, respectively.

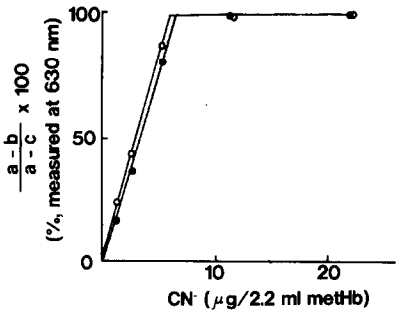

Fig. 5. Trapping with methemoglobin cyanide evolved in Conway dishes. Methemoglobin solutions which were collected from the center chamber of Conway dishes had their absorption spectra recorded between 530 nm and 650 nm. The optical densities at 630 nm of methemoglobin without cyanide (a), of methemoglobin with different concentrations of cyanide (b), and of methemoglobin with excess amounts of cyanide (c) were obtained. The ratio of (a−b)/(a−c) was expressed as a percentage and was plotted against cyanide concentrations (closed circles). For comparison, different concentrations of cyanide were added directly to methemoglobin solutions as in Fig. 1, and the same ratio was plotted against cyanide concentrations (open circles).

above 10.9 μM methemoglobin in heme. This result shows that cyanide concentrations can also be determined by adding a small volume of methemoglobin solution to solutions containing cyanide.

In order to estimate the cyanide concentrations in biological tissues, we performed experiments in Conway dishes where cyanide gas was evolved and trapped by methemoglobin in the center chamber. We measured the changes in the absorption spectra of methemoglobin in the center chamber of the Conway dishes after different amounts of cyanide had been evolved by sulfuric acid. Then, we determined the differences in absorbance at 630 nm of methemoglobin with and without cyanide, at different concentrations of cyanide (Fig. 5). It was found that cyanide which evolved from the outer chamber and was trapped by methemoglobin in the center chamber was linearly dependent on the concentrations of cyanide added. By using this method, the determination of as little as 0.4 μg cyanide in erythrocyte suspension was possible. Figure 5 shows that the recovery of cyanide was 80%.

6. Discussion

Aldridge [1] developed a method for detecting cyanide, in which cyanide is converted to cyanogen bromide, which reacts with a pyridine–benzidine reagent to form a dyestuff. Even though it is possible to detect cyanide in concentrations as small as 0.1 μg/ml, there are some disadvantages in this method; the intensity of color is stable only for 30 min, and benzidine is a strong carcinogenic reagent. Aldridge's original method was modified by replacing benzidine with *p*-phenylenediamine [7], or with tetramethylbenzidine [8]. To

stabilize the color and increase the intensity of the dyestuff, Nusbaum and Skupeko [9] extracted it with n-butanol. There are several alternative methods to detect cyanide, which include chloramine-T and pyridine [10], and chloramine-T and γ-picoline–barbituric acid reagent [11], but the cyanide products seem to be relatively unstable. Another colorimetric method involves the liberation of cyanide by aeration of the acidified sample in a Conway dish [5], however, it takes several hours for complete removal of hydrogen cyanide from the sample. Shanahan [12] improved the method for shortening the cyanide distillation time, using a cyanide distillation apparatus, but used pyridine–benzidine for detection of cyanide.

As we have shown using methemoglobin to trap cyanide (Figs. 1–4), the method has several advantages;

(a) methemoglobin reacts with cyanide in a 1:1 manner;

(b) the reaction finishes within 20 min at room temperature;

(c) the determination is done easily by measuring changes in the spectra of methemoglobin caused by the binding of cyanide; and

(d) A standard cyanide curve whose depiction is often time-consuming, is not necessary.

We added cyanide solution directly to methemoglobin solution in the ratio of 1:100 (Figs. 1 and 2). The detection limit of cyanide was about 0.2 μg/ml in water, because we could detect 0.43 μg cyanide in 2 ml methemoglobin solution when we added 20 μl cyanide (43.4 μg/ml) to 2 ml 55 μ*M* methemoglobin in heme (Fig. 3). On the other hand, when we added 40 μl concentrated methemoglobin solution (1.1 m*M* in heme) to 2 ml water containing 0.7 μg cyanide (i.e. 13.5 μ*M* cyanide and ratio 1:50), we also determined the cyanide concentration as 0.75 μg/2.04 ml from the changes in absorbance at 630 nm. Therefore, the sensitivity of this method for detecting cyanide is comparable to that of Aldridge [1].

Cyanide–methemoglobin complex is very stable at 4 °C for two days. Various species of hemoglobin are commercially available, and it is easy to obtain methemoglobin by oxidizing ferrous hemoglobin with ferricyanide. We checked the cyanide binding capacity of bovine methemoglobin, which was prepared by dissolving bovine hemoglobin powder (Sigma, St. Louis, MO) in 200 m*M* potassium phosphate solution, pH 7.0, oxidizing it with ferricyanide, and by passing it through a column of Sephadex G-25 (previously equilibrated with 200 m*M* potassium phosphate solution, pH 7.0). Though not shown in fig, we found that bovine methemoglobin binds cyanide in a stoichiometric manner, as is shown in human methemoglobin, indicating that bovine methemoglobin is also useful for the convenient detection of cyanide. The solution of methemoglobin can be stored at −80 °C for a year or so, and may be thawed before use.

Thus, the present method may be used to detect cyanide in drainage water from factories handling cyanide.

The disadvantages of using methemoglobin are that changes in absorption

spectra of methemoglobin are caused by changes in the pH of solutions and by the presence of a strong reducing agent such as dithionite. However, these disadvantages can be overcome by using 0.2 *M* phosphate buffer (pH 7.0) to stabilize the pH of the solution, and by adding an oxidant such as ferricyanide. In addition, the contamination by dithionite of solutions containing cyanide may occur only rarely.

Furthermore, we examined whether sodium hydroxide, which is often used as a cyanide trapping reagent in the center chamber of a Conway dish [5], could be replaced by methemoglobin. As shown in Fig. 5 cyanide evolved from the outer chamber was efficiently absorbed by methemoglobin placed in the center chamber. By using this method, we can determine the different concentrations of cyanide in red cell suspensions within two hours.

The recovery of cyanide from red cell suspension was nearly 80% of that expected (Fig. 5). This result may be explained by blood being coagulated by sulfuric acid, as suggested by McAuley and Revie [13]. The results also show that methemoglobin in Conway dishes can be used to detect cyanide in waste water, biological tissues and liquids.

7. Acknowledgement

This research was supported by grants from the Nissan Science Foundation (1989).

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