II.1.3 Cyanide

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Introduction

Hydrogen cyanide (HCN) is volatile (boiling point: 25.7°C) and weakly acidic (p*K*a: 9.2). It is bound with cytochrome oxidase to inhibit its activity and induce cellular anoxia; it shows an immediate toxic effects. The fatal dose of HCN is about 100 mg. Cyanide has been being involved in various incidents of suicides, homicides and accidents. It is relatively easy to obtain cyanide, because it is being widely used in metallurgy, metal-plating and other chemical industries. It is a typical poison to be analyzed with high priority. Cyanide is also included in some plants, such as some beans and Japanese plums, in the forms of its glycosides; by ingesting such plants, HCN is sometimes released from the glycosides in the stomach. In the tropical areas, it is also contained in cassaba; its poisoning by ingesting a large amount of cassaba is being a problem for health. HCN can be produced during imperfect combustion of nitrogen-containing compounds; it is also included in cigarette smoke and gases produced in a fire.

There are many kinds of qualitative and quantitative methods for analysis of cyanide in wide fields, reflecting the great needs of its analysis. The Japanese Industrial Standard (JIS) standardized a method of cyanide analysis for factory waste water [1]. Nonomura [2] published a review for analytical methods of cyanide in water specimens. In two books [3, 4] both edited by the Pharmaceutical Society of Japan, the analytical methods for cyanide are also presented. In the field of forensic toxicology, the review on cyanide analysis written by Maseda and Fukui [5] seems useful. Many years ago, Bark et al. [6] and Guatelli [7] presented reviews on fundamental tests for cyanide, such as color tests. Very recently, preliminary tests or simplified qualitative tests for cyanide have been described in a book [8] edited by the Department of Legal Medicine Hiroshima University School of Medicine. In this chapter, the methods using microdiffusion plus pyridine-pyrazolone reaction and using headspace gas chromatography (HS-GC) are presented for analysis of cyanide in human blood, which is useful in forensic toxicology.

Determination of blood cyanide by the microdiffusion/pyridine-pyrazolone method

Reagents and their preparation

- A-20 mg aliquot of chloramine T (sodium *p*-toluenesulfonchloramide trihydrate, to be stored in a refrigerator, Wako Pure Chemical Industries, Ltd., Osaka, Japan and other manufacturers) is dissolved in distilled water to prepare 10 mL solution (2 mg/mL) just before use. A 15.6 g aliquot of sodium dihydrogenphosphate dihydrate is dissolved in distilled water to prepare 100 mL solution (1 M, preservable in a refrigerator). A 5 mL volume of the chloramine T solution is mixed well with 15 mL of the phosphate buffer and kept cooled in ice (0 °C) until use (the mixture to be prepared freshly just before use).

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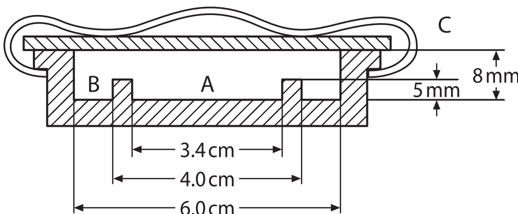
- A 0.1 g aliquot of 1-phenyl-3-methyl-5-pyrazolone (Wako Pure Chemical Industries, Ltd., and other manufacturers)[a] is dissolved in distilled water to prepare 100 mL solution (1 mg/mL, to be prepared freshly just before use). When it is not easily dissolved, the mixture solution can be warmed and stirred to obtain clear solution. A 20 mg aliquot of bis (1-phenyl-3-methyl-5-pyrazolone) (Wako Pure Chemical Industries, Ltd., and other manufacturers) is dissolved in 20 mL pyridine (1 mg/mL, to be prepared freshly). The above two solutions are combined just before use.
- Concentrated sulfuric acid is mixed slowly with a larger volume of distilled water to obtain 10-fold diluted solution (preservable at room temperature).
- A 1.76 g aliquot of *l*-ascorbic acid is dissolved in distilled water to prepare 10 mL solution (1 M, preservable in a refrigerator).
- One gram of NaOH is dissolved in distilled water to prepare 250 mL solution (0.1 M, preservable at room temperature in an airtight state).
- A 25.1 mg of potassium cyanide[b] (Wako Pure Chemical Industries, Ltd., and other manufacturers, analytical grade, designated as a poisonous substance by the Poisonous and Deleterious Substances Control Law) is dissolved in distilled water to prepare 10 mL solution (cyanide ion 1 mg/mL, preservable for a week in a refrigerator). The cyanide solution is diluted 500-fold with 0.1 M NaOH solution to prepare cyanide standard solution (2 μg/mL, to be freshly prepared).

Procedure

i. A specimen (1 mL blood and 0.03 mL of 1 M ascorbic acid solution are placed in the outer groove (B) of a Conway microdiffusion cell (*Figure 3.1*) (outer groove: wall height 8 mm, outer diameter 6 cm, inner diameter 4 cm; central round basin: wall height 5 mm, diameter 3.4 cm, Shibata Kagaku, Tokyo, Japan); 2 mL of 0.1 M NaOH solution for absorbing HCN gas is placed in the central round basin (A) and sealed airtightly with a glass plate cover smeared with glycerin at the joint part. By sliding the glass plate, a part of the roof of the outer groove (B) is opened; 0.5 mL of 10 % sulfuric acid is rapidly added to the blood specimen plus ascorbic acid, and the cell is again sealed immediately and fixed with a metal stopper. The Conway cell is gently swirled to well mix sulfuric acid and the specimen.

Figure 3.1



Schematic diagram of a cross section of the Conway microdiffusion cell. The bottom of the cell is compartmented by a circular wall of 5-mm height into a outer donut-shaped groove (B) and a central round basin (A). The glass plate cover is fixed with a metal stopper (C).

ii. The Conway cell is left at room temperature for 2 h (diffusion)[c].
iii. One milliter of the inner basin solution (0.1 M NaOH) is transferred to a glass tube and cooled with ice.
iv. A 0.2 mL volume of the chloramine T/phosphate buffer solution, which has been cooled with ice, is added to the above solution and cooled for 2 min (chlorocyanogen production).
v. A 3 mL volume of the pyridine-pyrazolone solution is added to the above mixture and left at room temperature for 40 min (coloration procedure)[d].
vi. The absorbance of the test solution is measured at 630 nm.
vii. Calculation: the external calibration method is employed; various concentrations of cyanide in 0.1 M NaOH are prepared, and 1 mL each is subjected to coloration and measurement procedures [procedure iv) to vi)] to construct a calibration curve (cyanide concentration *vs.* absorbance). Using the curve, a concentration value for a test specimen is calculated and doubled[e] to obtain a concentration in the blood specimen. When the absorbance obtained for a specimen is beyond the concentration range of the calibration curve, the inner basin solution is diluted appropriately and the same procedure is repeated.

Assessment and some comments on the method

The present pyridine-pyrazolone method is based on the König's reaction. The cyanide reacts with chloramine T[f] to produce chlorocyanogen, which opens the pyridine ring. The resulting aldehyde condenses with pyrazolone molecules to yield a blue compound[g], as shown in *Figure 3.2*. At the beginning of the color development, it becomes pink and then bleaches, followed by appearance of a blue color, which is intensified according to time intervals, reaches a stable stage for 2–3 h and then bleaches; therefore, the measurements of the absorbance should be made during the stable stage.

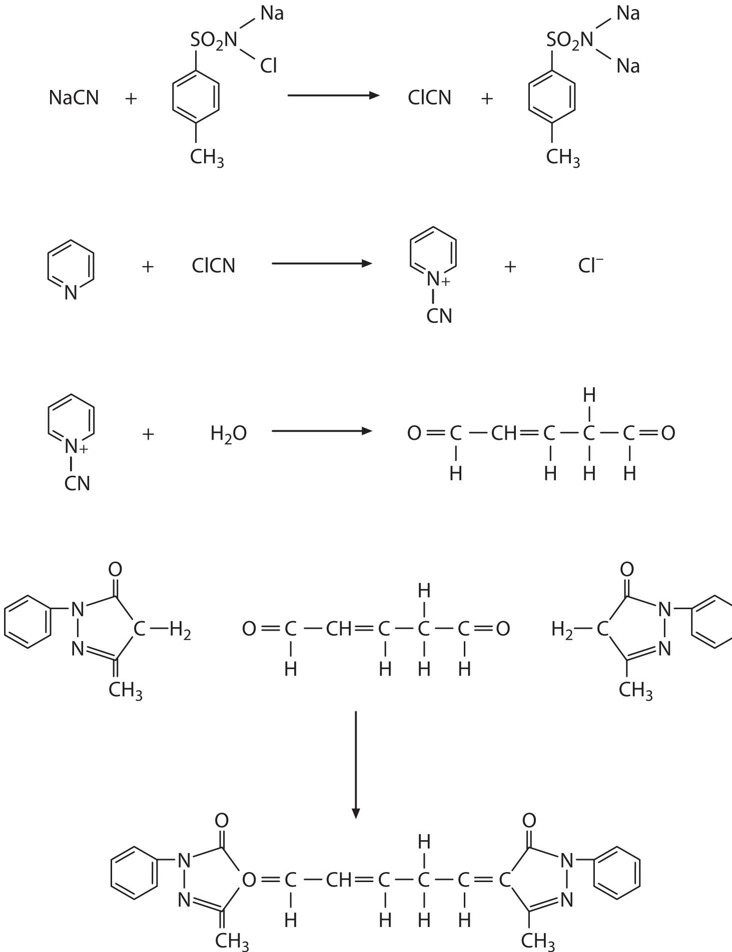
Figure 3.3 shows a calibration curve for cyanide. It is linear up to 1 µg/mL, but shows a minor trend of saturation above 2 µg/mL. Although the slope coefficient (A_{630}/µg/mL) of the curve varies according to a method of preparing reagents, room temperature and other analytical conditions, the variation is not greater than 1.5 %. The detection limit is about 10 ng/mL.

As shown in *Table 3.1* [9], cyanide can be detected from blood of healthy subjects. The endogenous levels are close to the detection limit of the present method.

The König's coloration method seems specific for cyanide, but thiocyanic acid (HSCN) markedly interferes with the above color development [10]. Thiocyanic acid also reacts with chloramine T to produce chlorocyanogen, which is not negligible. When a specimen containing thiocyanic acid is directly color-developed by the pyridine-pyrazolone method without extraction, it suffers from overestimated values of cyanide due to the false-positive reaction. Blood and serum contain much higher concentrations of thiocyanic acid than that of cyanide (*Table 3.1)*; in saliva, its concentrations are even higher. The concentrations of thiocyanic acid in blood and saliva of smokers are significantly higher than those of non-smokers; therefore, thiocyanic acid levels are being used as an indicator of smoking. To avoid such false-positive reaction, pretreatments of specimens, such as distillation, purging and diffusion, are essential to extract cyanide from the matrix. Thiocyanic acid is not volatile under acidic conditions, which enables the separation between thiocyanic acid and cyanide.

There is great variation in the level of cyanide in blood of healthy subjects [11]; the values are 3–75 ng/mL in non-smokers, and 9–180 ng/mL in smokers. However, the variation is not

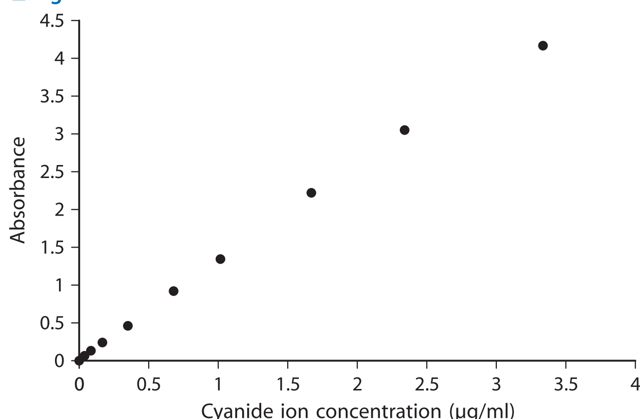
Figure 3.2



Color reaction mechanism for cyanide by the pyridine-pyrazolone method.

due to the individual difference, but due to different methods of analysis used. In every analytical method for cyanide in blood, a pretreatment of blood with relatively strong acid to denature hemoglobin to liberate HCN is included, prior to any type of detection. During the acid-treatment process, the oxygenated hemoglobin present in large amounts in blood (12–16 %) is rapidly denatured to produce large amounts of superoxide anion radical (O_2^-), which causes non-specific oxidation of compounds including thiocyanic acid [12]; thiocyanic acid is attacked by O_2 or its conjugated acid perhydroxyl radical (HOO•), resulting in liberation of cyanide from thiocyanic acid. Although the situation is different according to the concentrations of oxygenated hemoglobin, the kind of acid, its concentration and other pretreatment conditions, about 2.4% of total thiocyanic acid is usually converted to cyanide. By considering high concentrations of thiocyanic acid present in blood, it is easily concluded that a major part of cyanide detected from blood of a healthy subject is due to artificial production of cyanide by

Figure 3.3



Calibration curve for cyanide ion by the pyridine-pyrazolone method.

Table 3.1

Concentrations of cyanide and thiocyanic acid in blood and saliva of healthy human subjects*

Analyte	Concentration (ng/mL)		
	Total	Non-smoker	Smoker
The number of Specimen	40	20	20
Cyanide in blood**	5.7 ± 2.1	4.4 ± 1.1	7.0 ± 1.8
Thiocyanic acid in plasma	4,200 ± 4,490	1,940 ± 1,470	6,450 ± 5,340
Cyanide in saliva**	13.5 ± 10.9	9.9 ± 6.8	17.2 ± 13.5
Thiocyanic acid in saliva	63,700 ± 50,000	31,400 ± 23,500	96,000 ± 48,800

* The data were cited from reference [9] with slight modification.

** The cyanide concentrations are express as those in the form of cyanide ion.

oxidation of thiocyanic acid [11]. The high blood cyanide levels often observed in the literature seems to reflect such artifacts produced during pretreatments. For blood specimens of individuals (heavy smokers and tobacco amblyopia) containing high concentrations of thiocyanic acid, high concentrations of cyanide can be detected; this may cause misdiagnosis of cyanide poisoning. The artificial production of cyanide from thiocyanic acid can be suppressed by adding ascorbic acid to blood; ascorbic acid removes active oxygen and suppress the false positive reaction to give true cyanide levels in blood [11].

Cyanide in blood is relatively stable under storage in a refrigerator. After sampling blood specimens, the methemogloblin-reducing enzyme system in erythrocytes is gradually inactivated, causing gradual increase in methemogloblin concentrations. In blood specimens, high concentrations of cyanide can stably exist in the trapped form by methemogloblin. However, there is a report dealing with changes in blood cyanide concentration under storage in a refrigerator [13]. In a frozen state, blood cyanide seems very stable. Under storage at room temperature, cyanmethemogloblin is decomposed by the actions of denaturation, dissolution and putrefaction; therefore, there is a possibility of change in cyanide level in blood. The cyanide production by putrefaction should be also considered. The *in vitro* production

of cyanide can be observed, though it is not so marked; care should be taken for such phenomena.

As postmortem change of cyanide, spontaneous production of cyanide through denaturation blood by heat was reported in a fire death case; the produced level is below the fatal one and does not cause misdiagnosis for cyanide poisoning. When the cyanide-containing blood is heated strongly, the cyanide level is decreased [14].

The present method is applicable to any specimen other than blood, such as body fluids and organs (after homogenization). It is also applicable to non-biological specimens, such as drinks and foods in adulteration incidents. In such cases, care should be taken for the presence of nitrous acid in the specimen; nitrous acid can react with organic compounds in specimens to produce cyanide non-specifically. In the presence of an oxidant, such as hydrogen peroxide, thiocyanic acid contained in drinks or foods can be oxidized to yield cyanide. When a large amount of oxidant is present in them, it is difficult to suppress oxidative production of cyanide by adding ascorbic acid; it gives false-positive results for cyanide [12]. When the effects of an oxidant are suspected, 1 M sodium acetate buffer (pH 5) should be used in place of sulfuric acid in the microdiffusion procedure to avoid artificial production of cyanide.

Determination of blood cyanide by headspace-GC

Reagents and their preparation

- Concentrated phosphoric acid (85 %, w/v) is diluted 1.7-fold with distilled water (50 %, w/v, preservable at room temperature).
- A 1.76 g aliquot of *l*-ascorbic acid is dissolved in distilled water to prepare 10 mL solution (1 M, preservable in a refrigerator).
- A 25.1 mg aliquot of potassium cyanide (analytical grade, designated by the Poisonous and Deleterious Substances Control Law) is dissolved in distilled water to prepare 10 mL solution (cyanide ion 1 mg/mL, preservable for a week in a refrigerator). The cyanide solution is diluted 200-fold with distilled water just before use (5 µg/mL).

GC conditions

GC column[h]: a porous polymer-type fused silica wide-bore capillary column (HP PLOT Q, 30 m × 0.53 mm i. d., film thickness 0.25 µm, Agilent Technologies, Palo Alto, CA, USA).

GC conditions: an HP 6890 Series gas chromatograph (Agilent Technologies): injection mode: split; split ratio: 5; injection temperature: 200°C; detector: NPD; detector temperature: 250°C; carrier gas: He; its flow rate: 5 mL/min; column temperature: 120°C

Procedure

i. A 0.5 mL volume of a specimen (blood), 0.03 mL of 1 M ascorbic acid solution and 0.27 mL distilled water are placed in a glass vial with a screw cap (8 mL volume, external diameter

17 mm, height 6 cm, GL Sciences, Tokyo, Japan or other manufacturers) and capped air-tightly using a Tuf-Bond™ disc (PTFE/silicone septum)[i].

ii. A 0.2 mL volume of 50 % phosphoric acid solution is drawn into a tuberculin glass syringe attached with a 25 G × 1″ needle (0.50 × 25 mm, Terumo, Tokyo, Japan), injected into the vial and heated at 50° C for 30 min for headspace extraction of cyanide. In this case, the vial is put on an aluminum block (type D) heater (Reacti-Therm, GL Sciences), and the top of the vial is covered with cotton[j] upon heating.

iii. A 0.5 mL volume of the headspace vapor is drawn into a clean tuberculin glass syringe attached with a 25 G × 1″ needle[k,l] (0.50 × 25 mm, Terumo) and injected into GC immediately.

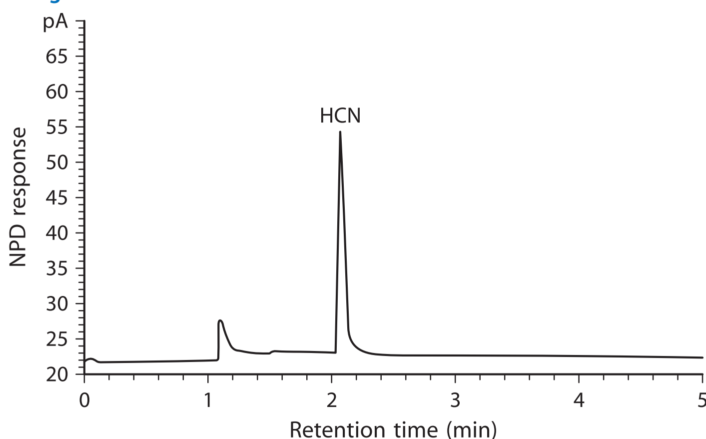
iv. Calculation: the external calibration method is employed; each of various concentrations of cyanide together with 0.5 ml each of blank blood, ascorbic acid solution and distilled water is placed in a vial, and the following procedure is same as described above. A calibration curve is constructed with cyanide concentration on the horizontal axis and peak area on the vertical axis[m]. The final volume of the solution mixture in the vial is 1.0 mL. Since a low level of endogenous cyanide is detected in the blank test, the cyanide peak areas obtained after subtracting the blank area are used for constructing the calibration curve. The cyanide concentration in a specimen can be easily calculated using the curve.

Assessment and some comments on the method

Figure 3.4 shows a GC chromatogram obtained from blood containing 200 ng/mL cyanide ion. Except the big peak of HCN (retention time, 2.1 min), small peaks due to alkyl nitrile compounds, such as acetonitrile (retention time, 5.7 min) can be detected. The detection limit is about 1 ng/mL with low split ratios, enabling measurements of endogenous cyanide in blood.

Darr et al. [15] first reported analysis of cyanide in blood by headspace GC using a column packed with a porous polymer packing material Porapak Q and an FTD (flame thermionic

Figure 3.4



GC chromatogram for HCN extracted from a blood specimen containing 200 ng/mL of cyanide by the headspace method [20].

detector, the same as an NPD) with a detection limit of 50 ng/mL. In GC with such a packed column, the resolution ability is frequently lowered by interference of volatile components contained in biomedical specimens; to solve this problem, tedious aging of the packed column at a high temperature is required after each measurement. A fused-silica capillary column with a similar porous polymer is resistant to degradation and enable repeated measurements without aging of the column. As headspace-GC analysis for HCN, the methods using a chlorination precolumn and an ECD [16] and using SPME [17] were reported. In addition, a sensitive headspace-GC method using a cryogenic oven trapping device was also reported [18]. The partition coefficient for HCN between aqueous and gas phases (at 50° C) is 76 [19], showing relatively low concentrations of HCN detectable in the headspace vapor. The toxic and fatal concentrations of cyanide in blood are several μg/mL or less. Therefore, a sensitive detector is required for analysis of cyanide in biomedical specimens [20]. Although toxic levels of cyanide in blood can be detected by GC with an FID, the test peak is usually interfered with by acetaldehyde [19]; the use of an NPD, an ECD (after halogenation) and mass spectrometry gives satisfactory results.

Toxic and fatal concentrations

The fatal concentrations of cyanide in blood were reported to be not lower than 3 [21] to 5 μg/mL [22]. In the acute poisoning by cyanide, the absorbed cyanide exists in blood plasma and is transported to vital organs (such as the respiratory center of the central nervous system) to exert its toxicity. According to time intervals, cyanide in plasma passes through erythrocyte membranes to be bound with methemogloblin and stays there in a relatively stable form. The hemoglobin concentration in blood is as high as 12–16%. Even if only one percent of the total hemoglobin is assumed to be present in the oxidized form (methemogloblin), entire amounts of cyanide present in blood at fatal levels can be trapped by methemogloblin. In blood plasma, cyanide is relatively unstable and disappears by absorption into erythrocytes or by being bound with proteins irreversibly. Theoretically, cyanide existing in blood plasma is directly related with its toxicity. However, a majority of cyanide being measured by the present methods does exist in a bound form with methemogloblin; further consideration about the relationship between blood cyanide concentration and its toxicity seems to be required.

The toxic concentrations in blood cyanide was reported to be 0.1–0.2 μg/mL [22]. However, in this report, the interference by thiocyanic acid cannot be excluded; the values should be reexamined.

Notes

a) A reagent mixture Cyanoline Blue® is commercially available from Dojindo Laboratories (Kumamoto, Japan). The pyridine-pyrazolone reagent can be simply prepared only by dissolving 0.27 g of the above mixture in 20 mL pyridine, followed by addition of 100 mL distilled water.

b) The extra-pure reagent contains almost 100 % potassium cyanide; however, it degrades during a long storage (*e.g.*, absorption of carbonic acid). When such a possibility arises, the potassium cyanide solution prepared should be titrated with silver nitrate aqueous solution using *p*-dimethylaminobenzylidenerhodanine as an indicator [1, 3].

c) In an emergent case, the diffusion procedure can be done in an incubator at 37° C. A quantitative extraction of cyanide from the acidic specimen into the alkaline absorbent solution can be achieved only in 15 min [23].
d) Also in an emergent case, the coloration procedure can be also shortened by incubating the reaction mixture at 37° C in an incubator for only 15 min [23].
e) Since a 1 mL aliquot of 2 mL alkaline absorbent solution is used, the value obtained using the external calibration curve should be doubled.
f) As a halogenating reagent, hypochlorite and bromine solution can be used except chloramine T.
g) Except the pyridine-pyrazolone reagent, pyridine-barbituric acid and 4-pyridinecarboxylic acid-pyrazolone reagents were developed as coloration reagents and are being practically used.
h) The porous polymer-type fused silica capillary columns are suitable for analysis of HCN. Except the HP PLOT Q column, the GS-Q column (wide-bore, J&W) is also being used [19].
i) Cyanide gas does not usually adsorb to the surfaces of glassware and rubber strongly. Although lipophilic volatile compounds are sometimes absorbed to the septa made of butyl rubber, the cyanide gas gives no problems even with such rubber septa.
j) During heating the vial at 50° C, the water vapor is aggregated on the septum surface to form water droplets, because of lower temperature on the top of the vial; cyanide gas can be absorbed into the water droplets, resulting in high concentration of cyanide in them, because the partition coefficient of cyanide between gas and aqueous phases is as high as 76 [19]. If the needle of a syringe touches the water droplets containing high concentrations of cyanide, serious contamination of the syringe by cyanide can occur. To avoid the formation of water droplets, the top of the vial should not be cooled, but heated at the same temperature by covering it with cotton.
k) The diameter of syringe needle is smaller the better, because the damage is less with a smaller needle diameter. With use of a larger-diameter needle of a gastight syringe, the septum is damaged, resulting in leakage of gas after injection several times only.
l) The glass syringe can be also heated by putting it on the aluminum block heater together with the glass vials to avoid the adsorption of water vapor together with cyanide gas to the syringe.
m) The calibration curve shows linearity from the detection limit up to about 100 μg/mL. The concentration in a specimen should be within the concentration range of the calibration curve. Usually, a calibration curve with a concentration range of 0–5 μg/mL is prepared for blood specimens.

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