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TECHNICAL NOTE

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Blood Cyanide Determination in Two Cases of Fatal Intoxication: Comparison Between Headspace Gas Chromatography and a Spectrophotometric Method*

ABSTRACT: Blood samples of two cases were analyzed preliminarily by a classical spectrophotometric method (VIS) and by an automated headspace gas chromatographic method with nitrogen-phosphorus detector (HS-GC/NPD). In the former, hydrogen cyanide (HCN) was quantitatively determined by measuring the absorbance of chromophores forming as a result of interaction with chloramine T. In the automated HS-GC/NPD method, blood was placed in a headspace vial, internal standard (acetonitrile) and acetic acid were then added. This resulted in cyanide being liberated as HCN. The spectrophotometric (VIS) and HS-GC/NPD methods were validated on postmortem blood samples fortified with potassium cyanide in the ranges 0.5–10 and 0.05–5 μg/mL, respectively. Detection limits were 0.2 μg/mL for VIS and 0.05 μg/mL for HS-GC/NPD. This work shows that results obtained by means of the two procedures were insignificantly different and that they compared favorably. They are suitable for rapid diagnosis of cyanide in postmortem cases.

KEYWORDS: forensic science, cyanide, headspace gas chromatography, Conway cell

Cyanide (CN) is a powerful, rapidly acting poison, death occurring within minutes of ingestion. Cyanide exerts its toxic effects by reacting with the trivalent iron of cytochrome oxidase, thus inhibiting electron transport and preventing cells from using oxygen (hypoxia), which results in rapid loss of vital functions (1,2).

Cyanide exposure is relatively common (2,4). Apart from sodium nitroprusside therapy (as a hypotensive agent) and ingestion of cyanide salt in the context of suicidal or homicidal attempts, the main sources of exposure are smoke from fires or cigarette smoking, accidental inhalation of hydrocyanic acid in the metal and plastic industries, and ingestion of various types of food such as cassava, cherry, or almond (2,3).

HCN, not being ionized and readily diffusible, is rapidly absorbed through biological membranes and diffuses throughout the body. Most of the cyanide concentrates in erythrocytes, presumably bound to methemoglobin. The major pathway of cyanide detoxification is by enzymatic conversion to thiocyanide and subsequent excretion by the kidney (1,4).

The postmortem specimen most frequently analyzed for cyanide in forensic toxicology is blood. Other sources are spleen, liver, and brain. Blood cyanide concentrations lower than 0.25 μg/mL are considered normal, and those above 0.25 μg/mL but below 2–3 μg/mL as elevated, but not ordinarily causing death. Concentrations above 3 μg/mL are consistent with death in the absence of other relevant and/or toxicological findings (4).

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In forensic toxicology, cyanide exposure is preliminarily determined by classical spectrophotometric techniques and then confirmed by chromatographic methods (2,5–9).

The aim of the present work was quantitative determination of blood cyanide in two cases of fatal intoxication. Blood samples of the two cases were analyzed in two different laboratories, first by a spectrophotometric method (VIS), performed in the laboratory of Froldi et al., and then by headspace gas chromatographic method with nitrogen-phosphorus detector (HS-GC/NPD), in the laboratory of Gambaro et al.

Case Histories

Case 1

A 26-year-old woman was taken to a hospital emergency department, presenting with slowed respiration, gasping breath and bradycardia. She died more than 1 h later. Symptomatology suggested cyanide poisoning.

Qualitative analysis by the Gettler and Goldbaum (10) technique revealed cyanide in stomach contents. Blood samples collected at autopsy were analyzed by VIS and results were confirmed by a gaschromatographic method.

Toxicological analysis on postmortem specimens was negative for common drugs and volatile compounds.

Case 2

The body of a 30-year-old man was found in his closed car in an isolated area. A bottle containing a colorless liquid was found nearby. Laboratory analysis of the liquid revealed cyanide. Forensic evaluation could not establish the time interval between ingestion and death.

The stomach contents, collected during autopsy, were analyzed by the Gettler and Goldbaum (10) technique. Postmortem blood samples were analyzed by VIS and results confirmed by a gaschromatographic method. Toxicological analysis was positive for cyanide and negative for common drugs and volatile compounds.

Blank Sample Collection and Storage

Samples were collected in hermetically sealed vials and stored at −20°C until analysis.

The concentrations of cyanide in blood decay rapidly in the first few hours after exposure. Literature data confirm that CN disappears quickly from blood, but does remain long enough to be detected in forensic or clinical samples which may not be available for analysis immediately after exposure (9).

The blood specimens used as blanks in this study were obtained from a pool of postmortem blood samples of nonsmoking adults or subjects who had not been injured by smoke inhalation. These specimens had been previously screened and confirmed to be devoid of cyanide, according to sensitivity of both analytical methods used. Blank blood samples, fortified with cyanide, were used as working standards for the validation and development of both methods.

Materials and Methods

Chemicals and Reagent

Potassium cyanide (KCN) standard was purchased from Carlo Erba Reagents (Rodano, Italy). Acetonitrile (IS), Chloramine T, H_2SO_4, NaOH, and HCl were purchased from J.T. Backer (Deventer, Holland).

Other chemicals used in this study were: Pyridine, Glacial acetic acid, and Barbituric acid, all purchased from Fluka (Buchs, Switzerland).

Pyridine-barbituric acid reagent was prepared by adding 15 mL of pyridine, 3 mL of concentrated HCl and 7 mL of H_2O to 3.0 g barbituric acid.

Water was obtained from a Milli-Q ultrapurifying system, 18.2 MΩ/cm (Millipore SA, Molscheim, France). All solvents and reagents were of analytical grade.

Spectrophotometric Method (VIS)

Glass Conway microdiffusion cells were used (18 × 70 mm o.d.; 8–10 × 41 mm o.d., inner chamber).

Adsorbing solution (2 mL, 0.1 M NaOH) was added to the inner compartment of each Conway cell, and the liberating solution (2 mL, 50% H_2SO_4) was added to the outer compartment. Blood samples (1 mL) were added to the opposite part of the outer chamber, as mixing had to be avoided. The cell was then quickly closed by a Teflon-lined screw cap and gently rotated to mix blood and liberating solution. After 30 min contact at 38°C, 1 mL of the inner chamber contents from each cell was taken up and transferred into a 10-mL volumetric flask. To each flask 3 mL of 1 M NaH_2PO_4 and 1mL of Chloramine-T (2.5 g/L) were added, mixed, and allowed to stand for 2–3 min. Pyridine-barbituric acid reagent (3 mL) was then added and the solution diluted to 10 mL with H_2O.

Absorbance was determined at 586 nm against a blank.

Spectrophotometric Analysis

VIS was Performed on a Varian CARY50 (Torino, Italy) Spectrophotometer. Standard cyanide solution was prepared by placing 25.0 mg of KCN in a 100-mL volumetric flask, to yield a solution with a concentration of 100 μg/mL of CN; 0.1 N NaOH was used as diluent. In another volumetric flask, 10 mL of this solution was transferred and added with 90 mL of 0.1 N NaOH, to yield a solution with a concentration of 10 μg/mL.

The standard cyanide solution was further diluted to yield six working solutions at concentrations in the range of 0.5–10.0 μg/mL.

Automated HS-GC/NPD Method

Apparatus—Automated headspace GC analysis was carried out on a ThermoFinningan Trace TG, equipped with an NP detector 850, and interfaced with an autosampler (all from ThF, Rodano, Italy). A 10-mL vial and a 2.5-mL Hamilton 1002 NTL headspace syringe (Hamilton Co, Reno, NV, U.S.A.) were used.

After addition of acetic acid to the sample vial, the autosampler moved the vial from the vial tray holder to the sample heater, where the vial was heated at 60°C with continuous agitation for 40 min. Using a thermostated syringe, 750 μL of headspace vapor was injected into the GC inlet at a split rate of 40:1. Injector temperature was 100°C.

Gas chromatographic separation took place in a PoraBOND U fused-silica capillary column (30 m × 0.32 mm i.d., 7 μm film thickness) (Varian, Torino, Italy). Purified helium was used as carrier gas, at constant pressure to assure a steady column flow rate of 2.0 mL/min. Detector gas flow rate was set at 60 and 3 mL/min for hydrogen and air, respectively.

Column temperature was programmed at 90°C for 5 min and increased by 10°C/min to a final temperature of 140°C. Detector temperature was 300°C.

In these chromatographic conditions, retention times (t_R) were about 1.9 and 4.2 min for cyanide and IS, respectively. (Fig. 1).

HS-GC/NPD Analysis

Standard cyanide solution was prepared by placing 25.0 mg of KCN in a 100-mL volumetric flask to yield a solution with a concentration of 100 μg/mL of CN; 0.1 N NaOH was used as diluent. In another volumetric flask, 5 mL of this was transferred and added with 95 mL of 0.1 N NaOH to yield a solution with a concentration of 5 μg/mL.

Standard cyanide solution was further diluted to yield six working solutions at a concentration in the range of 5.0–0.1 μg/mL. Approximately, 0.5 mL of each solution was transferred into a 10-mL vial together with 0.5 mL of IS and 0.5 mL of blank blood. Lastly, 50 μL of glacial acetic acid was then introduced through the silicone rubber with a microsyringe.

Internal standard solution was prepared by placing 1 mL acetonitrile in a 100-mL volumetric flask and diluting with water. This solution was then further diluted to yield a solution with a final concentration of 0.005 μg/mL.

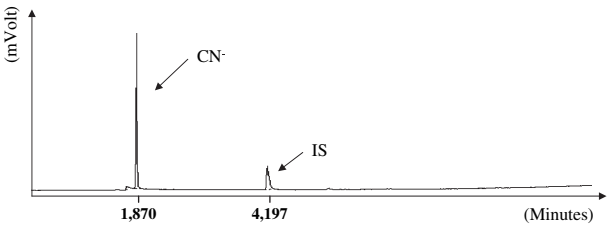

FIG. 1—*GC chromatogram of blank blood fortified with cyanide and IS.*

Preliminary analysis of samples revealed high concentrations of cyanide, so that the blood samples had to be suitably diluted to yield concentrations in a calibration range. 1mL of each blood samples was diluted in a 10-mL volumetric flask with water. Therefore, 0.5 mL each of diluted sample, IS, and 0.1 N NaOH were placed in a 10-mL glass vial, which was then capped with teflon-coated silicone rubber and sealed by crimping an aluminium cap. An aliquot (50 μL) of glacial acetic acid was introduced through the silicone rubber with a microsyringe. After each injection, the syringe was flushed many times with air to ensure no carry-over of residual cyanide. Blank air injections were also routinely run between samples to avoid this problem completely.

Results and Discussion

For UV/VIS measurements, the usual linear relationship follows the Lambert–Beer Law, which states that the absorbance of an analyte is directly proportional to its concentration.

In the VIS method, three calibration lines were constructed using six calibrators in the cyanide range 0.5–10 μg/mL. The concentrations for the first regression line were 0.525, 1.050, 2.625, 5.250, and 7.875 μg/mL, those of the second 0.515, 1.030, 2.575, 5.150, and 7.725 μg/mL, and those of the third 0.555, 1.110, 2.775, 5.550, and 8.325 μg/mL.

The regression lines for determination of linearity were $y = 0.2109x - 0.059$, $y = 0.1419x - 0.049$, and $y = 0.2059x - 0.051$, respectively. The correlation coefficients (R^2) of the three curves were 0.9989, 0.9994, and 0.9991. Method precision was evaluated by measuring intra-day and inter-day precision for cyanide. The intra-day coefficient of variation (CV%) was determined by performing three replicate analyses of each concentration on the same day. Inter-day CV% was determined by analyzing each concentration on three different days. Intra-day CV% was 8.1% and inter-day CV% 12.3%.

The limit of detection (LOD) was evaluated analyzing three blank samples and calculating the standard deviation of these responses. In our case, it was 0.2 μg/mL. The limit of quantitation (LOQ) is the lowest point of concentration on the curve, so that the Lambert–Beer law is still valid. In our case, the value was 0.5 μg/mL.

In the HS-GC/NPD method, sample cyanide concentration was calculated by the following ratio:

$$C_{CN} = (A_{CN}/A_{IS})/RR_{MEAN},$$

where A_{CN} is cyanide peak area and A_{IS} is IS peak area in the analytical sample and RR_{MEAN} is the mean of the RR calculated in the linearity study:

$$RR_{MEAN} = (A_{CN}/A_{IS})/C_{CN},$$

where A_{CN} is cyanide peak area and A_{IS} is IS peak area in working standard samples and C_{CN} is cyanide concentration (μg/mL) in working standard samples.

No interference at the retention time of the examined substances (CN and IS) was found on analysis of blanks (Figs. 2 and 3).

Calibration lines were constructed using at six calibrators over a concentration range of 0.05–5.0 μg/mL, and three independent determinations were made at each concentration ($n = 18$). The concentrations of the first and second calibration lines were 0.056, 0.112, 0.558, 1.116, 2.790, and 5.588 μg/mL, and those of the third line were 0.054, 0.108, 0.542, 1.084, 2.710, and 5.420 μg/mL.

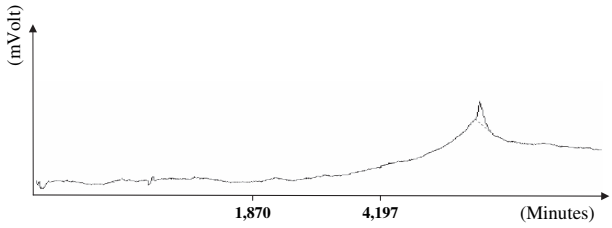

FIG. 2—*GC chromatogram of blank blood.*

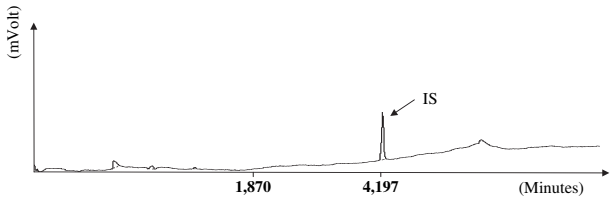

FIG. 3—*GC chromatogram of blank blood fortified with IS.*

Linear regression lines were obtained by plotting normalized peak areas (ratios of cyanide peak areas to IS peak areas) versus cyanide concentration by means of the least-squares method. The regression lines for determination of linearity were $y = 1.2142x - 0.0282$, $y = 1.2597x - 0.0371$, and $y = 0.8374x - 0.0627$. Correlation coefficients in the range 0.9754–0.9990 were obtained. The use of RR is suitable because the intercept of each calibration line was close to zero.

Method precision was ascertained by measuring intra-day and inter-day precision for cyanide. The study was carried out over a period of three days at six concentrations: intra-day precision was evaluated by RR_{MEAN} and coefficient of variation (CV%) of three replicate analyses of each working standard sample on the same day, inter-day precision was determined by analyzing six working standard samples on three different days. The resulting intra-day CV% was 13.8% and the inter-day CV% 18.6%.

Accuracy is the agreement between the measured and the true value. The percentage recovery of a standard samples provide the measure of method accuracy. The percentage recovery is calculated using the following formula:

$$Rec\% = \text{experimental value}/\text{theoretical value} \times 100,$$

where experimental value is the measured of standard sample; theorical value is the theorical concentration of standard sample.

Accuracy data were obtained by performing three replicate analyses of standard samples at six concentrations ($n = 18$) and the Rec% (mean) was 91.4%.

Limits of detection and LOQ were calculated from linear regression analysis. The LOD was 0.011 μg/mL and the LOQ 0.055 μg/mL. The calculated LOD value for our assay is below the lethal cyanide blood concentration, thus making the method described here suitable for detecting accidental or self-induced cyanide poisoning.

Blood samples from the two cases were first analyzed by VIS and subsequently confirmed by HS-GC/NPD.

In case 1, cyanide concentrations, determinated by the two methods, were 7.0 and 7.5 μg/mL, respectively. For case 2, they were 30.1 and 30.8 μg/mL. The results obtained by the two techniques compared favorably with each other.

The chromatographic procedure is easier to perform than the VIS procedure, and also has several other advantages:

sample preparation and analysis are easier and quicker; and the sample, once in the vial, does not require extraction or derivatization steps. Analysis performed with the fused-silica PLOT columns showed a very good separation for both substances, and no interference with endogenous blood components was observed.

Spectrophotometric data were confirmed by automated HS-GC/NPD results, showing that the gaschromatographic procedure is an effective alternative to VIS in determining cyanide in case of intoxication.

The automated HS-GC/NPD method is not linear for cyanide concentrations of >5 μg/mL, because high concentrations produce residuals in the headspace system, so that it was validated in the cyanide range 0.05–5 μg/mL.

As one important advantage of the VIS is the possibility of analyzing samples with high concentrations of cyanide, this method is suitable for rapid preliminary analysis. Instead, the automated HS-GC/NPD method is more sensitive, and is therefore suitable for determining cyanide in blood samples of fire victims with smoke inhalation and for confirming cyanide exposure in forensic toxicology.

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