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Rapid quantitation of cyanide in whole blood by automated headspace gas chromatography

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Abstract

Cyanide (CN), a chemical asphyxiant, is a rapidly acting and powerful poison. We have developed a sensitive, rapid, simple, and fully automated method for measuring CN in whole blood. The assay is based on the use of gas chromatography (GC) with nitrogen–phosphorus detection and acetonitrile as an internal reference. Following the automated addition of phosphoric acid to the blood sample, the released hydrogen cyanide is analyzed using a fully automated headspace GC system. The assay, validated on human blood samples spiked with potassium cyanide and on clinical samples from fire victims who had smoke inhalation injury, can detect CN at a wide range of concentrations (30–6000 μg/l) in about 17 min (including incubation and GC run time, and <2 min for manual sample preparation). This automated, high-throughput, simple, and sensitive method is suitable for the rapid diagnosis of CN in clinical and forensic specimens. Published by Elsevier Science B.V.

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1. Introduction

Cyanide (CN) is a potent and rapidly acting toxic agent. CN exerts its toxic effects by reacting with the trivalent iron of cytochrome oxidase, thus inhibiting electron transport and preventing the cells from using oxygen (hypoxia), which results in a rapid impairment of vital functions. After CN enters the body through ingestion, mucous membrane absorption, or inhalation, it passes into the bloodstream [1]. Most of the CN in blood concentrates in the erythrocytes [2] presumably bound to methemoglobin [3], an oxidized form of hemoglobin, which acts as a ''cyanide sink'' [2].

CN is widely used in industry (e.g. electroplating, metal refining), as a fumigant and insecticide, and in the process of soil sterilization [1]. Other sources of exposure to CN include cyanogenic glycosides found in digestible plants, automobile exhaust, and tobacco smoke [1,4]. CN is also produced endogenously in patients treated with sodium nitroprusside, a hypotensive agent [1]; CN has been found in the blood of fire victims with smoke inhalation injury [5–8]. Because of its rapid rate of action and availability, CN is of concern as a potential chemical terrorist agent. CN was used during World War II in the Nazi extermination camps [4]. In the U.S., during 1982–1991, 11 deaths were known to have resulted from

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deliberate tampering with over-the-counter medications (e.g. Tylenol and Sudafed); all involved CN [9].

Quantitation of human exposure to CN for clinical and forensic diagnosis is important. Several gas chromatography (GC) methods, including headspace GC with electron-capture detection (ECD) [10], GC with ECD and mass spectrometry detection [11], headspace GC with nitrogen–phosphorus detection (NPD) [12–16], headspace GC with NPD and cryogenic oven trapping [17], and solid-phase microextraction (SPME) coupled to GC with NPD [18], have been reported for the determination of CN in blood. Most of these methods have adequate sensitivity, but involve derivatization steps [10,11], or require relatively long incubation times (i.e. 15–60 min) [12–15,17,18].

In this paper, we describe the first fully automated headspace-GC method for measuring CN in whole blood. This sensitive and simple assay is not labor intensive, and is significantly faster than previously reported headspace methods [12,13,15,17].

2. Experimental

2.1. Sample collection and storage

Blood specimens for method development and validation were obtained from healthy nonsmoking adults. Clinical samples for method validation were obtained from excess blood specimens from patients who had been injured by smoke inhalation and were hospitalized at Grady Memorial Hospital's Burn Center in Atlanta, GA, USA. The information requested for each clinical sample included the nature of the fire exposure, the approximate time between exposure to smoke and collection of the blood, and whether the person had been started on cyanide-antidote medication at the time the blood was collected. No identifiable personal information was obtained, and blood samples were discarded after use. All of the blood samples used for this study were collected in tubes containing EDTA as the anticoagulant agent, and were kept at 4 °C until the measurements were made, normally on the same day of the blood collection or within 2 days (for the clinical samples).

2.2. Reagents

Potassium cyanide (KCN) and acetonitrile (ACN) standards were custom made for this project by Protocol Analytical Supplies, Inc. (Middlesex, NJ, USA). Phosphoric acid (85%) and sodium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA, USA). L-Ascorbic acid (99%+) was purchased from Aldrich (Milwaukee, WI, USA). Organic-pure water (OP H_2O, Type I) was prepared using a Solution 2000™ water purification system (Solution Consultants Inc., Jasper, GA, USA). The gases for GC analysis were obtained from Air Products (Atlanta, GA, USA). All chemicals and solvents were used without further purification.

2.3. Instrumentation and analysis

2.3.1. Automated headspace-GC analysis

The automated headspace-GC analyses were carried out on a HP 6890 gas chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with a NPD and interfaced to a CTC Combi PAL™ LEAP GC sampler (LEAP Technologies, Inc., Carrboro, NC, USA). The twin-tier PAL autosampler, mounted on top of the GC system, included a vial tray holder with a Peltier cooler; a sample heater block; and two movable heads each housing one syringe holder. These movable heads were programmed using the HP 6890 ChemStation software to perform motions in the *x*, *y*, and *z* axes and to actuate the syringe plungers. One head (which ran along a track on the top tier) held a 250-μl liquid-handling syringe (Hamilton Company, Reno, NV, USA) and was used to add sequentially ascorbic acid and phosphoric acid to the sample vials, kept at ca. 5 °C in the vial tray holder. The other head (which ran along a track on the bottom tier) housed the headspace syringe assembly; it was used to transfer the sample vials from the vial tray holder to the heater block for the incubation process and to inject the headspace vapor into the GC inlet. Following the automated addition of ascorbic and phosphoric acid to the sample vial, the twin-tier PAL autosampler was used to move the vial from the vial tray holder to the sample heater, where the vial was heated at 60 °C with continuous agitation (500 rpm) for 5 min. Using a 2.5-ml headspace syringe (Hamilton Company, Reno, NV, USA), a

1-ml volume of the headspace vapor was injected into the GC inlet, maintained at 150 °C in splitless mode (purge valve was opened 0.75 min after the injection). The gas chromatograph was equipped with an HP-PLOT Q (Agilent Technologies, Wilmington, DE, USA) capillary column (15 m×0.32 mm I.D., 20 μm film thickness). The helium flow-rate was maintained at 3 ml/min. The following oven temperature program was used: initial temperature 30 °C, hold for 1.5 min, ramp at 35 °C/min to 190 °C, hold at final temperature for 1 min (7.1 min total run time). The detector was held at 320 °C. To avoid the possibility of contamination of the syringes and therefore avoid carryover problems, the liquid-handling syringe was rinsed three times with OP H_2O after the addition of each acid, and the headspace syringe was flushed with ultra high purity helium for 4 min after the injection.

2.3.2. Automated SPME–GC analysis

We also carried out automated SPME analyses using the twin-tier PAL autosampler with the SPME fiber assembly housed on the movable head, which ran along a track on the bottom tier. We used a 70 μm Carbowax/DVB StableFlex fiber (Supelco, Bellefonte, PA, USA). Before initial use, the fibers were preconditioned in the GC inlet at 250 °C for 30 min. Fibers were replaced as needed. Following the automated addition of ascorbic and phosphoric acid to the sample vial, the twin-tier PAL autosampler was used to move the vial from the vial tray holder to the sample heater, where the vial was heated at 50 °C with continuous agitation (400 rpm) for 4 min. The SPME fiber was then exposed to the sample headspace for 15 s, retracted, and desorbed for 4 min in the GC inlet, which was fitted with a narrow-bore 0.75 mm I.D. SPME injection liner (Supelco, Bellefonte, PA, USA). The helium flow-rate was maintained at 1 ml/min. The oven temperature program used was: 30 °C, hold for 1.5 min, ramp at 35 °C/min to 190 °C, hold at final temperature for 2 min (8.1 min total run time).

2.3.3. Sample preparation

The manual steps of the sample preparation procedure were the following: 0.5 ml of blood were dispensed into a 5-ml ice-chilled vial, and 50 μl of 50 mg/l ACN (100 μl for the SPME analysis) were added to the blood to serve as an internal reference. Next, 100 μl of the appropriate KCN analytical standard or 100 μl of distilled water at pH 10 (for blanks and unknowns) were added to the blood, and the vials were immediately crimp-sealed and placed on the sample tray of the twin-tier PAL autosampler. The rest of the sample preparation procedure was fully automated: 150 μl of a solution of 50 mg/ml ascorbic acid (100 μl for the SPME analysis) followed by 200 μl of 50% phosphoric acid were added to each vial using the PAL system immediately before starting the incubation process.

Quality control (QC) materials were prepared following the procedure outlined in the above paragraph using 0.5 ml of 0.1 N NaOH [19] instead of blood, and 100 μl of the appropriate KCN standard to afford three QC materials at concentrations of 100, 3000, and 5500 μg/l. The QC samples were characterized to define the mean and the 95th and 99th confidence intervals of CN concentration by the analysis of at least 20 repeat measurements over an 8-week period.

2.3.4. Data analysis

All of the samples, blanks, standards, and QC materials were processed identically, and the data were evaluated for accuracy of integration. If necessary, data were manually reintegrated. We used the CN response factor (i.e. peak area ratio of CN to ACN) for quantification. Eight standard KCN concentrations (30, 60, 200, 500, 1000, 2000, 4000, and 6000 μg/l), which encompassed the entire linear range of the method, were used to construct a calibration curve of response factor versus standard concentration. Calibration curves were used for quantification of unknown samples. Because the detection limit was determined to be below the lowest standard, samples with values below the lowest standard were designated as nondetectable.

Data were automatically processed using the quantitation software of the HP 6890. The integrated peak areas for each analyte and retention times were saved in a report file in Excel format. An Excel macro, created in-house and incorporated into the GC acquisition method, automatically converted the Excel report files into report text files. These text files were imported into an R:BASE (Microrim, Inc., Redmond, WA, USA) database specifically created

for this analysis using an automated, custom-written routine. Statistical analysis of the data was carried out using Statistical Analysis System (SAS) software (SAS Institute, Cary, NC, USA).

3. Results and discussion

3.1. CN in blood

Most of the CN in blood concentrates in the erythrocytes [2] presumably bound to methemoglobin [3]. This bound form of CN may be released by heating or acid treatment [20]. Our method, like others before [10,12–15,17,18], used the conversion of CN to the volatile HCN upon acidification for the detection of CN in blood by GC. However, to our knowledge, ours is the first report of a fully automated and controlled addition of acid to achieve the conversion of CN into HCN in blood.

Up to 80% of small doses of CN are detoxified by the liver enzyme rhodanese to thiocyanate (SCN), which is readily excreted in the urine [1]. Interestingly, CN also may be produced from SCN in the presence of erythrocytes under acidic conditions [2,21], although the amount of CN that may be formed from a given amount of SCN is small (ca. 1%) [19,21].

Ascorbic acid prevents the conversion of SCN into CN [21]. CN may also form spontaneously during heating of blood at temperatures above 63 °C [20]. Therefore, to minimize the extraneous production of CN in blood during heating or from SCN upon denaturation of the hemoglobin by acid, we incubated the samples at 60 °C and added ascorbic acid to the blood before acidifying with phosphoric acid.

3.2. NPD chromatogram

Typical gas chromatograms of a spiked blood sample and a blank are shown in Fig. 1. The retention times of CN and ACN were 3.8 and 5.1 min, respectively. No interfering peaks and a very low background were observed across the whole linear range of the method. To monitor for contamination of the reagents or of the blood used to obtain the calibration curve, and to check for possible interferences, a 0.1 N NaOH reagent blank and a blood blank were processed daily along with the rest of the samples. No signal at the retention time for CN was ever detected in the reagent blank; only a very small signal corresponding to endogenous CN was found in the blood blank (Fig. 1A). These findings confirm that interferences with other nitrogen-containing compounds are not likely.

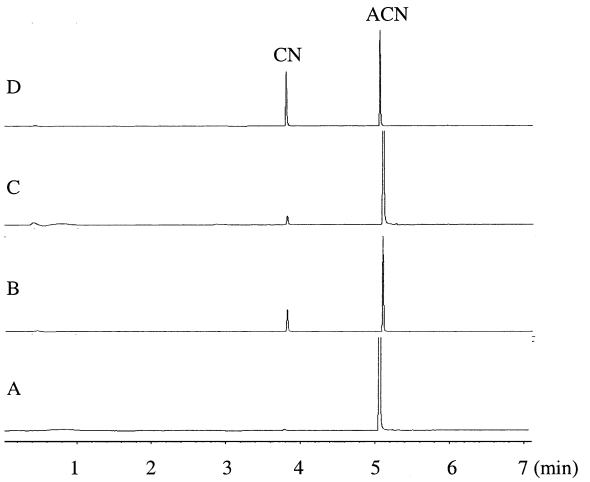

Fig. 1. Chromatograms for blood samples from a blank (non-smoker) (A), a specimen spiked with 500 μg/l KCN (B), a fire victim who suffered from mild smoke inhalation injury (C), and a fire victim who died as a result of the fire (D). The *y*-scale of chromatograms (A) and (C) had been expanded (×7 and ×6, respectively) to better depict the CN signal.

We initially tested a Supel-Q PLOT capillary column, used previously for the analysis of CN in blood [17,18], but the CN peak displayed considerable tailing under our experimental conditions. In contrast, a HP-PLOT Q column gave symmetric and narrow CN peaks (ca. 0.025 min peakwidth) with no tailing over the entire concentration range even with an initial oven temperature equal to 30 °C. This latter observation is of interest because in a recent report on the determination of CN in blood by headspace GC, sharp CN peaks were obtained only with cryogenic oven trapping and an initial oven temperature of −30 °C [17].

3.3. Calibration curve

A typical calibration curve of CN in blood is shown in Fig. 2. Each individual calibration curve showed excellent linearity (i.e. linear correlation

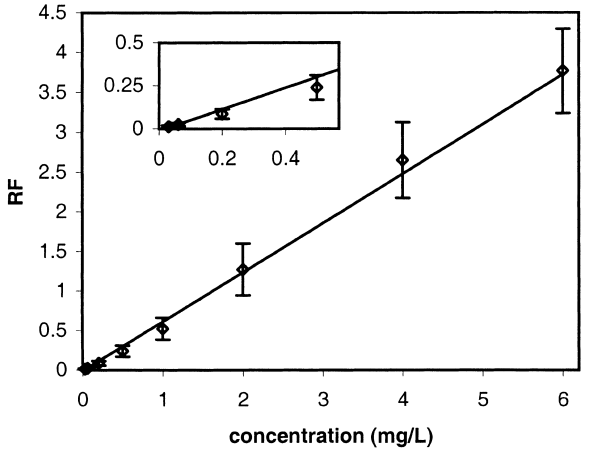

Fig. 2. Calibration curve based on replicate analyses of eight standard solutions across the linear range (±S.D.) with a linear correlation coefficient of 0.958. The values are weighted ($1/x$) by SAS software. The calibration curve spanned over three orders of magnitude and was linear across this entire range, including the lowest levels as shown in the figure inset.

coefficient generally >0.99) over three orders of magnitude. Interday variability of daily calibration curves was small; the relative standard deviation of the slope of nine calibration curves over 3.5 months was 10.6%. The calibration curve spanned three orders of magnitude with a working range from the lowest to the highest standard (30–6000 μg/l).

We evaluated the blood matrix effect on the calibration curve by analyzing the standards prepared in 0.1 N NaOH versus those spiked into whole blood. The standards spiked in blood produced calibration curves with slopes and intercepts not significantly different from those produced by the standards prepared in 0.1 N NaOH. These results suggest no interfering matrix effect for the range of CN concentrations measured.

3.4. Limit of detection

The replicate analysis of 27 blood samples spiked with low level standards (i.e. 30, 60, 200 μg/l) was used to calculate the limit of detection (LOD), equal to $3S_0$, where S_0 is the standard deviation value as the concentration approaches zero [22]. The calculated LOD was 13.8 μg/l, which compares well with values previously reported using GC methods (i.e. ~2 to 50 μg/l) [10,12,14,15,17,18].

The fatal threshold for CN exposure is difficult to assess because of factors such as route of exposure, variability of elimination half-lives, and storage conditions before analysis [4]. Lethal cases usually have CN blood levels >1000 μg/l, and levels >200 μg/l are considered toxic and dangerous [1]. Similarly, wide ranges, from low μg/l (ca. 5 μg/l) to ca. 80 μg/l, have been reported for endogenous levels of CN in blood [3,5,17,19,23–25]. The calculated LOD value for our assay is below the CN blood lethal level, thus making the method described here suitable for the detection of accidental or self-induced CN poisoning.

3.5. Accuracy of the method

The accuracy was established by determining the recovery of 15 spiked blood samples. To examine the consistency of the recovery over the range of levels encountered in blood, the measurements were taken by quintuplicate at three different concentrations on two separate days. The mean recoveries of CN in blood, expressed as a percentage of the expected value, at 200, 2000, and 4000 μg/l spiking levels were 96, 86, and 99%, respectively. The average mean recovery, a measure of the accuracy of the method, was 94%. The slope of a linear regression analysis of the calculated versus the expected concentration was 0.999 which confirmed the excellent accuracy of the method.

3.6. Sample analysis precision

The precision, determined by calculating the average coefficient of variation (CV) of nine repeated measurements on spiked blood (60, 1000, and 2000 μg/l) over an 8-week period, was 16%. This value, which reflects both intra- and inter-day variations, indicates the good reproducibility of the method.

3.7. Automated SPME analysis

We also applied the versatility of the PAL sampler to perform the fully automated SPME analysis of CN in blood using a Carbowax/DVB fiber capable of extracting a wide molecular range of analytes, including HCN from blood [18]. SPME is a rapid, sensitive, and solvent-free extraction method which

involves exposing a fused-silica fiber coated with a nonvolatile polymeric material to a sample or its headspace. During exposure, the volatile and semivolatile components of the sample with a chemical affinity for the SPME fiber coating are absorbed and retained on the fiber. Subsequently, the absorbed analytes may be thermally desorbed in the injector of a gas chromatograph for separation and quantitation [26]. Both the SPME and headspace variations of the method described in this paper gave comparable results in terms of sensitivity, throughput, and speed. However, in some cases, we observed that the SPME fiber darkened, the coating became powdery and was easily removed from the fused-silica. These are known potential problems associated with the stability of Carbowax-DVB fiber coatings [27]. Based on our results, we concluded that the headspace method was more robust and rugged than the SPME assay in the concentration range of interest for this study.

3.8. Stability of CN in blood

The levels of CN in blood decay rapidly in the first few hours after exposure [8,28]. We evaluated the stability of CN spiked in blood at 4 °C over 1 month by analyzing three samples: two spiked with KCN and one control (Fig. 3). The concentration of CN in the low spike sample (~400 μg/l) decreased by 30% after 1 day and by 50% after 6 days. Levels of CN indistinguishable from those of the nonspiked control were observed after 12 days. The concentration of CN in the high spike sample (~2700 μg/l) decreased by 20% after 1 day, by 30% after 2 days, and by 45% after 5 days. After 14 days, the CN levels dropped by 66% and remained relatively constant for the next 2 weeks. Our data confirm that CN disappears quickly from blood, but remains in the blood long enough to be detected in forensic or clinical samples that may not be available for analysis immediately after the exposure to CN, a situation that may be encountered after an incident affecting a large number of people.

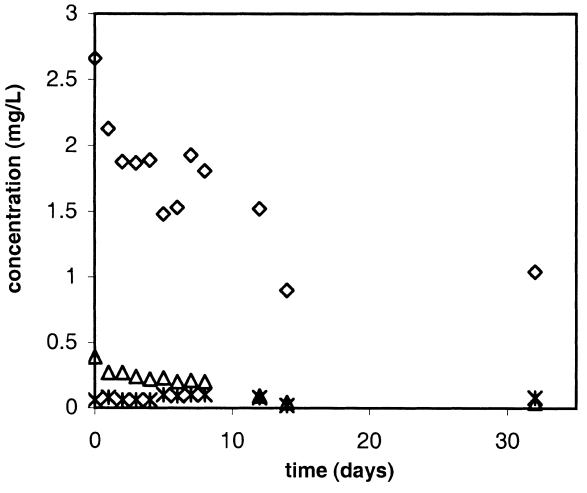

Fig. 3. Stability at 4 °C of cyanide spiked in whole blood: nonspiked control (*), low spike sample (~400 μg/l) (△), and high spike sample (~2700 μg/l) (◇).

3.9. CN in the blood of fire victims

We also measured CN in the blood of three fire victims with smoke inhalation (Figure 1). The blood samples were collected within 12 h after the fire, and cyanide-antidote medication was not used. We found a high concentration of CN (i.e. 1200 μg/l) in the blood of a person who died as a result of a trailer fire (Fig. 1D). We also detected much lower levels of CN (i.e. 103 μg/l) in the blood of a person with facial burns after a fire, and even a lower concentration of CN (i.e. 40 μg/l) in the blood of a person who suffered from mild smoke inhalation during a house fire (Fig. 1C). These results indicate that the assay is useful for detecting blood CN at levels above and below the toxic ranges for CN in whole blood, thus confirming the potential applicability of our method in clinical and forensic toxicology.

4. Conclusion

We have developed the first fully automated headspace-GC method for the rapid quantitative detection of CN in whole blood. Our assay is simple, sensitive, rugged, and accurate. It is also significantly less labor intensive (manual sample preparation <2 min/sample) and has a higher throughput (20 samples in ~6 h, including sample preparation and GC run) than previously reported GC methods. This automated and simple method is suitable for the rapid diagnosis of CN in clinical and forensic specimens.

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