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Determination of Cyanide in Biological and Non-biological Matrices by Headspace Gas Chromatography coupled to Flame-Ionization Detector

Technical Note

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Abstract

A simple, rapid and reliable method for quantitation of cyanide was developed on a headspace gas chromatograph coupled to a flame ionization detector using a HP-Innovax (Polyethylene glycol bonded) column on an Agilent 7890A GC. Cyanide in blood or other matrices was liberated by conversion of potassium cyanide to the volatile hydrogen cyanide (HCN) through addition of 5N sulfuric acid in a headspace vial and analyzed using an Agilent G1888 headspace auto-sampler. HCN gas diffuses into the headspace above the specimen in a sealed vial based on Henry's Law of partial pressure.

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This method showed good linearity (r^2= 0.9996) in the range of 8.0-60.0 μg/mL with 1.0 μg/mL of HCN as the limit of detection. The accuracy of the method at three different concentrations (8.0, 16.0, 50.0 μg/mL of HCN) was in the range of 95.0% to 101.9% and inter-day precision (%CV) ranged from 1.3% to 3.8%. Identical calibration curves with a coefficient of correlation above 0.990 were obtained when blood, gastric contents, liver tissue homogenate, urine and water were used as calibration standard matrices. Therefore, a single calibration curve is sufficient for diverse matrices and preparation of matrix-matched standards is not required. The method showed successful quantitation of Hydrogen cyanide in gastric contents, which is one of the most variable matrices in forensic toxicology. The method is well adopted for postmortem specimens because of its wide linear range, adaptability to various matrices, ease and rapidity of use.

تحديد السيانيد في العينات الحيوية وغير الحيوية بواسطة الحقن بالرأس الفراغي بتقنية كروماتوغرافيا الغاز المقترنة بكاشف التأين باللهب.

تم تطوير طريقة بسيطة وسريعة وموثوق بها من أجل التحديد

الكمي للسيانيد بواسطة الحقن بالرأس الفراغي بتقنية كروماتوغرافيا الغاز المقترنة بكاشف التأين باللهب باستخدام عمود فصل HP-In-novax (ارتباط البولي ايثيلين جلايكول) وجهاز كروماتوغرافيا الغاز Agilent 7890A GC. وقد تم تحرير السيانيد في الدم أو العينات الأخرى عن طريق التحويل من سيانيد البوتاسيوم لسيانيد الهيدروجين المتطاير (HCN) من خلال إضافة حامض الكبريتيك 5N في قارورة الرأس الفراغي وتحليلها باستخدام حاقن الرأس الفراغي الآلي G1888 من Agilent.

ينتشر غاز HCN في فراغ قارورة الرأس الفراغي المغلقة بشكل محكم فوق العينة بناء على قانون هنري للضغط الجزئي. ولقد أظهرت الطريقة خطية جيدة (r2 = 0.9996) في المجال 8.0-60.0 $\mu g/mL$ ، وكان حد الكشف الأدنى LOD لغاز HCN 1.0 $\mu g/mL$. تراوحت صحة الطريقة التحليلية accuracy عند ثلاثة تركيزات مختلفة (8.0، 16.0، 50.0 $\mu g/mL$) من HCN بين 95.0%-101.9%، بينما تراوحت دقة الطريقة (CV% inter-day precision) بين 1.3%-3.8%. وقد تم الحصول على منحنيات المعايرة مع معامل ارتباط فوق 0.990 عند تطبيق هذه الطريقة على كل من عينات الدم، محتويات المعدة، الكبد، البول، والماء والتي استخدمت كعينات معايرة قياسية.

وكنتيجة لهذه الدراسة فإن منحنى معايرة واحد يكفي للعينات المختلفة، وليس هناك حاجة لتحضير معايرة خاصة لكل عينة. وأظهرت الطريقة نجاحاً في التحديد الكمي لسيانيد الهيدروجين في عينات محتويات المعدة، والتي تعد واحدة من العينات الأكثر تغيراً في علم السموم الجنائي. ولقد تم اعتماد الطريقة بشكل جيد لعينات ما بعد الوفاة نظراً لمجالها الخطي الواسع، ومناسبتها لمختلف العينات، وسهولة وسرعة الاستخدام.

Introduction

Poisoning is a major medical issue in developing countries. Survival rate is very low with cyanide poisoning [1]. Cyanide belongs to the class of potent and fast-acting Chemical Asphyxiants that block the transport of oxygen into cells [2]. Exposure to cyanides is relatively common and may be accidental, occuring due to diet, industry, the enviroment, fumigation, mining, tobacco smoking and smoke from combustion. Exposure may also be intentional as in cases of suicide and homicide [3]. Endogenous production of cyanide from Cyano-genetic glycosides (bitter almonds, apple seeds) and hypotensive prescription medications like Sodium Nitroprusside also occurs. Average endogenous levels of cyanide in blood in

smokers and non-smokers are 0.123 µg/mL and 0.059 µg/mL respectively [1]. Toxic concentration of cyanide in blood ranges from 2-3 µg/mL [1]. Lethal concentrations of cyanide are 5-100 µg/mL in blood, 5 µg/mL in urine, 2-4000 µg/mL in gastric contents and as high as 43 mg/Kg in the liver [4-6]. The blood concentration found in suicide cases ranges from 1-53 µg/mL and in fire cases, the average blood concentration is reported to be 1.12 µg/mL [1].

Analytical methods for the determination of cyanide include titration, colorimetric techniques, spectrophotometry, ion selective electrodes and headspace gas chromatography with various detectors like TCD, NPD, Dual NPD/FID, ECD and MS to detect and quantify hydrogen cyanide gas in postmortem biological specimens [7-18]. Determination of cyanide in body fluids by photometry followed by classical Conway micro-diffusion is a time consuming method [1]. The use of a cyanide Ion Selective Electrode is convenient and fast but not free of interferences [19]. Amperometry is also very sensitive and popular but the response of the electrode varies with time [20]. Frequent reconditioning and recalibration is required for the electrode which affects the analysis time [19]. Methods using HPLC, LC/MS or GC/MS require derivatization, therefore they are not only expensive but time consuming. The method developed by Dumas et al. and Murphy et al. [10, 20] on GC-MS with a headspace technique is fast and sensitive but not well adopted for forensic toxicological analysis of postmortem specimens due to a narrow linear range and limited application on non-traditional matrices. Further in these methods, isotopically labeled cyanide was used as internal standard, which is expensive. Moreover, these methods have been validated only for aqueous solutions or well-preserved clinical specimens but not for postmortem specimens [21]. Manipulative techniques like extraction and/or micro-diffusion before analysis are unable to rapidly determine the cyanide concentration in biological samples [19].

Quantification of hydrogen cyanide by Headspace GC-FID alone has not been reported yet. In the present study, we employed Headspace GC-FID for the detection and quantification of hydrogen cyanide gas in postmortem specimens. This method is well adapted to various matrices i.e., blood, gastric contents, liver tissue homogenates, urine and water. Although FID is less sensitive for Hydrogen Cyanide as compared to NPD and MS, it is easy to operate, economical, has longer life and requires less maintenance. This method is well suited to forensic laboratories that

cannot utilize expensive techniques (GC/MS, GC/NPD). This study aimed to detect and quantify cyanides in diverse biological matrices.

Materials and Methods

2.1 Reagents:

Potassium cyanide (Reagent grade, 97%) was obtained from Acros Organics, New Jersey, USA. Analytical grade sulfuric acid, sodium hydroxide pellets and hydrochloric acid were purchased from Fisher Scientific. Synthetic drug-free blood and urine were obtained from Immunalysis Corporation, USA. 0.1N hydrochloric acid was used as the simulated gastric content.

2.2 Analytical Methods:
2.2.1 Headspace GC/FID Analysis:

An Agilent 7890A Gas Chromatograph coupled to a Flame Ionization Detector with a split injector and Agilent G1888 Headspace auto-sampler was used for the analysis. The loop, oven and transfer line temperatures of the headspace auto-sampler were set to 80°C, 70°C and 90°C respectively. The multiple headspace extraction mode was turned off. Injection time and oven stabilization time were 1.0 min with a loop equilibration time of 5 seconds. Loop fill time and vial pressurization time were set to 0.20 min with a vial equilibration time of 10 min. The low shaking mode was turned on in order to facilitate the liberation of HCN gas from matrices.

The separation in the gas chromatograph was accomplished on an HP-Innovax (PEG) capillary column (30m Length, 320μm internal diameter, 0.5μm film thickness). Injections were made in the split mode with the split ratio of 0.1:1. The injector was held at 200°C and at a pressure of 4.7543 psi. An Agilent split liner without glass wool was used. Septum purge flow was 3mL/min and split flow was 0.09479 mL/min. The nitrogen carrier gas (99.999% pure, Noor Chemicals Private Limited, Pakistan) flow to the column was set to 1.4 mL/min. The Gas saver mode was turned off in order to allow more nitrogen to run through the liner to displace any residual HCN, hence reducing the carry-over. The initial GC oven temperature was 50°C which was then ramped at a rate of 10°C/min to 70°C and held for 6.7 minutes. Maximum oven temperature was set to 265°C with an equilibration time of 0.5 min. The slow fan was disabled. The FID heater temperature was set to 250°C. The Hydrogen gas (fuel gas in FID produced

from HydroGen PH200 H2 generator by Peak Scientific, Scotland, UK) and air flow rates were set to 30 mL/min and 400 mL/min respectively with a make-up flow of 25 mL/min. The run time was 8.7 min and the retention time of HCN was 7.4 ± 0.1 min.

2.2.2. Preparation of stock solution of potassium cyanide (KCN):

A 2.5 mg/mL KCN stock standard solution was prepared by dissolving 25.0 mg KCN in 5 mL of deionized water in a 10 mL volumetric flask. One drop of 1N sodium hydroxide solution was added to alkaline the solution in order to prevent liberation of HCN from the stock solution of KCN. The final volume of the solution was made using the blank blood. Theoretically, 2.5 mg/mL KCN solution will liberate 1.04 mg/mL HCN gas (assuming that all KCN is converted to HCN) on acidification. This stock solution was stored in the refrigerator (2-8°C) and used to prepare various concentrations of HCN in the rest of the study.

Similarly, another stock solution of KCN (2.5 mg/mL) was prepared by using separate weighing of KCN in a second 10mL volumetric flask. This stock solution was used for the preparation of positive quality control samples.

2.2.3. Preparation of the Sample:

In a 20 mL headspace vial (Agilent technologies, USA), 1.0 mL of the sample (unknown or spiked matrix) was added. The vial was quickly sealed, after the addition of 1.0 mL of 5N sulphuric acid, by crimping the cap. The sealed vial was subjected to analysis in a sequential analysis mode of HS-GC/FID. Data analysis was performed using an Agilent GC Chemstation along with an external calibration method to quantify HCN gas.

2.3. Experimental methods:
2.3.1. Linearity:

For linearity study, five concentrations (8.0, 12.0, 16.0, 30.0, 60.0 μg/mL of HCN) were prepared in blank blood using KCN stock solution. Each concentration was run in triplicate for five days and concentration versus mean peak area curve was plotted. r^2 value was used to evaluate linearity.

2.3.2. Carry-over:

The appearance of unintended analyte in the air blank sample after the analysis of the positive control sample was evaluated. Carry-over after the highest concentration should

not exceed 10% of the signal of the lowest concentration. Air blanks were analyzed after each concentration of HCN (8.0, 12.0, 16.0, 30.0 and 60.0 μg/mL) to check carry-over.

2.3.3. Accuracy (% Recovery) and Precision (Reproducibility):

Three concentrations of HCN (8.0, 16.0, 50.0 μg/mL) were prepared by fortifying the blank blood with KCN stock solution. Each concentration was analyzed in triplicate on five different days (fifteen measurements for each concentration) spread over a period of 14 days. During this period, the system was subjected to routine maintenance (i.e., changing the GC Inlet liner and GC column) between different sets of injections in order to evaluate the reproducibility of this method. % recovery and % CV were calculated to evaluate the accuracy and precision respectively.

2.3.4. Limits of Detection and Quantitation (LOD & LOQ):

Ten different concentrations of HCN (0.5, 1.0, 2.0, 3.0, 4.0, 8.0, 12.0, 16.0, 30.0, 60.0 μg/mL) were prepared by fortifying the blank blood with KCN stock solution. Each concentration was analyzed in triplicate to evaluate the LOD and LOQ of HCN.

2.3.5. Robustness:

To evaluate the robustness of the method, key parameters of the analytical procedure were changed. Robustness towards the injection time was tested by changing 1.0 min injection time to 0.5 min. Robustness towards oven temperature was checked by changing the temperature gradient to an isothermal program at 70°C. Robustness towards the analyst was checked by carrying out the analysis by three untrained analysts.

2.3.6. Specificity towards other volatile analytes:

To validate the method against other volatile compounds, which are routinely tested in postmortem specimens, 1.0 mL of blank blood fortified with KCN stock solution at the target concentration 8.0 μg/mL HCN was added in each of the six headspace vials. 10 μL of ethanol, acetone, methanol, isopropanol and n-propanol were added in separately labeled headspace vials. 20mg of aluminium phosphide was added in the sixth headspace vial. All six vials were immediately sealed after the addition of 1.0 mL sulphuric acid (5N) and analyzed to evaluate interferences.

2.3.7. Matrices other than blood:

Calibration curves using four calibration levels (8.0, 16.0, 30.0 and 60.0 μg/mL of HCN) and a positive quality control sample (12.0 μg/mL of HCN) were prepared in different spiked matrices: Blank blood, gastric contents, liver tissue homogenate, urine and water. These concentrations in different matrices were analyzed to evaluate their calibration curves on the basis of r^2 values and % recoveries of positive control samples.

Results

3.1. Linearity:

The developed method combines the headspace analysis with a gas chromatograph to yield a simple chromatogram (Figure 1) with a peak of HCN detected by FID at the retention time 7.429 min. The calibration plot of average peak areas of five separate runs versus concentrations was linear from 8.0μg/mL to 60.0μg/mL and fits a least squares regression curve. Correlation coefficient (r^2) was 0.9996 (Figure 2). The value of $r^2 > 0.99$, also observed
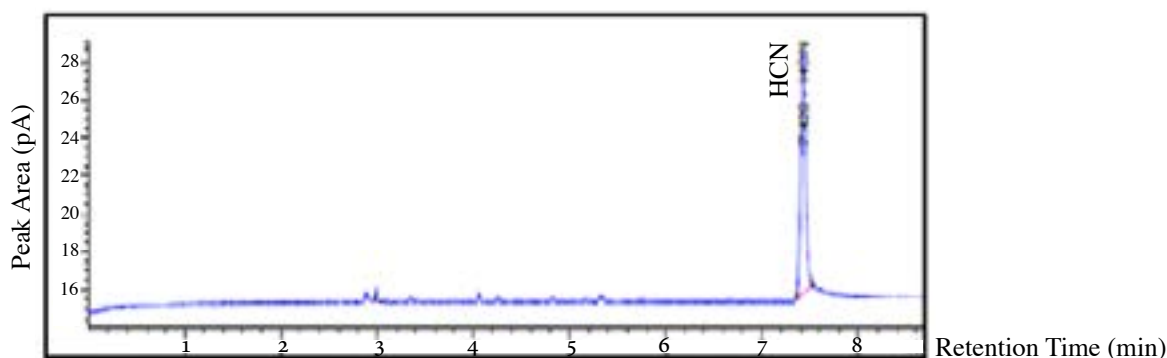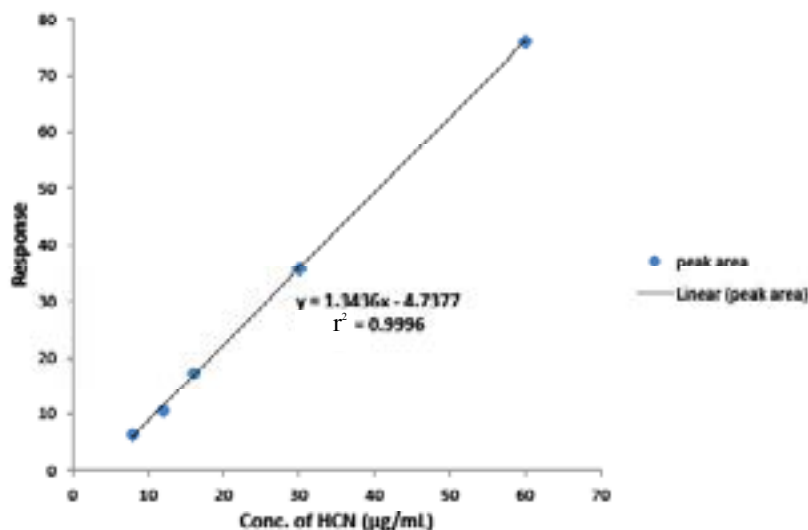

Figure 1- *Gas Chromatogram obtained for fortified blood sample (8.0 μg/mL HCN)*

Figure 2- *Average peak areas versus concentrations curve showing a high degree of linearity for the large dynamic range (8.0-60.0 µg/mL HCN)*

in other studies, indicated a strong linearity in our tested concentration range [22].

3.2. Carry-over:

All concentrations of HCN (8.0-60.0 µg/mL) were checked and carry-over of HCN gas was not observed even after analyzing the highest concentration of HCN (i.e., 60.0 µg/mL).

3.3. Accuracy and Precision:

An accuracy of 95.0% at 8.0 µg/mL, 101.9% at 16.0 µg/mL and 98.3% at 50.0 µg/mL HCN concentrations (n=5) were observed (Table 1). These results were within acceptable accuracy range (± 20% of the target concentration). The day-to-day relative standard deviation ranged from 1.3% to 3.9% and was within the acceptable range of % CV (≤ 5%). These results showed that the

Table 1- *Accuracy and Precision (n=5)*

Sr. No.	Concentration (µg/mL)	Days	Concentration Recovered (µg/mL)	Mean Conc. Recovered (µg/mL)	Standard deviation	Accuracy (%)	% CV
1	8.0	1	7.32	7.56	0.29	95.0	3.8
		2	7.53				
		3	7.93				
		4	7.25				
		5	7.77				
2	16.0	1	16.05	16.30	0.21	101.9	1.3
		2	16.32				
		3	16.20				
		4	16.62				
		5	16.30				
3	50.0	1	48.15	49.13	0.67	98.3	1.4
		2	49.65				
		3	48.72				
		4	49.37				
		5	49.73				

method is accurate and precise.

3.4. Limits of Detection and Quantitation (LOD & LOQ):

The limit of detection (LOD) determined was 1.0 µg/mL of HCN. The peak produced at the retention time of HCN (7.4 min) was reproducible at this concentration. The limit of quantitation (LOQ) was 4.0 µg/mL of HCN.

3.5. Robustness:

The method proved to be robust with respect to headspace injection time (0.5min injection time showed deviations less than 5% from the expected concentrations) and oven temperature. The QC samples analyzed by three different untrained analysts were within the acceptable recovery range (± 20% of the target concentration of HCN). Therefore, this method is robust and does not require extensive training of analysts.

3.6. Specificity towards other volatile analytes:

Retention times of ethanol, methanol, acetone, isopropanol, n-propanol and phosphine gas (poisonous gas released from aluminium phosphide on acidification) were considerably different from the retention time of HCN gas (Table 2). No interference was observed in quantification of HCN in the presence of these analytes.

Table 2- *Specificity towards other volatiles*

Sr. No.	Compound Name	Retention Time (min)
1	Hydrogen Cyanide	7.429
2	n- Propanol	7.326
3	Acetone	3.847
4	Ethanol	5.137
5	Isopropanol	5.004
6	Methanol	4.596
7	Phosphine	2.790

3.7. Matrices other than blood:

In forensic toxicology cases, different types of matrices are submitted for determination of poisons like cyanide. When cyanide is ingested as a solid or as a liquid, gastric contents may prove useful for toxicological analysis. Quantification of cyanide in gastric contents by headspace GC/FID is not reported in the literature. All the calibration curves for spiked matrices: Blank blood, gastric contents, liver tissue homogenate, urine and water showed good linearity with r^2 values greater than 0.990 (Table 3). These results suggest that cyanide can be quantified in diverse matrices using a single calibration curve. Therefore, valuable time of analysts can be saved, which would otherwise be wasted in the complicated preparations of matrix-matched standards.

Table 3- *Accuracy and Precision (n=5)*

Sr. No.	Matrix Used	r^2 value	Target Positive Control Conc. of HCN (µg/mL)	Recovery Range (± 20% of Target HCN conc. in µg/mL)	Conc. of HCN obtained (µg/mL)
1	Blood	0.9995			10.99
2	Gastric contents	0.9993			11.13
3	Water	0.9999	12.00	9.60-14.40	11.98
4	Liver Tissue	0.9905			9.87
5	Urine	0.9989			11.21

Discussion and Conclusion

A simple, rapid and reliable Headspace GC-FID method was developed and validated for qualitative and quantitative determination of hydrogen cyanide (HCN) in blood, urine, liver tissue, gastric contents and water. The method had a linear dynamic range from 8.0-60.0 µg/mL for postmortem specimens with a LOD of 1.0 µg/mL for HCN. No extraction is required for the sample preparation before analysis. The use of a Headspace Auto-sampler further shortens the analysis time. The

simplicity and short analytical time make this method more favorable than other analytical techniques available for analysis of HCN. The versatility of the technique allows the analysis of HCN gas in almost all biological and non-biological matrices. The method is robust, accurate, precise and convenient to perform. This method is well adopted for forensic toxicology to determine toxic or lethal levels of cyanide. The operating conditions are well suited for the analysis of other volatiles like ethanol, methanol, Isopropanol, n-propanaol, acetone as well as other poisonous gases like phosphine in biological or non-biological matrices. Further refinement and validation studies of this method with actual postmortem samples are underway.

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