Isotope Dilution-Mass Spectrometry Determination of Blood Cyanide by Headspace Gas Chromatography

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Abstract

A direct and sensitive method for the determination of blood cyanide by isotope dilution was developed. The blood is placed in a headspace vial, and K\(^{13}\)C\(^{15}\)N is added as internal standard. Addition of phosphoric acid liberates the cyanide as HCN. The detection is accomplished by mass spectrometry after a fine mass calibration tuning. The detection limit obtained is 0.3 pmol/L. The within- and inter-run coefficients of variation are 4.4% (for a concentration of 2.5 pmol/L) and 3.9% (for a concentration of 4.7 pmol/L), respectively. The observed recovery is 98%. A round-robin exercise was carried out to compare the performance of this method with others currently in use in other clinical laboratories.

Introduction

Cyanide exposure is still relatively common. Apart from sodium nitroprusside therapy (hypotensive agent) and ingestion of cyanide salts or cyanide producing chemicals (acetonitrile) in the context of suicidal or homicidal attempts, the main sources of exposure is smoke from fires or cigarette smoking, accidental inhalation of hydrocyanic acid in metals or plastics industries, and various types of foods such as cassava, cherry, or almond (1).

A potent and rapidly acting toxic agent, cyanide prevents binding of oxygen by inhibiting cytochrome oxidase, a cellular respiratory enzyme (2). Normal blood cyanide levels are usually less than 5 µmol/L (including smokers). Blood concentrations in the range of 75 µmol/L are usually associated with severe intoxication, and levels above 100 µmol/L are often fatal.

Numerous analytical methods have been proposed in the literature for the determination of blood cyanide. Although generally not very sensitive and time consuming, the classical spectrophotometric assay (1,3,4) has been the standard method of analysis for years. With the need for more appropriate methods aimed at quick results turnaround time, newer and more sensitive methods have since been developed using headspace injection (5-10) combined with gas chromatography–nitrogen-phosphorus detection (GC–NPD). Methods using derivatization prior to GC analysis (11-13) have also been proposed in attempts to further improve sensitivity. Recently, a method by high-performance liquid chromatography–mass spectrometry (HPLC–MS) was published (14). Although the method makes use of an isotopically labeled internal standard for added precision, the cyanide in the sample needs to be derivatized prior to analysis.

We are describing a new method that is rapid, sensitive, precise, rugged, and specific to cyanide. This method takes advantage of isotope dilution–MS combined with headspace GC to quantify blood cyanide levels. One determination can be accomplished within 20 min. Isotope dilution obviates the necessity of running a calibration curve each time a sample needs to be tested. The response factor of the cyanide relative to its isotopically enriched analogue (internal standard) is all that is required for accurate quantitation. Cyanide present in the sample is released as hydrogen cyanide following acidification. The equilibrated gas is then simply injected into the GC column. Cryo-focusing was found to be unnecessary.

Results from a round-robin exercise among five external laboratories are reported and compared with results obtained with this method.

Experimental

Chemicals

Potassium cyanide (KCN 25 g) was purchased from Sigma-Aldrich (#20781-0, Oakville, ON, Canada). Isotopically labeled potassium cyanide (K\(^{13}\)C\(^{15}\)N) was purchased from Cambridge Isotope Lab (#CNLM-1961, Andover, MA). Sodium hydroxide was obtained from J.T. Baker (#3722-01, Ville Mont-Royal, QC, Canada). Ascorbic acid (#25556-40) was obtained from Sigma-Aldrich, and phosphoric acid (85%) was from Fisher (A-242-1, Nepean, ON, Canada).

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Materials
The following materials were purchased from Chromato-
graphic Specialities (Brockville, ON, Canada): 30-m x 0.32-
mm capillary column GS-GASPRO (#J-113-4332); headspace
vials (22 mL) (#C223720-C); vial septums (#C669120M) and
aluminum caps (#51200210). Disposable plastic syringes and
needles were obtained from TERUMO (Quebec City, QC,
Canada).

Instrumentation
An HP-5890 GC coupled to an HP-5972 mass selective de-
tector was purchased from Hewlett-Packard (Mississauga, ON,
Canada). The MS detector was linked to a data-handling system
with Chemstation integration software (Agilent) for data ac-
quision and storage.

Analytical procedure
With each batch of samples, a reagent blank and a reference
material were run. One milliliter of EDTA anti-coagulated
whole blood was transferred into a 22-mL headspace vial,
and 250 µL of internal standard (K13C15N at 40 µmol/L in
0.1M NaOH) was added. Septa and aluminum caps were
gently placed on top of the vials without crimping to prevent
any possible external contamination. The vials were then
placed in a -80°C freezer for 5 min. After this time, they
were removed from the freezer and ascorbic acid (100 mg)
was added using a pre-calibrated spatula. Ascorbic acid was
used to prevent conversion of thiocyanates, if already present
in the sample, to cyanide. Two-hundred fifty microliters of a
1:1 mixture of phosphoric acid 85% and water was added to
each vial. The vials were rapidly crimped to prevent any loss
of cyanide and placed in a water bath at 60°C for 30 s, just
long enough for the blood to thaw. The vials were shaken on
a vortex mixer for approximately 10 seconds and were incub-
at 60°C for 15 min. Immediately after the incubation step,
the vials were again shaken on a vortex mixer during 15
s and 400 µL of the gas phase was sampled for injection into
the chromatographic system.

Chromatographic and MS parameters are given in Table I.

MS calibration
The auto-tune software function using PFTBA as calibrant is
not entirely appropriate to achieve the maximum sensitivity of
the instrument when looking at low range masses.
After normal tuning is performed and while the calibrant gas
is turned off, the repeller and lens parameters are optimized to
reach maximum intensities at masses 17 (water), 28 (nitrogen),
and 32 (oxygen). With this special tuning procedure, the re-
sulting sensitivity at low mass is greatly enhanced compared to
the normal auto-tuning mode.

Data analysis
Cyanide concentrations are evaluated using the technique of
isotope dilution at the mass ratio 27/29 (CN/13C15N). The
amount of K13C15N added to the samples corresponds to 10
µmol/L. It was observed that 13C15N contributes slightly to
mass 27, which is the one used to determine CN. Therefore, an
isotope contribution of 1% of mass 29 needs to be subtracted
from the intensity at mass 27 before calculating the mass ratio.

Method validation
The method was validated according to ISO 17025. The de-
tection and quantitation limits were determined by running 10
consecutive aliquots of a blood sample taken from a non-
smoker and spiked at 2 µmol/L with sodium cyanide (NaCN).
The limits of detection and quantitation were calculated as 3
and 10 standard deviations of the mean, respectively.
The intrarun coefficient of variation was estimated from the
analysis of the same 10 replicates.
The inter-run coefficient of variation was estimated by run-
ing 10 aliquots on different days of a blood sample taken
from a smoker with an average cyanide concentration of
4.7 µmol/L.
The average percent recovery was determined by spiking
base blood with 2 µmol/L of cyanide. The method performance
parameters are listed in Table II.
The accuracy of the method could not be evaluated due to
the absence of appropriate certified reference materials.
However, we carried out a round-robin exercise among five

<table>
<thead>
<tr>
<th>Table I. Instrumental Conditions</th>
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<tr>
<td>HP 5890</td>
</tr>
<tr>
<td>Gas Helium</td>
</tr>
<tr>
<td>Injector temp. (°C)</td>
</tr>
<tr>
<td>Detector temp. (°C)</td>
</tr>
<tr>
<td>Oven max. (°C)</td>
</tr>
<tr>
<td>Oven program</td>
</tr>
<tr>
<td>Total run time</td>
</tr>
</tbody>
</table>

| HP 5972                     |
| Solvent delay               | 5.5 min |
| SIM ion (H-CN)              | 27; (RT = 6.65 min) |
| Confirmation mass           | 26 |
| SIM ion (H13C15N)           | 29; (RT = 6.65 min) |

<table>
<thead>
<tr>
<th>Table II. Method Performance Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection limit</td>
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<tr>
<td>Quantitation limit</td>
</tr>
<tr>
<td>Intrarun coefficient of variation</td>
</tr>
<tr>
<td>Inter-run coefficient of variation</td>
</tr>
<tr>
<td>Recovery</td>
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<tr>
<td>Linearity</td>
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</tbody>
</table>

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<tr>
<th>Table III. Round-Robin Test Material</th>
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</thead>
<tbody>
<tr>
<td>CN-1</td>
</tr>
<tr>
<td>(blood) Blood from a non-smoker that has been spiked (Baseline level unknown)</td>
</tr>
<tr>
<td>CN-2</td>
</tr>
<tr>
<td>(solution) Standard solution of KCN in 0.1N NaOH</td>
</tr>
</tbody>
</table>
Results and Discussion

This analytical procedure was initially set up on an automatic headspace sampler (HS-10 from Perkin-Elmer, Inc.). During the course of method development, it was observed that cyanide tended to adsorb onto the needle surface, causing irregular carryover. Even though the needle was consistently flushed with the carrier gas and the temperature maintained at 60°C, the problem persisted. It was then decided to switch to manual injection with disposable syringes and modified terumo needles. Disposable needles have very sharp edges. While piercing the septum during the injection, the needle breaks off pieces of septum that tend to clog the needle. In order to avoid this, the needle tip is slightly bent using crimps to make it similar to “point #5” style needles commercially available but not disposable.

Optimum equilibration time of cyanide within the headspace vial was determined over the course of 2 h. A blood sample obtained from a non-smoking individual was spiked to 40 μmol/L with KCN. The initial equilibration temperature used was 60°C. Cyanide was determined at various timed increments (single determination) until a maximum peak or plateau could be observed. Results are shown in Figure 1.

In the context of emergency testing, there is no need to prolong the time of equilibration unnecessarily. Results show a maximum at 45 min; however, a steady-state is observed at around 103,000–104,000 in peak area, which is the reading we get at 15 min. The ideal time was set at 15 min.

Similarly, the equilibration temperature was optimized at the set equilibration time (single determination). Results, shown in Figure 2, show a maximum reading at 60°C. A typical chromatogram is shown in Figure 3.

Standard curve

Theoretically, isotope dilution has the advantage of generating bias-free results that are independent of the matrix. Most common headspace methods use no internal standard (5, 8–12). Some, however, use acetonitrile or propionitrile (7).

To demonstrate the effectiveness of using isotope dilution, we have compared aqueous and blood standard curves using isotope dilution and acetonitrile as internal standard.

Results are summarized in Figures 4 and 5 (single determination).

Table IV. Methodology Used by Participants

<table>
<thead>
<tr>
<th>Method</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>Autosampler</td>
<td>Headspace</td>
<td>Headspace</td>
<td>Spectro</td>
<td>GC</td>
<td>Spectro</td>
</tr>
<tr>
<td>Injector</td>
<td></td>
<td></td>
<td></td>
<td>No information</td>
<td>Headspace</td>
<td>No information</td>
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<tr>
<td>Detector</td>
<td>Fluorescence</td>
<td>MS</td>
<td>NP</td>
<td>Visible</td>
<td>NP</td>
<td>Visible</td>
</tr>
<tr>
<td>Linearity</td>
<td>0.03–2 μg/mL</td>
<td>0.01–2.6 μg/mL</td>
<td>0.1–1 μg/mL</td>
<td>0.05–2 μg/mL</td>
<td>0.03–4 μg/mL</td>
<td>0.1–6 μg/mL</td>
</tr>
<tr>
<td>Detection limit (DL)</td>
<td>0.03 μg/mL</td>
<td>0.01 μg/mL</td>
<td>0.1 μg/mL</td>
<td>0.03 μg/mL</td>
<td>0.014 μg/mL</td>
<td>0.1 μg/mL</td>
</tr>
<tr>
<td>Precision</td>
<td>9.7% (60 × DL)</td>
<td>4.4% (5 × DL)</td>
<td>11.4% (21 × DL)</td>
<td>14% (6 × DL)</td>
<td>16% (6 × DL)</td>
<td>6% (10 × DL)</td>
</tr>
<tr>
<td>IS</td>
<td>None</td>
<td>K¹³C¹⁵N</td>
<td>Acetonitrile</td>
<td>None</td>
<td>Acetonitrile</td>
<td>None</td>
</tr>
<tr>
<td>CRM</td>
<td>KCN (Aldrich)</td>
<td>None</td>
<td>NaCN (Aldrich)</td>
<td>None</td>
<td>None</td>
<td>NaCN (Sigma)</td>
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<tr>
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<td>ID</td>
<td>Multi level</td>
<td>External</td>
<td>No information</td>
<td>Standard curve</td>
</tr>
</tbody>
</table>
Clearly, the technique of isotope dilution is matrix independent. One can readily analyze blood or any other biological fluids without worrying about calibration curves. When acetonitrile is used as the internal standard, the analysis becomes matrix dependent judging from the difference in the slopes.

Method performance parameters

The method performance parameters are listed in Table II. One blood sample and one aqueous solution containing cyanide were distributed among five external laboratories that test for cyanide under routine settings.

Results of the participating laboratories are described in Table V. Our laboratory is Lab B.

In general, results for the blood sample are relatively good. Two distributions can be observed for the aqueous sample. The methods of Labs D and F would appear to be the least sensitive.

Lab A shows good performance for the blood; however, this lab shows a bias for aqueous samples that is perhaps due to the lack of an appropriate internal standard in the HPLC method. Our laboratory (Lab B) seems to show the best performance judging from the theoretical values. It seems also to be the only one showing no bias between aqueous and blood samples. Lab C possibly has a bias due to the calibration, which would explain high results for the two samples. It is known that the use of acetonitrile as an internal standard generates a positive bias on aqueous samples with this method. Lab D uses the standard calorimetric method. This method does not seem to be adequate to determine low concentrations. Also, a bias due to the type of calibration can be observed. Lab E uses a method very similar to that of Lab C. A calibration bias could be observed for the blood. Again, we know that the use of acetonitrile as an internal standard generates a positive bias on aqueous samples with this method, but curiously, results for the aqueous sample are lower. Lastly, Lab F uses the standard colorimetric method with its known disadvantages (lack of sensitivity and precision).

Conclusions

We have described a reliable and sensitive method that requires no analyte derivatization or special sample pre-treatment. Using the technique of isotope dilution, the standard calibration curve is no longer necessary. Results can be

![Figure 3](https://academic.oup.com/jat/article-abstract/29/1/71/735337)

**Figure 3.** Peak profile of a cyanide spiked blood sample equivalent to 10 μmol/L. Peak identification: 1, pentane and 2, cyanide.

![Figure 4](https://academic.oup.com/jat/article-abstract/29/1/71/735337)

**Figure 4.** Aqueous and blood standard curves using acetonitrile as internal standard.

![Figure 5](https://academic.oup.com/jat/article-abstract/29/1/71/735337)

**Figure 5.** Aqueous and blood standard curves using the technique of isotope dilution.

<table>
<thead>
<tr>
<th>Table V. Results* Obtained from the Participants</th>
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<tbody>
<tr>
<td>Lab Code</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
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<tr>
<td>D</td>
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<td>E</td>
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<td>F</td>
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* μmol/L.
generated within 20 min, and the method is also applicable to other biological fluids.

Results observed with the round-robin exercise confirms the excellent performance of this method.

Further work will be needed to automate the method using an headspace autosampler. Although some data are available in the literature (15–21), a stability test for cyanide in whole blood will need to be performed under various conditions (temperature, time, etc.) to confirm the stability/instability of the analyte. Consequently, it would be interesting to compare blood cyanide levels between smokers and non-smokers with this method.

References


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