Clinical Chemistry 52:3
458–467 (2006)

Drug Monitoring and Toxicology

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

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Background: **Cyanide (CN) is a lethal toxin. Quantification in blood is necessary to indicate exposure from many sources, including food, combustion byproducts, and terrorist activity. We describe an automated procedure based on isotope-dilution gas chromatography–mass spectrometry (ID GC/MS) for the accurate and rapid determination of CN in whole blood.**

Methods: **A known amount of isotopically labeled potassium cyanide ($K^{13}C^{15}N$) was added to 0.5 g of whole blood in a headspace vial. Hydrogen cyanide was generated through the addition of phosphoric acid, and after a 5-min incubation, 0.5 mL of the headspace was injected into the GC/MS at an oven temperature of −15 °C. The peak areas from the sample, $^1H^{12}C^{14}N^+$, at *m*/*z* 27, and the internal standard, $^1H^{13}C^{15}N^+$, at *m*/*z* 29, were measured, and the CN concentration was quantified by ID. The analysis time was 15 min for a single injection.**

Results: **We demonstrated method accuracy by measuring the CN content of unfrozen whole blood samples fortified with a known amount of CN. Intermediate precision was demonstrated by periodic analyses over a 14-month span. Relative expanded uncertainties based on a 95% level of confidence with a coverage factor of 2 at CN concentrations of 0.06, 0.6, and 1.5 μg/g were 8.3%, 5.4%, and 5.3%, respectively. The mean deviation from the known value for all concentrations was <4%.**

Conclusion: **The automated ID GC/MS method can accurately and rapidly quantify nanogram per gram to microgram per gram concentrations of CN in blood.**

Cyanide (CN) is a lethal toxin with many potential sources of exposure present in our environment. In nature, substances yielding CN are present in cassava plants and in certain seeds, such as the pits of wild cherries, peaches, and apricots *(1)*. The presence of CN in tobacco smoke represents an important health problem *(2)*. The volatile acid hydrocyanic acid (HCN)[1] is used to prepare acrylonitrile, an important component in the production of acrylic fibers, synthetic rubber, and plastics *(1)*. Gaseous HCN, produced when many synthetic and natural (e.g., wool and silk) products burn, is a threat to fire fighters and fire victims *(3)*. Large quantities of cyanides are used in many chemical processes, including fumigation, case hardening of iron and steel, electroplating, and the concentration of ores *(1)*. Because of its availability and history of military use, CN has been identified as a potential chemical terrorism agent *(4, 5)*.

Reported blood CN concentrations of clinical and forensic relevance vary greatly in the literature. In a recent review, Lindsay et al. *(6)* reported baseline blood CN concentrations ranging from 0.003 to 0.075 mg/L for nonsmokers and from 0.007 to 0.177 mg/L for smokers. In a single study conducted on victims of residential fires, investigators found that blood CN concentrations of survivors averaged 0.562 mg/L (n = 66), whereas concentrations for fire fatalities averaged 3.0 mg/L (n = 43), but variability in the reported CN concentrations was nearly 100% relative *(7)*. Part of the variability in the reported results is attributable to the short half-life of CN in blood as a result of the body's ability to detoxify CN, and another part of the variability is attributable to the instability of CN in blood that is stored for even short periods of time *(6, 7)*. Variance in the literature exists even for blood CN concentrations considered to be lethal, with values ranging from 1 to 3 mg/L *(6, 7)*. Thus, a useful analytical method for the determination of CN in blood must be able to measure from microgram per liter to

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Received September 23, 2005; accepted December 23, 2005.
Previously published online at DOI: 10.1373/clinchem.2005.061002

[1] Nonstandard abbreviations: HCN, hydrocyanic acid; ID GC/MS, isotope-dilution gas chromatography/mass spectrometry; and SIM, selected-ion monitoring.

milligram per liter concentrations of CN as rapidly as possible.

Many methods for the determination of CN in blood have been reported. Most procedures involve the liberation of CN^- from the blood matrix as gaseous HCN by the addition of acid. The evolved HCN is either absorbed in a basic solution or sampled directly from the headspace before detection. Detection has been based on colorimetry *(8)*, fluorometry *(2, 9)*, HPLC-mass spectrometry (MS) *(10)*, and gas chromatography (GC) with electron capture *(11, 12)*, nitrogen-phosphorus *(5, 13–15)*, and MS detection *(16, 17)*. The determination of CN by equilibrium headspace analysis appears most efficient, with analysis times ranging from 17 to 40 min per sample; however, accurate analysis depends on a matched, interference-free response between CN and an internal standard. Acetonitrile (CH_3CN) and propionitrile (CH_3CH_2CN) have been used as internal standards for headspace analysis of CN in blood *(5, 14, 15)*. Seto et al. *(14)* were not satisfied with CH_3CN as an internal standard. They found differences in the vaporization behavior of CH_3CN relative to HCN for different temperatures and matrices, as well as a large amount of CH_3CN in their control blood; they therefore resorted to a direct calibration method instead of an internal standard method. On the basis of reactivity, boiling point, and elution time differences alone, the response of HCN relative to CH_3CN or CH_3CH_2CN can be expected to vary significantly.

We present a method for the determination of CN in whole blood based on isotope-dilution (ID) GC/MS with headspace analysis using $K^{13}C^{15}N$ as the internal standard. Application of $K^{13}C^{15}N$ as an internal standard for headspace analysis decreases the analysis time and improves accuracy because the internal standard and analyte are chemically identical, enter the headspace under the same conditions, and elute from the GC column at the same time. Use of $K^{13}C^{15}N$ was first reported by Tracqui et al. *(10)* for the determination of CN in blood by HPLC-MS. Their procedure required derivatization of the samples, and analyses could be accomplished in 40 min. Recently Dumas et al. *(17)* published a method based on headspace GC/MS using $K^{13}C^{15}N$ as the internal standard. Their results show that GC/MS analysis of CN can be accomplished in half the time required by the HPLC method. The procedure of Dumas et al. had not been published at the start of our research. Our research was initiated to provide value assignment and stability testing data for a set of frozen blood-based CN standards. The standards are being produced by the Centers for Disease Control and Prevention to evaluate the measurement capabilities of members of the Laboratory Response Network for Chemical Terrorism, a network of public health laboratories equipped to respond quickly to acts of chemical terrorism *(18)*. The method we developed, although similar to that of Dumas et al. *(17)* in the use of headspace GC/MS with $K^{13}C^{15}N$ as the internal standard, differs in several ways. We use cryogenic oven cooling, which increases the sensitivity for HCN and decreases the cycle time of the GC. Sample analysis and cool down of the oven for the next injection is accomplished in 15 min. The method has been automated, allowing for unattended sample analysis. This is of particular relevance to a potential chemical terrorism incident involving CN, which would require increased sample analysis capacity. In this report, we present a full evaluation of the accuracy and day-to-day precision of our automated procedure.

Materials and Methods

MATERIALS

Labeled $K^{13}C^{15}N$ (atom fraction, 99% ^{13}C, 99% ^{15}N) was obtained from Sigma Aldrich. Natural $K^{12}C^{14}N$ standard solution was obtained from Spex CertiPrep. HPLC-grade water and 85% *o*-phosphoric acid were obtained from Fisher Scientific. Analytical reagent-grade sodium hydroxide was obtained from Mallinckrodt. Human whole blood from 3 nonsmokers was purchased from Interstate Blood Bank at the start of the study and stored at 4 °C for the duration of the study. Approximately 450 mL of blood was obtained from each donor. The blood obtained from the first donor contained sodium heparin as the anticoagulant, blood from the second donor contained sodium citrate as the anticoagulant, and blood from the third donor contained CDPA-1 (citrate-phosphate-dextrose-adenine) as the anticoagulant.

PROCEDURES

Preparation of internal standard and calibration. A stock solution containing ~500 μg/g of the labeled $K^{13}C^{15}N$ internal standard was prepared gravimetrically by dissolving the potassium cyanide in 0.1 mol/L NaOH. Masses were obtained by use of a balance with a readability of 0.01 mg. Concentrations are reported on a mass basis because greater accuracy can be obtained from gravimetric measurements than is typically achievable with volumetric techniques. Working internal standard solutions containing $^{13}C^{15}N$ mass fractions of ~3 μg/g were prepared periodically by gravimetric dilution of the stock solution with 0.1 mol/L NaOH. The concentration of $^{13}C^{15}N$ in the internal standard stock solution was calibrated by reverse ID MS against a standard solution of natural $K^{12}C^{14}N$. In this procedure, a known amount of natural CN is added to a weighed aliquot of the labeled internal standard solution, and the resulting mass ratio is measured in the GC/MS. Six calibration mixtures were prepared. The calibration mixtures contained a constant $^{13}C^{15}N$ concentration of 3 μg/g and $^{12}C^{14}N$ concentrations of 0.05, 0.25, 1, 2.5, 7.5, and 14.5 μg/g. Once the calibration mixtures were prepared, they were not subject to bias from CN loss because the ratio of natural/labeled CN, established on first mixing, defined the concentration. Measurements were performed on 0.1-g aliquots of each of the calibration mixtures, diluted to 0.5 g with 0.1 mol/L NaOH in a 5-mL headspace vial and processed in the same manner as samples (described below). The resulting

6 calibration samples had a constant internal standard concentration of 0.6 μg/g $^{13}C^{15}N$ and nominal $^{12}C^{14}N$ concentrations of 0.01, 0.05, 0.2, 0.5, 1.5, and 2.9 μg/g. The measured 27/29 peak-area ratios were used to calculate the labeled internal standard stock solution concentration for the given set of GC/MS operating conditions. This procedure is intended to compensate for the response factor of the instrument across a wide dynamic range. The concentration of the 3 μg/g $^{13}C^{15}N$ working internal standard solution used for sample analysis was calculated by dividing the measured stock solution concentration by the gravimetric dilution factor. The concentration of the working internal standard solution was checked periodically by use of fresh calibration mixtures prepared from a 0.1-g aliquot of the working internal standard solution and a known amount of natural CN added from a fresh dilution of the natural standard solution. The mean (SD) concentration of the natural CN standard solution used for calibration of the labeled internal standard stock solution was certified to be 1003 (5) mg/L. This was converted to a mass-fraction basis after the density of the solution was determined to be 1.0222 (0.0020) g/mL (n = 4). The concentration of the natural standard solution was verified by titration with high-purity silver nitrate with potassium iodide used as indicator, as described by Rodkey and Collison *(19)*.

Automated sample analysis. CN-fortified blood samples were prepared in 5-mL headspace vials by addition, by mass, of a 0.1-g aliquot of the appropriate diluted natural standard solution to 0.5 g, by mass, of human whole blood stock. Baseline blood samples were prepared by addition of 0.5 g by mass of the human whole blood stock to a 5-mL headspace vial. A 0.1-g aliquot of the working internal standard solution was added by mass to the CN-fortified and baseline samples, and the vials were sealed with aluminum crimp caps that contained a blue Teflon-faced septum and magnetic insert. Samples were placed in the Pelletier cooled (5 °C) tray of a Gerstel dual-rail MPS 2 PrepStation. The PrepStation was configured to accept 10- and 20-mL vials. To accommodate the 5-mL headspace vials, the tray was elevated slightly from its standard position. The PrepStation was used to automatically generate and inject HCN from the samples into the GC/MS instrument. Samples were processed sequentially. HCN was produced by the automated addition of 0.2 mL of 50% mass concentration phosphoric acid solution. The phosphoric acid solution was prepared by diluting 59 mL of 85% *o*-phosphoric acid to 100 mL with HPLC-grade water. After addition of the acid, the sample vial was automatically transferred to a heated agitator and incubated for 5 min at 42 °C. Metal inserts were placed inside the incubator to accommodate the 5-mL vials. A 0.5-mL aliquot of the headspace was automatically sampled by a heated (47 °C) gas-tight syringe and injected into the GC/MS, and the GC/MS analysis cycle was initiated. Additional samplings of the headspace from the same vial were analyzed at 15-min intervals. For this work, the headspace was sampled twice, and the mean 27/29 area ratio was used for calculations. After the first injection, the sample vial was transferred back to the cooled tray, and the headspace syringe was flushed with high-purity helium for 1 min. Once the syringe flush was complete, the vial was transported back to the heated agitator for the remainder of the GC cycle, in preparation for the next injection. A 0.5-mL aliquot of air from an empty vial was injected periodically to check for carryover between samples containing different concentrations of CN. No carryover was observed. A slight increase in the background at *m/z* 29 was occasionally observed. This could be removed by rinsing the headspace needle and syringe with acetone and drying thoroughly before reuse.

Investigation of the effect of ascorbic acid

Because ascorbic acid has been used in several procedures for the determination of CN in blood to prevent interference from thiocyanate (SCN) *(5, 15, 17, 20, 21)*, we conducted a preliminary investigation of the effect of its presence and absence on results for baseline CN concentrations and on samples fortified with a known amount of CN. Samples with no ascorbic acid present were prepared as described above. Samples with ascorbic acid were prepared in the following manner. The 0.5-g blood sample (baseline or fortified) was added to the headspace vial, and the vial was sealed. A 0.15-mL volume of an aqueous solution containing an L-ascorbic acid concentration of 50 g/L was added via the septum, followed by the working internal standard solution. Samples were processed by the automated sample analysis procedure described above. Experiments were conducted on refrigerated as well as frozen blood samples because our eventual goal was to analyze frozen CN-fortified blood-based standards.

GC/MS

GC/MS analysis was performed with a Hewlett Packard Model 6890 GC/Model 5973 MSD controlled by Chemstation software (Rev. BA) and equipped with an HP-PLOT Q-bonded polystyrene-divinylbenzene phase (15 m × 0.32 mm; 20-μm film thickness) column (Agilent Technologies). The 0.5-mL headspace sample was injected into the GC port in the splitless mode, with a 0.75-min purge delay. The injection port was equipped with a direct 2-mm i.d. deactivated liner and was maintained at 100 °C. The helium carrier gas was maintained at a constant flow rate of 2.1 mL/min. The transfer line was held at 230 °C. The initial oven temperature of −15 °C was obtained by cryogenic cooling with liquid nitrogen. The oven was held at −15 °C for 1.5 min after the sample injection and then ramped at 40 °C /min to 240 °C, where it was held for 1 min. The analysis time was 7.63 min with a total cycle time (analysis and cool down) of ~15 min per injection. The retention time of HCN was ~4.7 min. Data were collected by selected-ion monitoring (SIM) of *m/z* 27 ($^{1}H^{12}C^{14}N^{+}$) and *m/z* 29 ($^{1}H^{13}C^{15}N^{+}$) at dwell times of 30

ms each. Chromatograms were integrated manually. Peak area data for *m/z* 27 and *m/z* 29 were copied into a commercial spreadsheet program, and the 27/29 peak area ratio was calculated.

CALCULATION OF CN CONCENTRATION

The following functional relationship was used to calculate the ID GC/MS CN concentrations:

$$c_{\text{Sample}} = \left\{ \frac{m_{\text{IS}} \times c_{\text{IS}}}{\text{Ab } 27_{\text{Sample}}} \times \frac{\left[\left(\frac{27}{29}\right)_{\text{IS}} - \left(\frac{27}{29}\right)_{\text{Blend}} \right]}{\left[\left(\frac{27}{29}\right)_{\text{Blend}} \times \left(\frac{29}{27}\right)_{\text{Sample}} - 1 \right]} - blk \right\} \times \frac{M_r\text{CN}}{m_{\text{Sample}}}$$

Where

c_{Sample} = the measured sample CN mass fraction in units of μg/g

m_{IS} = the mass (g) of added labeled working internal standard solution

c_{IS} = the mean labeled working internal standard concentration in units of μmol $^{13}C^{15}N$/g measured by reverse ID GC/MS

Ab 27_{Sample} = the molecular fraction of natural $^{1}H^{12}C^{14}N$ in the sample

$\left(\frac{27}{29}\right)_{\text{IS}}$ = the measured $^{1}H^{12}C^{14}N/^{1}H^{13}C^{15}N$ ratio in the pure labeled internal standard

$\left(\frac{27}{29}\right)_{\text{Blend}}$ = the measured $^{1}H^{12}C^{14}N/^{1}H^{13}C^{15}N$ peak-area ratio in a sample to which the labeled internal standard has been added

$\left(\frac{29}{27}\right)_{\text{Sample}}$ = the natural $^{1}H^{13}C^{15}N/^{1}H^{12}C^{14}N$ ratio in the sample

blk = the baseline concentration of CN in the blood stock in units of μmol

M_r CN = the relative molecular mass of cyanide

m_{Sample} = the sample mass (g)

Of the 9 variables listed in the equation, only 3, the sample mass (m_{Sample}), the mass of the internal standard (m_{IS}), and the $(27/29)_{\text{Blend}}$ ratio, need be measured for every sample. The remaining variables are measured periodically or are constants. Measurement procedures for the labeled internal standard solution concentration (c_{IS}) and the baseline CN concentration in the blood stock (*blk*) are described above. The 29/27 ratio of the pure internal standard solution, $(27/29)_{\text{IS}}$, was measured by GC/MS at the start of the investigation in separate aliquots containing 1.2 μg of $^{13}C^{15}N$, four times more than the amount added to samples to overwhelm any natural CN blank from the measurement system. The 29/27 ratio of natural CN in the sample $(29/27)_{\text{Sample}}$, the molecular fraction at *m/z* 27 from natural CN in the sample (Ab 27_{Sample}), and the relative molecular mass of cyanide (M_r CN) are constants. The molecular fraction at *m/z* 27 from natural CN in the sample (Ab 27_{Sample}), was calculated to be 0.98517 by multiplying the atom fraction of ^{1}H by the atom fraction of ^{12}C and the atom fraction of ^{14}N. Atom fractions of the elements were obtained from Coplen et al. *(22)*. In like manner, the molecular fraction at *m/z* 29 from natural CN in the sample was calculated to be 0.0000428 by summing the individual atom fractions for $^{1}H^{13}C^{15}N$, $^{2}H^{12}C^{15}N$, and $^{2}H^{13}C^{14}N$. The 29/27 ratio of natural CN, $(29/27)_{\text{Sample}}$, was calculated to be 0.0000434 by dividing the molecular fraction calculated for *m/z* 29 by the molecular fraction calculated for *m/z* 27.

CALCULATION OF EXPANDED METHOD UNCERTAINTY

The expanded ID GC/MS method uncertainty was computed from the individual type A and type B uncertainty estimates for each component of the ID equation. Type A components included the standard uncertainty of the $(27/29)_{\text{Blend}}$ ratio measurement, the standard uncertainty of the enriched internal standard solution calibration (c_{IS}), and the standard uncertainty of the CN baseline measurements in the blood stock. Type B errors included uncertainty components for the following: assay and aliquoting of the CN standard solution used for the internal standard calibration (c_{Nat}), mass measurements (m_{Sample} and m_{IS}), isotopic composition, and relative molecular mass of CN in the sample (Ab 27 and M_r CN), the 27/29 ratio in the enriched internal standard $(27/29)_{\text{IS}}$, and the 29/27 ratio in a natural sample $(29/27)_{\text{Sample}}$. Type B uncertainties were modeled by a uniform probability distribution, and standard uncertainties for type B components were derived by dividing by the square root of 3. Standard uncertainties were multiplied by sensitivity coefficients. Sensitivity coefficients were derived by evaluation of the partial derivatives of the ID equation with respect to each of the measured quantities *(23)*. Uncertainties were combined according to ISO guidelines as the square root of the sum of the squares *(24)*. The expanded uncertainty was calculated based on $k = 2$.

Results

OVEN TEMPERATURE

SIM chromatograms obtained at *m/z* 27 and *m/z* 29 for 0.6 μg/g solutions of CN in 0.1 mol/L NaOH, using the HP-PLOT Q column without cryogenic cooling at an oven temperature of 33 °C and with cryogenic cooling at oven temperatures of −15 °C and −32 °C, are shown in Fig. 1. As indicated in Fig. 1, symmetric and narrow peaks were obtained only by reducing the column temperature below ambient. Peaks other than HCN appear in the SIM chromatograms because they have mass spectra fragments at the masses of interest, primarily at *m/z* 29. Peaks were identified by obtaining spectra for the same sample in the scan mode from 25 to 200 atomic mass units (amu) and comparing with the NIST mass spectral database. Match factors were better than 600. A SIM chromatogram ob-

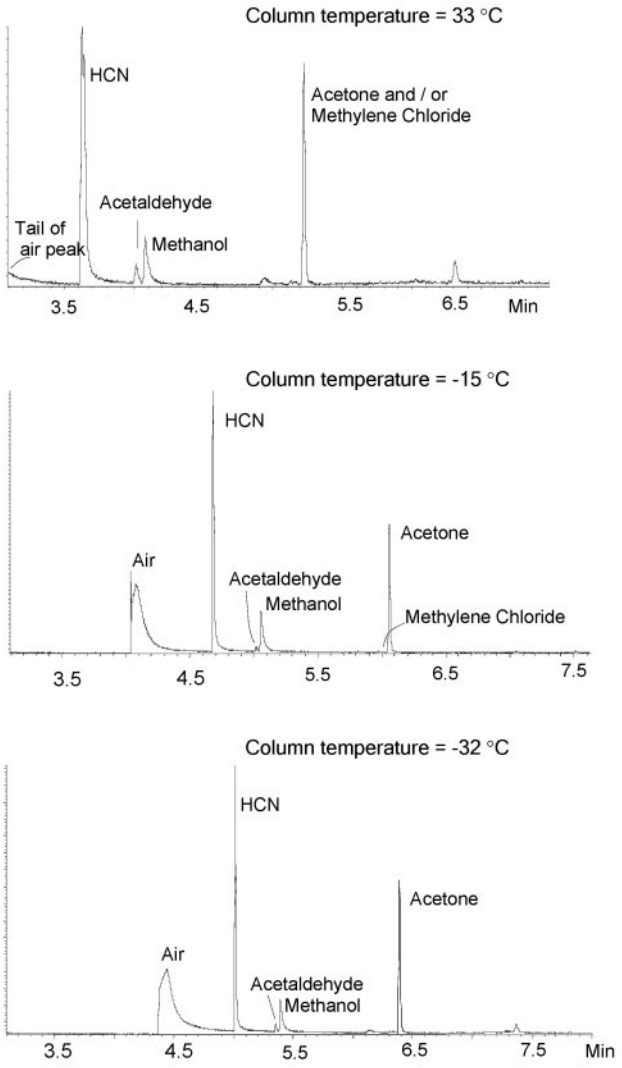

Fig. 1. SIM chromatograms obtained at *m/z* 27 and *m/z* 29 for 0.6 μg/g solutions of CN in 0.1 mol/L NaOH with an HP-PLOT Q column without cryogenic cooling at an oven temperature of 33 °C (*top*), and with cryogenic cooling at oven temperatures of −15 °C (*middle*) and −32 °C (*bottom*).

tained at *m/z* 27 and *m/z* 29 for a fortified blood sample containing a CN mass fraction of 0.5 μg/g and to which enriched $^{13}C^{15}N$ had been added at a mass fraction of 0.6 μg/g is shown in Fig. 2. The chromatogram was obtained at an oven temperature of −15 °C. The inset in Fig. 2 shows the peaks obtained for the $^{1}H^{12}C^{14}N^{+}$ ion at *m/z* 27 and the $^{1}H^{13}C^{15}N^{+}$ ion at *m/z* 29. The scale of the *y* axis of the inset is the same as the *y* axis of Fig. 2. As for Fig. 1, peaks were identified by obtaining spectra for the same sample in the scan mode. HCN is well resolved from the components of air, acetaldehyde, methanol, ethanol, and acetone peaks. Of the identified constituents, only acetaldehyde and ethanol were specific to the blood stock; the remaining peaks were present in blank solutions and represent laboratory background.

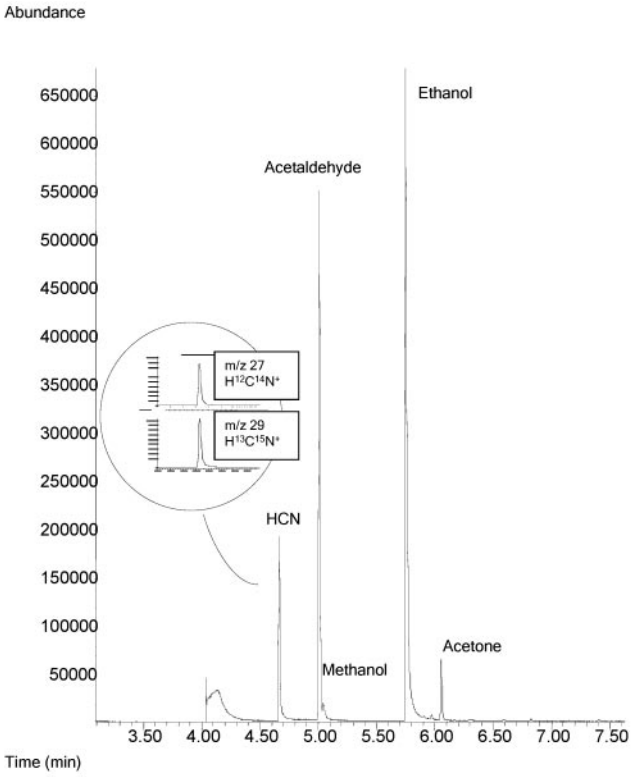

Fig. 2. SIM chromatogram obtained at −15 °C for a fortified blood sample containing a CN mass fraction of 0.5 μg/g and supplemented with enriched $^{13}C^{15}N$ at a mass fraction of 0.6 μg/g.

The *inset* shows the peaks obtained for the $^{1}H^{12}C^{14}N^{+}$ ion at *m/z* 27 and $^{1}H^{13}C^{15}N^{+}$ ion at *m/z* 29.

INCUBATION TEMPERATURE

The signal generated in the headspace varied with incubation temperature; we therefore investigated several incubation temperatures. The results for CN-fortified blood samples incubated at temperatures of 32, 42, and 52 °C are shown in Fig. 3. Each sample contained the same amount of natural and labeled CN, and the headspace was sampled every 15 min starting at 5 min. Both the peak

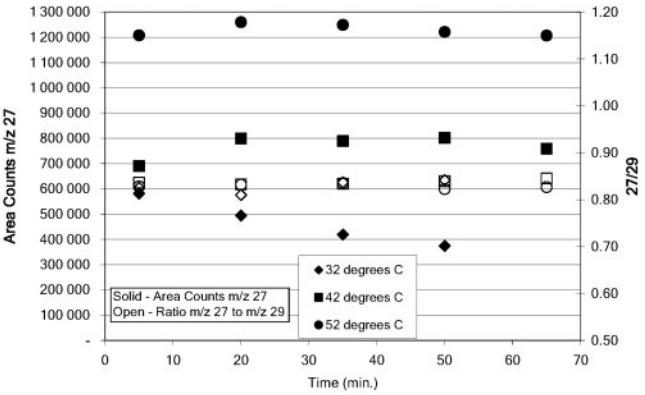

Fig. 3. Peak areas at *m/z* 27 (*filled symbols*) and peak-area ratios of *m/z* 27 to *m/z* 29 (*open symbols*) at incubation temperatures of 32 °C (*diamonds*), 42 °C (*squares*), and 52 °C (*circles*).

areas obtained for *m/z* 27 (left-hand *y* axis; solid symbols) and the ratio of peak area at *m/z* 27 to peak area at *m/z* 29 (right-hand *y* axis; open symbols) are shown vs incubation time for the 3 temperatures. As indicated by the open symbols, the measured 27/29 ratio did not vary with temperature or with incubation time, despite variation in the area counts over time. For example, area counts at *m/z* 27 for the sample incubated at 32 °C varied from 580 000 at 5 min to 370 000 at 50 min, a 44% change, but the measured 27/29 ratio varied by <4% over the same time period. Constant peak-area ratios were obtained within 5 min of headspace generation, indicating that equilibration of the natural CN and labeled isotope analog occurred before generation of the headspace. Although higher signals were generated at 52 °C, it was desirable to keep the incubation temperature as low as possible because of reports of the artifactual production of CN at increased temperatures *(20)*. As a result, an incubation temperature of 42 °C was chosen because of the stabilities of the signal and ratio, which were better than those obtained at 32 °C and similar to those obtained at 52 °C. Typically, 600 000 area counts were obtained at *m/z* 27 for a CN mass fraction of 0.5 μg/g in blood incubated at 42 °C. The mean (SD) background at *m/z* 27 and 29 was 4000 (1500) area counts (n = 5).

USE OF ASCORBIC ACID

At baseline CN concentrations, the effect of ascorbic acid use on the measured CN concentration was small. The mean (SD) baseline CN concentration measured for blood stored at 4 °C was 0.0133 (0.0013) μg/g (1s; n = 3) without the addition of ascorbic acid and 0.0083 (0.0019) μg/g (1s; n = 3) for the same blood stock with the addition of ascorbic acid. The mean baseline CN concentration measured for a different blood stock stored at −20 °C was 0.0919 (0.0038) μg/g (1s; n = 6) without ascorbic acid and 0.0854 (0.0085) μg/g (1s; n = 6) with the addition of ascorbic acid. However, results for blood samples fortified with 0.3 and 1.5 μg/g CN and stored at −20 °C were 20% higher when ascorbic acid was used vs when it was not. In addition, a signal suppression effect was observed with the use of ascorbic acid. Area counts for the internal standard at *m/z* 29 were 20% lower for refrigerated baseline samples in the presence of ascorbic acid and as much as 40% lower for frozen samples.

LINEARITY

We compared linear regression lines generated with blood-based standards with regression lines based on calibration standards prepared in 0.1 mol/L NaOH. The slope, *y*-intercept, and correlation coefficient are listed in Table 1 for measurements with and without the blood matrix. Calibration standards were produced by adding a 0.1-g aliquot of the premixed calibration standards (described above) to 0.5-g samples of blood or 0.1 mol/L NaOH. Measured area counts at *m/z* 27 were corrected for endogenous $^{12}C^{14}N$ in the blood and for the small amount of $^{12}C^{14}N$ in the internal standard before construction of the regression line. The regression line was constructed by plotting the blank-corrected 27/29 peak-area ratio on the *y* axis and the ratio of the amount of $^{12}C^{14}N$ standard to the amount of $^{13}C^{15}N$ internal standard on the *x* axis. ANOVA at a 95% level of confidence indicated that the slopes and intercepts for the blood-based curves were not statistically different from those measured without the blood matrix. These data indicate there is no matrix-related bias provided the CN endogenous to the blood is taken into account. Furthermore, the system was linear over the range 0.01–2.9 μg/g, CN concentrations that are encountered in most clinical and forensic situations *(6)*.

INTERNAL STANDARD COMPOSITION

The ratio of $^{1}H^{12}C^{14}N$ to $^{1}H^{13}C^{15}N$ in the pure labeled internal standard, $(27/29)_{IS}$, was measured multiple times at the start of the investigation. The mean (SD) measured ratio $(27/29)_{IS}$ was 0.0052 (0.0010) (n = 9). This ratio indicates that there is more natural CN present in the material than reported on the product label, which lists both the atom fraction of ^{13}C and ^{15}N as 0.99. To test for possible contamination during preparation and measurement, we dissolved and assayed separate aliquots of different amounts of the labeled internal standard salt. In each case, a measured $(27/29)_{IS}$ ratio within 20% of the mean value was obtained. Thus, the mean measured ratio of 0.0052 was used in the sample calculations rather than the ratio calculated based on atom fractions listed on the product label. Error in the sample concentration resulting from uncertainty in the $(27/29)_{IS}$ ratio was minimized because, despite the presence of some natural CN in the labeled internal standard, the material was still highly enriched.

Table 1. Commutability and linearity of GC/MS for CN in blood and 0.1 mol/L NaOH.

		Blood			0.1 mol/L NaOH		
Day	CN concentration range, μg/g	Slope	*y*-intercept	r^2	Slope	*y*-intercept	r^2
1	0.01–2.9	0.9382	0.0137	0.99988	0.9336	0.0066	0.99998
2	0.01–2.9	0.9188	0.0098	0.99994	0.9054	0.0271	0.99977
	Mean	0.929			0.920		
	SD	0.014			0.020		

LABELED INTERNAL STANDARD CONCENTRATION (c_{IS})

As stated, the concentration of CN in the internal standard stock solution was measured by reverse ID MS. Here the ID equation was solved for c_{IS}, and the variables subscripted with "Sample" were applied to the natural CN standard (c_{Nat}). Results for the 6 calibration mixtures measured near the start (calibration 1), middle (calibration 2), and end (calibration 3) of the investigation along with the gravimetric dilution factors used to obtain the working internal standard solution concentrations are listed in Table 1 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue3/. Variation among the 6 calibration mixtures was less than 5% relative. The concentrations of the internal standard solutions remeasured near the middle and end of the investigation agreed with the initial measurement to within 2%.

BASELINE CN OF BLOOD STOCKS (*blk*) AND LIMIT OF DETECTION

The baseline CN concentration in each blood stock was measured and tracked over its period of use. Typical measured 27/29 area ratios for these samples ranged from 0.02 to 0.03. The mean (SD) endogenous blood CN concentration measured over a 4-week period for the first donor was 0.0177 (0.0010) μg/g (n = 6). As the blood was repeatedly sampled over a 4-month period, the CN concentration was observed to double; therefore, blood from the second donor was used. The mean (SD) CN concentration measured in blood from the second donor was 0.0144 (0.0024) μg/g (n = 4). The blood CN concentration from this source was also found to increase after several months of use to 0.0271 (0.0037) μg/g (n = 3). The CN concentration measured for blood from the third donor was 0.0152 (0.0025) μg/g (n = 3) and was tracked for less than 1 month. The baseline CN content of the blood was measured during each analysis period and used for the corresponding sample calculations. The detection limit, calculated as 3 SD of the measured CN baseline concentrations, ranged from 0.003 to 0.007 μg/g.

ACCURACY, REPEATABILITY, AND TIME-DIFFERENT INTERMEDIATE PRECISION

We assessed the repeatability of the method by analyzing 4 replicate samples of refrigerated human whole blood fortified with a known amount of CN at nominal concentrations of 0.06 μg/g (low), 0.6 μg/g (medium), and 1.5 μg/g (high). Samples were prepared and analyzed within a 2-h period. The results are presented in Table 2. The baseline CN content of the blood stock was subtracted from the reported value, giving mean corrections of 25%, 3%, and 1% at 0.06, 0.6, and 1.5 μg/g, respectively. The relative standard deviation (1s; n = 4) was better than 2% for the medium- and high-concentration samples and better than 5% for the low-concentration samples. In all cases, results were within 4% of the added amount of CN.

We estimated the time-different intermediate precision of the ID GC/MS procedure by use of the standard deviation of the percentage difference between the measured and added CN for fortified samples analyzed periodically over a 14-month span. The GC/MS instrument was used to measure analytes other than CN throughout this period. Refrigerated blood samples fortified with a known amount of CN were analyzed, as were samples containing a known amount of CN in 0.1 mol/L NaOH. All samples were freshly prepared by adding the appropriate amount of CN to a 0.5-g aliquot of blood or 0.1 mol/L NaOH as specified, so as not to confound the results with stability issues of CN in stored samples. Samples prepared in this way did not contain identical amounts of CN. Results for the low, medium, and high CN concentrations are presented in Tables 2, 3, and 4, respectively, in the online Data Supplement. Each table contains the sample analysis date, blood/NaOH sample mass, internal standard mass, the measured $(27/29)_{Blend}$ ratio for 2 headspace samplings at incubation times of 5 and 15 min, the measured CN concentration, the concentration of added CN, and the calculated percentage difference between the measured and added CN concentrations. Measured CN concentration results were calculated from the mean of the two $(27/29)_{Blend}$ ratio measurements, but as evidenced by the general good agreement of the 2 measurements, it was not necessary to sample the headspace twice. The baseline CN content of the blood stock was subtracted from the measured value of blood-based samples before comparison with the added CN concentration. The standard deviation of the percentage difference between the measured and added CN at 0.06 μg/g for blood-based samples was 7.7% (n = 21), and the mean difference was 2.4% lower than the added amount of CN. Part of this variability can be attributed to the correction for baseline CN in the blood stock. The standard deviation for NaOH-based controls with CN concentrations ranging from 0.06 to 0.25 μg/g was 5.1% (n = 8) with a mean difference 0.64% lower than the added amount. The standard deviation at 0.6 μg/g CN for blood-based controls was 2.0% (n = 15), and the mean difference was within 1% of the added amount of CN.

Table 2. Repeatability and accuracy of ID GC/MS determination of CN in human whole blood.

Sample	Low concentration (0.06 μg/g)	Medium concentration (0.6 μg/g)	High concentration (1.5 μg/g)
1	0.0555	0.6311	1.5548
2	0.0602	0.6151	1.5363
3	0.0575	0.6176	1.5088
4	0.0612	0.6191	1.5167
Mean	0.0586	0.6207	1.5291
SD	0.0026	0.0071	0.021
RSD,[a] %	4.4	1.2	1.4
Difference from added CN, %	−2.3	3.5	1.9

[a] RSD, relative standard deviation.

Similar results were obtained for the NaOH-based controls at this concentration. Finally, the standard deviation for blood-based controls at 1.5 μg/g CN was 2.4% (n = 15), and the mean difference was within 1.5% of the added CN amount. Similar results were obtained for NaOH-based controls at 3.0 μg/g CN.

EXPANDED METHOD UNCERTAINTY

The uncertainty components used to calculate the expanded method uncertainty for the determination of CN by ID GC/MS at 0.06, 0.6, and 1.5 μg/g are presented in Table 3. The standard uncertainty of the $(27/29)_{Blend}$ ratio measurement was based on the mean standard deviation of measurements at 5 and 15 min for each concentration. The standard uncertainties of the enriched internal standard solution calibration (c_{IS}) and the CN baseline measurements were based on the analyses with the highest standard deviation. Uncertainty in the assay and aliquoting of the CN standard solution was estimated from the uncertainty of the silver nitrate titration assay, the solution density measurement, and weighing uncertainties of the dilutions. Uncertainty of the mass measurements of the samples and the internal standard solution used for addition experiments was estimated based on the smallest mass measured and observed balance stability. Uncertainties in the isotopic composition, the 29/27 ratio in a natural sample, and the relative molecular mass were estimated based on uncertainties reported by Coplen et al. *(22)*. Uncertainty in the 27/29 ratio of the enriched internal standard was estimated from replicate measurements of large aliquots of the pure internal standard. Combining the uncertainty estimates for each of these components, we calculated the expanded uncertainties for the determination of CN by ID GC/MS at CN concentrations of 0.06, 0.6, and 1.5 μg/g to be 8.3%, 5.4%, and 5.3%, respectively.

Discussion

Calafat and Stanfill *(5)* were the first to use an HP-PLOT Q column for CN analysis. They reported that CN could be analyzed without the use of cryogenic cooling. Likewise, Dumas et al. *(17)*, using a GS-GASPRO column, found that cryogenic cooling was unnecessary. This was not our experience. The HCN peaks obtained without cryogenic cooling with the HP-PLOT Q column were wide and often split. We found cryogenic cooling to be advantageous for cooling the oven in a rapid and reproducible fashion and for producing narrow peak profiles. Ishii et al. *(15)*, using a Supel-Q PLOT column, found that a temperature of −30 °C was required for their work, but we obtained good peak profiles at −15 °C.

Seto *(20, 21)* found that use of the antioxidant ascorbic acid can prevent the conversion of thiocyanate (SCN) to CN under the acidic conditions typically used to generate HCN. For baseline CN concentrations, our findings show that the measured CN concentration was 0.005 μg/g lower for refrigerated blood samples in the presence of ascorbic acid. Results reported by Seto *(21)* for the CN concentration of transfusion blood samples measured without the addition of ascorbic acid were 0.61 μmol/L

Table 3. Individual components of uncertainty for the ID GC/MS determination of CN concentrations of 0.06, 0.6, and 1.5 μg/g.

			CN concentrations					
			0.06 μg/g		0.6 μg/g		1.5 μg/g	
Source	Equation component	*df*[a]	c_i[b]	u_i[c]c_i, % relative	c_i	u_ic_i, % relative	c_i	u_ici, % relative
Type A								
Ratio measurement	$(27/29)_{Blend}$	2	1.04	2.0	1.005	0.69	1.002	0.30
IS concentration	c_{IS}	5	1	1.9	1	1.9	1	1.9
Blank correction	*blk*	3	1	2.4	1	0.24	1	0.096
Combined type A (u_ic_i), % relative				3.7		2.0		1.9
Type B								
CN standard concentration	c_{Nat}	∞	1	1.7	1	1.7	1	1.7
Sample mass	m_{Sample}	∞	1	0.023	1	0.023	1	0.023
IS mass	m_{IS}	∞	1	0.11	1	0.11	1	0.11
Ab 27; M_r CN	Ab 27_{Sample}; M_r	∞	1	0.029	1	0.029	1	0.029
IS composition	$(27/29)_{IS}$	∞	0.05	0.72	0.01	0.14	0.0023	0.033
Sample composition	$(29/27)_{Sample}$	∞	6×10^{-6}	2×10^{-7}	4×10^{-5}	1×10^{-6}	9×10^{-5}	2×10^{-6}
Combined type B (u_ic_i), % relative				1.9		1.7		1.7
Coverage factor				2		2		2
Expanded uncertainty (95% CI),[d] % relative				8.3		5.4		5.3

[a] Degrees of freedom.
[b] Sensitivity coefficient.
[c] Relative standard uncertainty.
[d] CI, confidence interval.

(0.016 mg/L), similar to the CN concentration we measured in our blood stock in the absence of ascorbic acid. However, in the presence of ascorbic acid, Seto *(21)* measured a CN concentration that was 0.011 mg/L lower, twice the effect we observed with the use of ascorbic acid. Seto *(21)* reported suppression in peak area in the presence of ascorbic acid and ascribed the lower observed peak areas to suppression of SCN conversion to CN. We observed suppression in the peak area measured for the internal standard at *m/z* 29 as well as for the sample at *m/z* 27, indicating that signal suppression in the presence of ascorbic acid may not be attributable solely to suppression of the formation of CN from SCN. The 20% increase in the CN concentration we measured for frozen fortified CN samples in the presence of ascorbic acid was troubling, and as a result, ascorbic acid was not used further. The absence of an antioxidant appeared to have less than a 4% effect on the accuracy of the results obtained for refrigerated blood samples fortified with known amounts of CN at nominal concentrations of 0.06, 0.6, and 1.5 μg/g, although results at 0.06 μg/g did show greater variability. Our findings are in agreement with the conclusion of Ishii et al. *(15)* that the use of ascorbic acid may be necessary for baseline studies, but it is not necessary for samples containing toxic concentrations of CN.

The ID equation, expressed in terms of ratios, highlights the great advantage of ID, that the accuracy of the result is independent of absolute analyte recovery *(25)*. ID is based on the equilibration of a known amount of a standard containing enriched isotopes of the analyte of interest with the naturally occurring isotopes of the sample. The ID equation compensates for the small amount of natural CN that is present in the internal standard solution and the small amount of labeled CN that occurs naturally. The ID equation for the calculation of CN concentration is derived from a simple mass balance between the measured $^{1}H^{12}C^{14}N/^{1}H^{13}C^{15}N$ ratio in a sample to which the labeled internal standard has been added and the moles of $^{1}H^{12}C^{14}N$ and $^{1}H^{13}C^{15}N$ in the sample and the internal standard. For CN, the measured $^{1}H^{12}C^{14}N/^{1}H^{13}C^{15}N$ ratio [referred to as $(27/29)_{Blend}$] has the following relationship with the moles of $^{1}H^{12}C^{14}N$ (referred to as 27) and moles of $^{1}H^{13}C^{15}N$ (referred to as 29) in the sample and internal standard:

$$\left(\frac{27}{29}\right)_{Blend} = \frac{(27)_{Sample} + (27)_{IS}}{(29)_{Sample} + (29)_{IS}}$$

This expression can be solved for $(27)_{Sample}$ and algebraically rearranged to give the expression in terms of ratios that appears in the *Materials and Methods* section. Conversion from mole fraction to mass fraction is accomplished by incorporating the relative molecular mass of CN (M_r CN) and the molecular fraction of natural $^{1}H^{12}C^{14}N$ in the sample (Ab 27_{Sample}).

Expanded uncertainty estimates for the determination of CN by ID GC/MS at 0.06 μg/g are dominated by the uncertainty of the correction for baseline CN, and at 0.6 and 1.5 μg/g by a conservative estimate of the uncertainty of the CN assay standard concentration and the uncertainty in the internal standard concentration. We evaluated the internal standard concentration over a wide range of blend ratios. This provides a measure of the uncertainty associated with influences on the instrument response, such as detector saturation and spectral background. Uncertainty could be reduced if calibration samples were prepared with 27/29 blend ratios closely matched (one slightly higher and one slightly lower) to the 27/29 blend ratio of the sample being measured rather than use of the mean of a broad range of ratios. However, in an emergency situation in which samples containing various CN concentrations will be received, there will be no time to closely match the calibration blend with the unknown sample blend; therefore, a measure of the uncertainty associated with the GC/MS measurement of a broad range of ratios is useful. Our uncertainty estimates are based on results calculated using the mean $(27/29)_{Blend}$ ratio measured for 2 headspace samplings at incubation times of 5 and 15 min. Uncertainties can be expected to be slighter larger if a single measurement at 5 min incubation time were used.

In summary, we have developed and critically evaluated an ID GC/MS method for the determination of CN in blood. This method is applicable to the range of CN concentrations encountered in both clinical and forensic applications, from the determination of baseline CN concentrations to increased concentrations attributable to smoking, food consumption, drug therapy, fire exposure, and both accidental and deliberate exposure/consumption. Use of the isotopic analog as an internal standard requires less equilibration time and chromatographic run time and provides high accuracy and precision. On average, results within 4% of the amount of added CN were obtained for concentrations ranging from 0.06 to 1.5 μg/g with analysis times of 22 min/sample for 2 samplings of the headspace. The limit of detection of the ID GC/MS procedure for the determination of CN in blood is estimated to be 0.007 μg/g.

We thank R. Kobelski, R. Trimpert, P. Holt, W. McShane, and E. Hamelin of the Emergency Response and Air Toxicants Branch, Centers for Disease Control and Prevention (Atlanta, GA) for their advice and support. We also acknowledge E. Pfannkoch of Gerstel Inc. (Baltimore, MD) for excellent customer service. Certain commercial equipment, instruments, or materials are identified in this report to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for this purpose.

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