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Stephanie L. Youso
South Dakota State University

Gary A. Rockwood
U.S. Army Medical Research Institute of Chemical Defense

John A. Lee
South Dakota State University

Brian A. Logue
South Dakota State University, brian.logue@sdstate.edu

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Determination of cyanide exposure by gas chromatography–mass spectrometry analysis of cyanide-exposed plasma proteins

Stephanie L. Youso[a], Gary A. Rockwood[b], John P. Lee[a], Brian A. Logue[a,*]

[a] Department of Chemistry and Biochemistry, South Dakota State University, Shepard Hall 121, Box 2202, Brookings, SD 57007, USA
[b] US Army Medical Research Institute of Chemical Defense, 3100 Ricketts Point Road, Aberdeen Proving Ground, MD 21010-5400, USA

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ABSTRACT

Exposure to cyanide can occur in a variety of ways, including exposure to smoke from cigarettes or fires, accidental exposure during industrial processes, and exposure from the use of cyanide as a poison or chemical warfare agent. Confirmation of cyanide exposure is difficult because, *in vivo*, cyanide quickly breaks down by a number of pathways, including the formation of both free and protein-bound thiocyanate. A simple method was developed to confirm cyanide exposure by extraction of protein-bound thiocyanate moieties from cyanide-exposed plasma proteins. Thiocyanate was successfully extracted and subsequently derivatized with pentafluorobenzyl bromide for GC–MS analysis. Thiocyanate levels as low as 2.5 ng mL^{-1} and cyanide exposure levels as low as 175 μg kg^{-1} were detected. Samples analyzed from smokers and non-smokers using this method showed significantly different levels of protein-bound thiocyanate ($p < 0.01$). These results demonstrate the potential of this method to positively confirm chronic cyanide exposure through the analysis of protein-bound cyanide in human plasma.

1. Introduction

Cyanide is a toxic chemical that is used in many industries, including gold and silver mining, pesticide production and handling, electroplating, and chemical manufacturing. Exposure to cyanide is possible through a number of routes, including the ingestion of certain foods (e.g., almonds or cassava root) [1,2], exposure to smoke from cigarettes or fires [3,4], accidental exposure during industrial processes, and from the use of cyanide as a poison or chemical warfare agent [5]. Acute cyanide exposure causes incapacitation of oxygen utilization, disruption of electron transport, tissue hypoxia, and can result in death [6]. Chronic exposure to cyanide can also lead to a battery of adverse health conditions [7]. Chronic cyanide exposure can increase the size of the thyroid gland leading to thyroid malfunction, degenerate cerebral white matter, and significantly increase the incidence of other neuronal disorders, such as ataxic neuropathy [8]. These health conditions can have major consequences and may persist for many years following the actual occurrence of cyanide exposure. Although the effects of chronic cyanide exposure are documented, there is little information to indicate the level of cyanide exposure that can stimulate harmful health effects in humans [9].

Detection and quantification of cyanide or its metabolites from biological fluids is important to confirm exposure and permit adequate time for treatment; however, cyanide's volatility and reactivity make it a difficult analyte to reliably detect and quantify [10–13]. Cyanide has a half-life of less than 1 h in biological fluids [14], making direct detection difficult and sometimes unreliable [15,16]. Therefore, reliable determination of cyanide exposure normally involves the detection of a more stable cyanide biomarker. The major metabolite of cyanide, thiocyanate, is formed by reaction of a sulfur donor (e.g., thiosulfate) with cyanide and the enzyme rhodanese [16,17]. Thiocyanate is considered more stable than cyanide *in vivo*, but it can be introduced by routes other than cyanide metabolism [1,2], making it difficult to use as a marker of cyanide exposure. Cyanide also forms a minor metabolite, 2-amino-2-thiazoline-4-carboxylic acid, or ATCA, by reacting with cystine [18]. This metabolite is relatively stable and has good potential as a biomarker for cyanide exposure [11–18], but there is little information on its relevance as a marker of cyanide exposure. Cyanide can also react with disulfide bonds in proteins to form protein-bound thiocyanate [19]. Because certain proteins have long half-lives [20–22], the analysis of thiocyanate protein adducts has the potential to be a long-term repository for information regarding cyanide exposure. Fig. 1 illustrates the reaction of cyanide with cysteine residues to form a stable thiocyanate (–SCN) moiety in a protein [19] and the proposed extraction of the thiocyanate adducts under basic conditions.

* Corresponding author. Tel.: +1 605 688 6698.
E-mail address: Brian.Logue@sdstate.edu (B.A. Logue).

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Although there are a number of methods to detect free thiocyanate from various biological fluids [13,23,24], there has been very little investigation into the isolation and detection of protein-bound thiocyanate adducts. By isolating plasma proteins and extracting protein-bound thiocyanate, confirmation of cyanide exposure may be possible. The method presented provides a simple analytical method to isolate and analyze protein-bound thiocyanate adducts. This method was used to determine chronic low-level cyanide exposure through the analysis of smoker and non-smoker plasma samples.

2. Experimental

2.1. Materials

2.1.1. Reagents

Sodium carbonate (Na_2CO_3), sodium cyanide (NaCN), and tetrabutylammonium sulfate (TBAS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). An isotopically labeled internal standard, $NaSC^{13}N^{15}$, was obtained from Isotec (Miamisburg, OH, USA). The derivatization agent, pentafluorobenzyl bromide, PFBBr, was acquired from Thermo Scientific (Hanover Park, IL, USA). All aqueous solutions were prepared with deionized, distilled water (18 MΩ cm) from a Labconco Water Pro PS system. All organic solvents used in these experiments were at least HPLC grade.

An aqueous stock solution of thiocyanate (100 mM) was prepared and diluted to the desired concentration for each experiment. A stock solution of 10 mM cyanide was prepared by dissolving solid NaCN in 10 mM sodium hydroxide to prevent the formation of gaseous hydrogen cyanide.

2.1.2. Biological fluids

Non-sterile, swine plasma with EDTA anti-coagulant was obtained from Pelfreeze Biologicals (Rogers, AR, USA) and stored at −80 °C until used. Human plasma samples were isolated from smoking and non-smoking volunteers (six each). Blood samples were collected from volunteers and mixed with an EDTA anti-coagulant to prevent clotting. Plasma was isolated from the blood samples by centrifugation and stored at −80 °C until analysis. Collection and analysis of human samples was approved prior to this study by the Human Subjects Advisory Committee at South Dakota State University, the Avera Medical Institute (Sioux Falls, SD), and the Human Research Protection Office of the U.S. Army Medical Research and Materiel Command (Ft. Detrick, MD).

2.2. Methods

2.2.1. Formation and extraction of plasma protein adducts

To mimic cyanide exposure, swine plasma was incubated with aqueous NaCN at room temperature for 3 h to allow the formation of thiocyanate–protein adducts. Plasma proteins were isolated by precipitation using acidified acetone; the resulting precipitate was washed with acidified acetone and diethyl ether three times each. The precipitated proteins were dried and then dissolved in an aqueous 0.1 M carbonate buffer solution at a pH of 10 to initiate hydrolysis of the thiocyanate adducts (Fig. 1). The solution was shaken at room temperature for 1 h to extract the protein-bound thiocyanate.

2.2.2. Internal standard

After hydrolysis, isotopically labeled thiocyanate (30 μL of 100 μg mL^{-1} $NaSC^{13}N^{15}$) was added to the buffered protein solution to act as an internal standard. Because the internal standard only differs from the analyte by two mass units, there is a slight possibility of error due to mass carryover of thiocyanate isotopes (the natural occurrence of the interfering isotope relative to the predominant form is approximately 0.003%). Therefore, a significantly larger amount of internal standard relative to the analyte was used to minimize any interference.

2.2.3. Analysis of extracted thiocyanate

The extracted thiocyanate was analyzed by GC–MS after derivatization based on the method reported by Kage et al. [25]. Briefly, 200 μL of the extracted protein solution was mixed with 10 mM PFBBr in ethyl acetate and 20 mM of TBAS in saturated sodium borate. The samples were heated at 80 °C for 1 h, shaken, and then centrifuged to separate the organic and aqueous layers. The organic layer was then collected and analyzed using electron ionization GC–MS.

An Agilent GC–MS system equipped with a 6890N gas chromatograph and a 5975B inert XL electron ionization (EI)/chemical ionization (CI) mass selective detector was used for the analysis of derivatized thiocyanate. The column consisted of a DB-5MS bonded phase column (30 m × 0.25 mm I.D., 0.25 μm film thickness; J & W Scientific, Santa Clara, CA) with helium carrier gas set at a flow rate of 0.1 mL min^{-1}. Initially, the temperature was set at 90 °C for 1 min, and then increased 10 °C min^{-1} to 200 °C. The temperature was then quickly increased to 270 °C at 60 °C min^{-1}. The derivatized thiocyanate molecular ion and internal standard molecular ion of

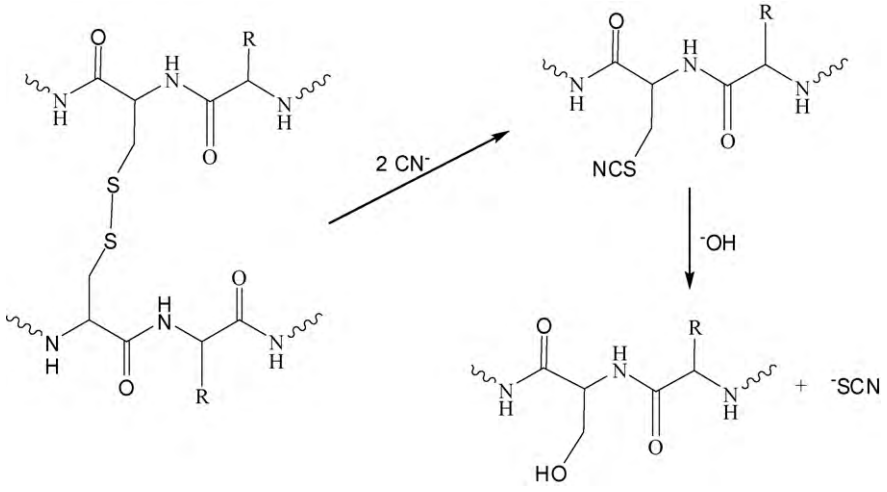

Fig. 1. The reaction of cysteine residues with cyanide to form thiocyanate adducts on proteins and the subsequent removal of thiocyanate under basic conditions.

239 m/z and 241 m/z, respectively, were monitored in selected ion monitoring mode. Ions with 181 and 161 m/z were also monitored for peak confirmation. A dwell time of 100 ms was used for each of the monitored ions.

3. Results and discussion

3.1. Extraction optimization

Thiocyanate adducts were successfully extracted from cyanide-exposed swine plasma through base hydrolysis. The pH, duration of the hydrolysis, and temperature of hydrolysis were optimized for the extraction of thiocyanate and were found to maximize extraction when performed at room temperature for 1 h in aqueous solutions buffered at pH 10. To determine the optimal time for thiocyanate hydrolysis, the duration of the hydrolysis was varied from 2 min to 12 h (Fig. 2). Within the first 30 min of hydrolysis, the concentration of extracted thiocyanate increased rapidly and then becomes essentially constant. The variability of the extraction was relatively large during the first 30 min of hydrolysis, but decreased significantly after 30 min. The effect of pH on the hydrolysis was also examined at various values ranging from 8 to 11. As seen in Fig. 3, the largest amount of thiocyanate was extracted at a pH value of 10 and the error for each pH value was low. Finally, the temperature of the hydrolysis was examined at room temperature (21 °C), 30 °C, and 50 °C. No significant difference in the amount of extracted thiocyanate was found between these temperatures (data not shown).

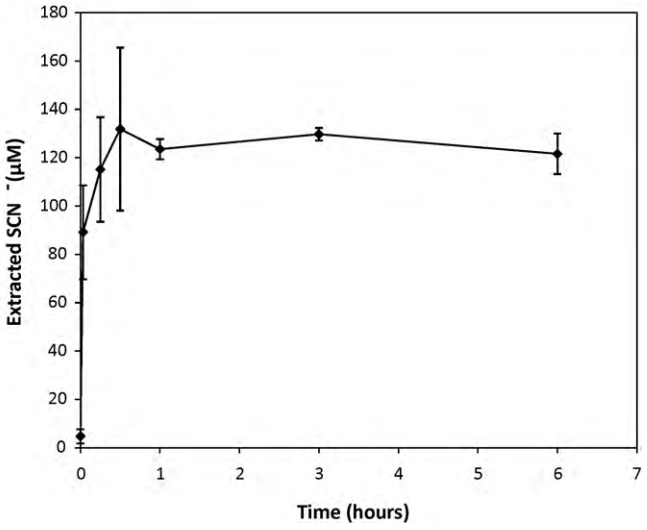

Fig. 2. Thiocyanate extracted from plasma proteins as a function of time (2 min to 6 h). Error bars denote standard deviation.

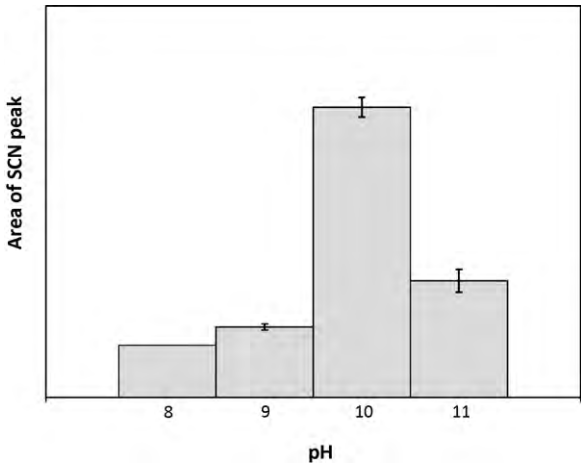

Fig. 3. The concentration of thiocyanate released from plasma proteins as the pH of hydrolysis was varied from 8 to 11. Error bars denote standard deviation.

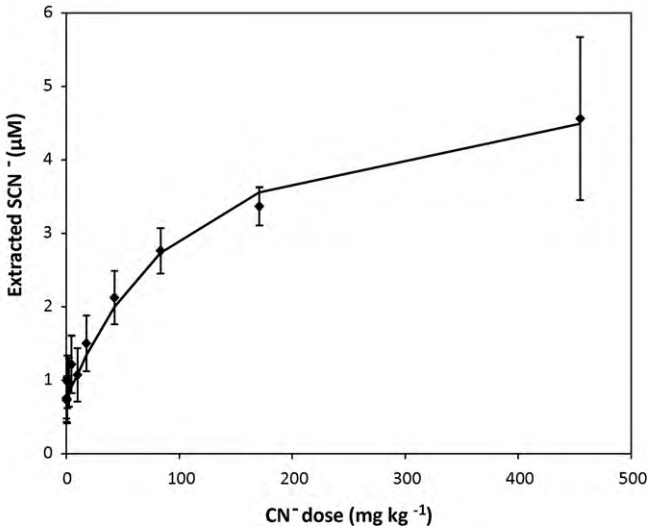

Fig. 4. The amount of thiocyanate extracted from plasma proteins as a function of the cyanide dose. The solid line was calculated with a Langmuir model for the binding of cyanide by plasma proteins (T_{sites} = 4.8 μmol; $K = 7.0 \times 10^{-4}$). Error bars denote standard deviation.

3.2. Detection limit and linearity

The detection limit was estimated to be 2.5 ng mL^{-1} of thiocyanate in the extraction matrix, at a signal-to-noise ratio of 3:1. This is comparable to the 1 ng mL^{-1} detection limit of aqueous thiocyanate reported by Kage et al. [25]. The linearity of thiocyanate analysis in the extracted protein matrix was tested from 1 μM to 100 μM and was found to be linear (R^2 = 0.996).

3.3. Dose–response study

Pig plasma was exposed to aqueous cyanide solutions at concentrations ranging from 2 μM to 5 mM. The resulting dose response can be seen in Fig. 4. As the dose of cyanide increases, the amount of bound cyanide, and subsequently the amount of thiocyanate extracted increases. As expected, thiocyanate levels did not increase in a linear manner. After an intial quick rise in the amount of extracted thiocyanate (i.e., protein-bound cyanide), the amount of extracted thiocyanate did not increase at the same rate relative to the cyanide dose. The nonlinear relationship between the dose of cyanide and the extracted thiocyanate implies there is maximum number of disulfide bonds in the protein that can be saturated with the cyanide to form cyanide adducts. It should be noted that the small amount of thiocyanate extracted from plasma samples that were not exposed to cyanide is due to naturally occuring cyanide (i.e., exposure to cyanide through diet and environmental exposure).

A Langmuir model was employed to investigate the relationship between the protein-bound cyanide relative to the concentration of cyanide available in solution (Eq. (1))

$$[CN^-]_{bound} = \frac{T_{sites} K [CN^-]_{dissolved}}{1 + K[CN^-]_{dissolved}} \quad (1)$$

The $[CN^-]_{bound}$ is the concentration of cyanide bound to the plasma proteins and assumed to be equivalent to the thiocyanate

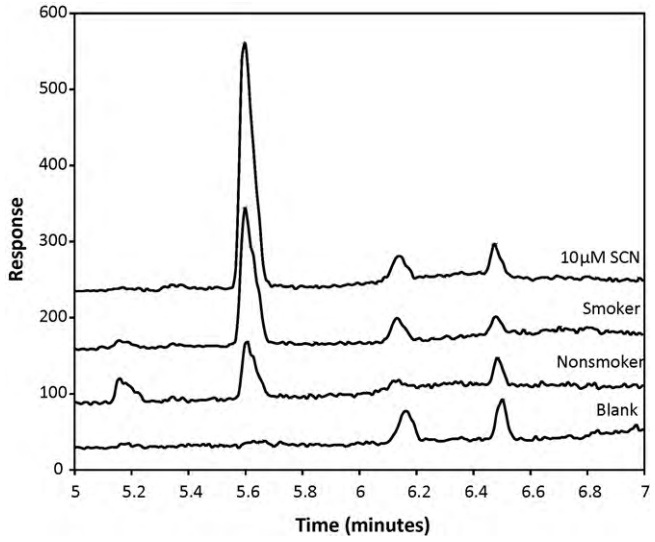

Fig. 5. The chromatograms of extracted thiocyanate from the plasma proteins from a non-smoker and a smoker, along with a 10 μM thiocyanate standard and a blank (water).

extracted. $[CN^-]_{dissolved}$ represents the aqueous cyanide concentration in the presence of the plasma proteins. The value for $[CN^-]_{dissolved}$ was calculated by subtracting the cyanide bound to the proteins from the concentration of cyanide that was initially introduced to the plasma sample. T_{sites} represents the amount of sites available to form thiocyanate adducts with cyanide and K represents the equilibrium constant of cyanide binding.

Using Eq. (1), the values of K and T_{sites} were adjusted to best describe the experimental data and $[CN^-]_{bound}$ was then plotted against the experimental values in Fig. 5. The values of K and T_{sites} were found to be approximately 7.0×10^{-4} and 4.8 μmol, respectively. If T_{sites} is assumed to be the number of disulfide sites available, this is significantly less than the actual amount of disulfide moieties in plasma proteins, reported to be approximately 55.3 μmol per 1 g of protein in swine plasma [26]. This suggests that not all disulfide bonds react with cyanide. This may be due to inaccessibility of disulfide bonds to cyanide due to their location within the protein. Incomplete extraction of the bound thiocyanate could also contribute to a lower calculated value of T_{sites}.

3.4. Analysis of human plasma

The proposed method's applicability for cases of chronic exposure was tested by analyzing human plasma samples obtained from smokers and non-smokers. Twelve samples were obtained from human volunteers in total; six samples from cigarette smokers and six samples from non-smokers. The plasma proteins were analyzed for bound cyanide by the optimized extraction procedure. Representative chromatograms of 10 μM standard thiocyanate solution as well as extracted thiocyanate from plasma samples of a smoker and a non-smoker are provided in Fig. 5. PFBBr derivatized thiocyanate elutes at 5.6 min with good resolution.

A significant difference was observed in the mean concentration of thiocyanate extracted from the plasma proteins of smokers compared to that of the non-smokers. Although there is some endogenous thiocyanate bound to human plasma proteins, as indicated by the low levels of thiocyanate found in the plasma of non-smokers, there were considerably elevated amounts of thiocyanate bound to plasma proteins of smokers. In non-smokers, the average concentration of protein-bound thiocyanate was found to be approximately 35.4 ng mL^{-1} with a standard deviation of 7.5 ng mL^{-1}. The amount of thiocyanate bound to the plasma proteins of smokers was significantly elevated ($p < 0.01$); an average concentration of 89.1 ng mL^{-1} with a standard deviation of 38.2 ng mL^{-1} was found. Therefore, the analytical method was successful at determining cases of chronic, low-dose cyanide exposure.

4. Conclusions

The method reported provides a technique to extract and analyze thiocyanate bound to plasma proteins using GC–MS. Currently, there are already a number of successful methods to analyze free thiocyanate from biofluids, however, the simple method presented here specifically targets thiocyanate moieties that are bound to disulfide bonds in proteins and it successfully established significantly elevated levels of thiocyanate adducts from proteins of smokers relative to non-smokers.

Currently, a more expansive study of human plasma proteins in smokers and non-smokers is underway to determine endogenous concentrations of bound thiocyanate, as well as to establish the variance between smokers relative to non-smokers. The concentrations found will be used to demonstrate the applicability of this method in cases of cyanide exposure in humans. Additionally, future work includes the study of the lifetime of thiocyanate adducts formed with plasma proteins by examining the existence of these adducts over time.

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