Fluorometric Determination of Cyanide in Biological Fluids with Pyridoxal*

Ronald L. Morgan and James L. Way**
Department of Pharmacology, Colleges of Pharmacy and Veterinary Medicine, Washington State University, Pullman, Washington 99164

Abstract
Investigations of the physiological disposition of cyanide during thiocyanate therapy has necessitated the development of a convenient and sensitive method for cyanide analysis. A fluorometric method involving the catalytic conversion of pyridoxal to 4-pyridoxaldehyde was adapted for use with biological fluids by employing microdiffusion analysis. The presence of the cyanide antagonist, sodium thiosulfate, interferes with the formation of the fluorophore. In order to circumvent this interference, the pH of the diffusion media was altered to selectively diffuse cyanide. After testing various acidifying agents, an acetate buffer (pH = 5.2) was determined to be satisfactory. The fluorometric method was then correlated with the classical colorimetric procedure by an in vivo study. Blood from mice treated with sodium nitrite and sodium thiosulfate, prior to receiving potassium cyanide, was analyzed by both procedures and no significant difference was demonstrated between the results of the two methods of analysis.

Introduction
Interest in the pharmacokinetic investigation of cyanide prompted the need for a rapid, convenient procedure to determine low concentrations of cyanide in a large number of samples in biological fluids. A fluorometric method has been reported (1) to be approximately ten times more sensitive than the present colorimetric method (2). This method can also be more convenient than the classical colorimetric method. The report herein describes the adaptation of this fluorometric procedure to biological fluids, using microdiffusion methods (3) with minor modifications in the presence of sodium thiosulfate, a common cyanide antagonist. Analysis for cyanide was found to be strongly inhibited in the presence of thiosulfate or sulfite ion. This interference was circumvented by altering the pH value of the diffusing media. Since the pKa of HCN is 9.2 and the pKa of the polythionic acids produced from thiosulfate ion is approximately 2.0 (4,5), a pH value was obtained which permitted diffusion of HCN while inhibiting the diffusion of the interfering ions.

Materials
Pyridoxal hydrochloride was purchased from Calbiochem (La Jolla, California) and potassium cyanide was purchased from Fisher Chemicals (Fairlawn, New Jersey). Sodium thiosulfate, sodium acetate, sodium carbonate, and sodium phosphate salts were obtained from J.T. Baker Chemicals (Phillipburg, New Jersey). The 1-phenyl-3-methyl-5-pyrazolone and chloramine T were obtained from Eastman Organic Chemicals (Rochester, New York) and the pyrazolone derivative was recrystallized from 95 percent alcohol. Synthesis of the bis(1-phenyl-3-methyl-5-pyrazolone) was conducted by the method of Knorr (6). Pyridine and the sulfuric acid were purchased from Mallinckrodt, Inc. (St. Louis, Missouri). All other chemicals used in this study were analyzed reagent grade of the highest purity available.

Methods

Microdiffusion Procedure
A 2.0 mL aliquot of 0.1 N NaOH was place into the center well of a Conway microdiffusion cell (A.H. Thomas Co., Philadelphia, Pennsylvania) and 0.2 to 1.0 mL of a Sample containing potassium cyanide, and in some cases sodium thiosulfate, was placed in the outer well. Ground glass covers were coated with Lubriseal (A.H. Thomas Co.) and placed slightly ajar over the cells. Subsequently, a 1.0 mL quantity of an acidifying solution was added and the cell was immediately sealed with the glass covers. The diffusion was allowed to proceed at room temperature for three hours.

Colorimetric Method
Aliquots of 1.0 mL of 0.1 N sodium hydroxide solution were removed from the center well of the Conway microdiffusion dish and added to test tubes maintained at 0-2°C, which contained 0.2 mL of chloramine T-phosphate reagent (0.25% chloramine T and 1.0 M NaH₂PO₄, 1:3 v:v). After approximately three minutes, 3.0 mL of pyridine-pyrazolone
reagent [saturated aqueous solution of L-phenyl-3-methyl-5-pyrazolone and 0.1% bis (L-phenyl-3-methyl-5-pyrazolone) in pyridine, 5:1 v/v] was added, the tubes were agitated, incubated at room temperature for 1 hour to allow for color development, and determined in a Gilford Spectrophotometer. The absorbance at 620-nm for each sample was determined and compared against that of a reagent blank. Cyanide concentrations in the samples were then calculated from a daily calibration curve made with freshly prepared standard solutions of potassium cyanide, subjected to identical microdiffusion and colorimetric procedures.

Fluorometric Method

Aliquots of 1.0 mL of 1.0 N sodium hydroxide solution from the center well were placed into a test tube containing 3.5 mL of 0.2 M sodium phosphate buffer, pH = 7.5. This solution was agitated and 0.5 mL of the pyridoxal-HCl (6 x 10^-3 M) was added. The resulting solution was heated to 50°C for 60 minutes and the reaction was stopped by adding 1.0 mL of 0.2 M solution of Na2CO3. The fluorescent intensity was determined on an Aminco-Bowman spectrophotofluorometer, using 432 nm for emission and 365 nm for excitation. Cyanide concentrations were determined by comparing the fluorescent intensities of the samples against a calibration curve of standard potassium solutions.

Effect of Varying pH Values on Cyanide Analysis in the Presence of Sodium Thiosulfate

Interference of sodium thiosulfate with the fluorometric method for cyanide was investigated by modifying the microdiffusion procedure to minimize the diffusion of SO2. The solution to be analyzed consisted of potassium cyanide (0.4 μmole/mL) and sodium thiosulfate (15 μmole/mL). This study employed various buffers with pH values ranging from 2.5 to 12 in the microdiffusion procedure. Acidifying agents employed consisted of a 1.0 M solution of either glycine, acetate, or phosphate buffer. The optimum of a pH value of 5.2 was determined from the plot in Figure 3. Polythionic acids formed from the thiosulfate anion, upon the acidification, have pKₐ values of approximately 2.0 (4); while the pKₐ value for hydrogen cyanide was approximately 9.2 (5). Therefore, at a pH value of 5.2, the polythionic acids remained primarily ionized; whereas the hydrogen cyanide was predominately unionized and was diffused. Using this modification, thiosulfate interference was circumvented in this microdiffusion procedure. The standard curves with and without sodium thiosulfate were identical as shown in Figure 4. In addition, through comparison of calibration curves, this method was found to be approximately five times more sensitive than the colorimetric method (2).

Cyanide Determination in Mice

Swiss-Webster mice, weighing from 25 to 35 g, were fasted overnight. Subsequently, the mice were divided up into four groups of six mice each and injected with sodium nitrite (100 mg/kg, s.c.) and sodium thiosulfate (1 g/kg, i.p.) at 45 minutes and 15 minutes, respectively, prior to the administration of potassium cyanide (30 mg/kg, s.c.) The groups of mice were sacrificed by decapitation and their blood was collected and frozen over dry ice at the times indicated on Figure 5. After the last group was sacrificed, the blood samples were
thawed and subjected to the modified microdiffusion procedure. When the microdiffusion was complete, samples from the central well of the Conway cell were analyzed, using fluorometric and colorimetric methods. Blood cyanide concentrations obtained by the two different methods were plotted against time (Figure 5) and were found not to be significantly different, according to the students' test (p = 0.05) (7). Similar results obtained by both methods indicate that the fluorometric method is comparable to the colorimetric procedure and is applicable for cyanide analysis in biological fluids.

Discussion

A simple, rapid, and sensitive fluorometric method for cyanide determination has been adapted for biological fluids in the presence of a cyanide antagonist, sodium thiosulfate. This method is based on the catalytic conversion of pyridoxal to 4-pyridoxylactone (1). Sodium thiosulfate interferes with this method due to the sulfite interaction with the aldehydic moiety. Sulfite is formed as a result of the liberation of SO₂ from unstable polythionic acids upon acidification of the solution of sodium thiosulfate (8,9,10). Interpretations of the cyanide concentrations in blood during therapy with sodium thiosulfate may tend to misconstrue the efficacy of thiosulfate ion in the antagonism of cyanide intoxication (10). Modification of the microdiffusion procedure described herein circumvents this interference and adapts this method for use in biological fluids when cyanide antagonists are present. Under conditions where no thiosulfate ions are present, 15% H₂SO₄ may be used (3) as the acidification agent.

There are two distinct advantages to this fluorometric method, over the colorimetric method for determination of cyanide in biological fluids. Under our conditions, the fluorometric method is approximately five times more sensitive than the colorimetric method (2). In addition, this method requires fewer and more stable reagents and less preparation time than does the colorimetric method; thereby reducing the tediousness of the cyanide analysis. Simplicity and sensitivity would make this method useful in clinical applications, pharmacokinetic analysis and chronic studies. It should be pointed out that in long-term preliminary studies with dogs, the apparent blood cyanide concentrations gradually increased more with 15% sulfuric acid than with pH 5.2 buffered solution containing sodium dodecylbenzylsulfate (10).

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References


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