MODIFIED PICRATE METHOD FOR DETERMINATION OF CYANIDE IN BLOOD

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ABSTRACT

Cyanide (CN^-) is widely distributed in the ecosystem and has been associated with toxic effects in humans and animals. Most outbreaks of CN^- poisoning in animals result from ingestion of plants containing cyanogenic glycosides. Various analytical techniques for estimating cyanide in blood are available. A simple picrate method was developed to determine blood CN^- in goats. This assay is a modification of commonly available methods using picrate paper and those using Conway diffusion cells. Cyanide in blood was measured during and after IV administration of KCN at 0.6 mg/min for 1 h. Blood CN^- levels in rabbits were determined after oral administration of KCN for 10, 20, 30 and 40 days. The CN^- concentration in blood of goats was time-dependent and continued rising during infusion followed by gradual decline after infusion stopped. A calibration curve set by dissolving various concentrations of KCN in distilled water showed a linear relationship between CN^- concentration and absorbance (R=0.995) ranging from 0.3-120 mg CN^-/L. Blood CN^- levels in rabbits showed time-dependent increase with maximum concentration (1.34 mg/L) at 40 days. This is a simple and inexpensive tool for the determination of blood CN^- in the laboratory and under field conditions as well.

Keywords: KCN; goats; rabbits; picrate paper; blood.

INTRODUCTION

Cyanide (CN^-) is widely distributed in the ecosystem and has been associated with toxic effects in humans and animals. Cyanide toxicity may be the result of intake from food sources, environmental pollution, intentional ingestion, chemical warfare, occupational exposure, homicide, and sometimes through the use of drugs such as nitroprusside and laetrile (Way, 1984; Watts, 1998).

Cyanogenic glycosides are naturally present in many plants, which become a source of CN^- following enzymatic hydrolysis. These may include cultivated forage plants of high nutritive value for man and animal e.g., *Linum* sp., *Manihot* sp., *Phaseolus lunatu*s, and *Sorghum* sp. (Poulton, 1983). Different plant species contain specific glucosides i.e. Linamarin (linseed), Dhurrin (sorghum) and Amygdaline (bitter almond). Their cyanogenic components may vary widely depending upon the season and the part of the plant, with young growing leaves having the highest concentrations. The minimum lethal dose of HCN is ~2 mg/kg BW in sheep and cattle. Plants containing 200 ppm are likely to be toxic, and highly poisonous plant samples may contain up to 6000 ppm of cyanogenic glycosides (Radostits *et al*., 2000).

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Cyanide intake has been associated with syndromes of the central nervous system. Ataxia has been observed in sheep, cattle, and horses grazing sorghum, and urinary incontinence is reported in horses (McKenzie and McMicking, 1977; Bradley *et al*., 1995). Cases of CN^- poisoning of varying degrees of severity and mortality in ruminants after ingestion of cyanogenic glycosides containing plants, especially sorghum species, are reported in Pakistan. Due to the lack of simple and economic diagnostic techniques under field conditions, field veterinarians depend upon the history of feeding and clinical signs to diagnose CN^- poisoning (pers. com. Nasir A).

Analytical techniques available for determination of CN^- in plants and biological fluids include amperometery (Park *et al*., 1997), voltammetery (Tatsuta *et al*., 2001) polarography (do Nascimento *et al.,* 1998), potentiometery (Sequeira *et al*., 1999), piezoelectricity (Gomes *et al*., 1998), gas chromatography (Cardeal *et al*., 1995), visible spectrophotometry (Laforge *et al*., 1994), mass spectrometry (Tracqui *et al*., 2002), HPLC (Akiyama *et al*., 2006) and flow injection (Recalde-Ruiz *et al*., 2000).

The variety of analytical methods reported for CN^- in blood may indicate the difficulty of its analysis in that there is no universally preferred method (Hughes *et al*., 2003). Available methods are expensive, laborious, and require technical expertise and sophisticated equipment.

None of these methods lend themselves in the field. Therefore simple and inexpensive assay is needed such as the one using picrate paper that we have developed and which is described here.

MATERIALS AND METHODS

Experimental animals

Two adult mixed-breed goats weighing 29 and 33 kg were purchased from the local market and fed green fodder (barseem) and concentrate (ICI Wanda, ICI, Pvt. Pakistan) twice a day. Both were de-wormed with oxfendazole (Oxafax, GlaxoWellcome, Pakistan) and acclimatized for two weeks prior to the commencement of the experiment. Animal care and the experimental protocol applied were approved by the Ethical Committee of the University of Veterinary and Animal Sciences, Lahore.

The hair over the jugular vein were shaved and skin was swabbed with methylated spirit. An intravenous catheter (18 g x 25 mm, V. Flon, J.P Medical Appliances Co. Italy) was inserted into the jugular vein and sutured in place (Hughes *et al*., 2003). Potassium cyanide (KCN) solution was prepared by dissolving KCN (15% w/v) (Merck, Germany) in normal saline (Immunasol-NS, AZ Pharmaceuticals Co. Ltd. Pakistan) and was administered intravenously by infusion set (SY' AH Impex, Pakistan) at 0.6 mg/min for 60 min to both goats. During infusion the goats were monitored for signs of adverse reactions, including restlessness, anxiety, rapid ventilation, and increased heart rate (Hughes *et al*., 2003). Blood samples were collected for CN^- assay before infusion (0 h), during infusion (10, 20, 30, 45, and 60 min) and following cessation of infusion (10, 20, 30, 45, and 60 min, 2, 3, 4, and 5 h). The samples were placed in heparinized vacutainer tubes (Chengdu Rich Science Industry Co. Ltd. China) and refrigerated at 4°C for analysis within 24 h.

Twelve adult local breed rabbits (average weight 1.45 kg), supplied by the University Diagnostic Laboratory, University of Veterinary and Animal Sciences, Lahore were housed individually in metal cages and fed concentrate and barseem. The rabbits were de-wormed with oxfendazole (Oxafax, ICI, Pvt. Pakistan) and acclimatized for two weeks prior to the commencement of the experiment. They were divided into two groups, 6 control and 6 experimental. The experimental group was given KCN orally at 3 mg/kg/day diluted in distilled water for 40 days, while the control group received distilled water. The KCN solution was prepared fresh daily.

Blood samples were collected from all rabbits at 0, 10, 20, 30, and 40 days for estimation of CN^-. The area over the jugular vein was shaved and swabbed with methylated spirit. A 3 mL blood sample was drawn into heparinized (green topped) vacutainer using a 24 X 1 in needle. Cyanide assay was conducted within 3 h of collection.

Preparation of reagents and picrate paper

All reagents were prepared and stored at 27°C with the exception of picrate papers, which were stored at -18°C. A 1.9 M sulfuric acid solution was prepared by dissolving 10.12 ml concentrated sulfuric acid (Merck, Germany) in 100 ml distilled water. Picric acid solution was prepared by adding 1.4 g picric acid (BDH, Laboratories) to 100 ml of 2.5% sodium carbonate solution (Bradbury *et al*., 1999). Picrate papers were prepared from 3MM Whatman chromatography paper cut into 10 x 30 mm strips, dipped in picric acid solution, air dried and attached to a 10 x 50 mm plastic strip with hobby glue as described by Bradbury *et al*., (1999).

Determination of cyanide

Initially, high concentrations (60-120 mg/L) of KCN solutions with or without blood were mixed with 0.1-0.5mL of 1.9 M sulfuric acid in 25 x 50 mm flat-bottomed polystyrene vials with a polyethylene screw cap. A picrate paper was placed in the vial which was then tightly closed and incubated at 27°C overnight. A color change from yellow to brown was considered a positive reaction.

A calibration curve for CN^- was prepared from standard solutions of CN^- prepared by dissolving KCN (Merck, Germany) in distilled water with CN^- concentrations of 0.31, 0.62, 1.25, 2.5, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120 mg/L. These solutions were run through the entire procedure described below for the calibration curve. To minimize error, micropipettes (Socorex Swiss) were used to measure the volumes. The calibration curve was linear (R= 0.995), in the range 0.3-120 mg CN^-/L (Fig. 1). To check the accuracy of the method a series of recovery experiments using various concentrations of CN^- (5, 10, 15, 25, 40 mg CN^-/L) in whole blood *in vitro* were performed. Precision of the procedure was checked by repeated measurements of standard CN^- solutions and CN^- added whole blood samples. Each sample was repeated six time using general method.

This method is a modification of picrate paper kits available for determination of CN^- in plant materials (Bradbury *et al*., 1999) and those using diffusion apparatus to detect CN^- in blood (Gangeloo *et al*., 1980; Laforge *et al*., 1994; Hughes *et al*., 2003). Each of the goat and rabbit blood samples (1 mL) was placed into a 25 x 50 mm flat bottomed polystyrene vial with a polyethylene screw cap followed by the addition of 0.1 mL of 1.9 M sulfuric acid. The vial was swirled to mix the contents. A picrate paper was added immediately and the vial was closed tightly. After incubation for 8, 16, 24 h at 27°C the vials were opened, and the picrate papers were removed from the plastic strips and eluted in 5 mL distilled water in separate test tubes for 30 min with

frequent stirring (Bradbury *et al.*, 1999). A blank was prepared similarly in which 1 mL distilled water was used in place of a blood sample. The absorbance of the colored solutions thus obtained was screened against visible range to know its λmax, which was found to be 510 nm. Thereafter the absorbance of the color solutions was measured at 510 nm with a spectrophotometer (Cecil CE-2041, 2000 series) against the blank.

Safety measures

To counter adverse reaction to CN^-, two antidotes, 10% sodium nitrite (Avonchem Ltd. UK) and 20% sodium thiosulfate (Merck, Germany) solutions were prepared in distilled water. These were to be administered in sequence with sodium nitrite solution at 20 mg/kg IV followed by sodium thiosulfate solution at 600 mg/kg IV (Soto-Blanco and Gorniak, 2004).

RESULTS

The calibration curve was linear (R=0.995) in the range of 0.3-120 mg CN^-/L with total standard deviation of 0.78% (fig. 1). The curve was described by the equation:

Cyanide (mg/L of blood) = 51.602 x absorbance

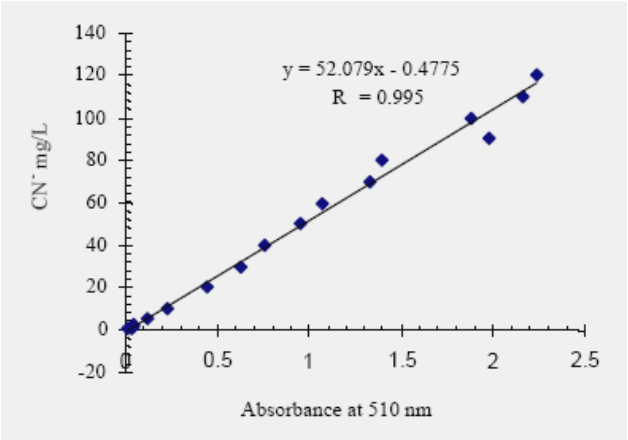

Fig. 1: Calibration curve for cyanide (CN^-) at a range of concentrations in distilled water.

In standard CN^- solutions a color change in picrate paper from yellow to brown was observed at a concentration of 3 mg CN^-/L and above. This method was sufficiently sensitive to quantify the low concentrations (0.3mg CN^-/L) of CN^- found in ruminant blood after administration of compounds containing CN^-. Recovery of CN^- from whole blood containing 5, 10, 15, 25 and 40 mg CN^-/L was (mean±SD) 4.7±0.22, 9.8±0.24, 15.2±0.27, 23.4±0.42 and 37.9±0.37 mg/L of blood, respectively.

In goats infused with KCN, the CN^- concentration in blood was time-dependent and continued rising during infusion, gradually declining after infusion ceased after 1 h (fig. 2). Blood CN^- increased from background levels to between 0.36 and 3.4 mg/L during the period of infusion and decayed exponentially toward control values once infusion stopped. No clinical signs of toxicity or other adverse reactions were seen either during or after infusion. The effect of reaction time for estimation of CN^- was also determined.

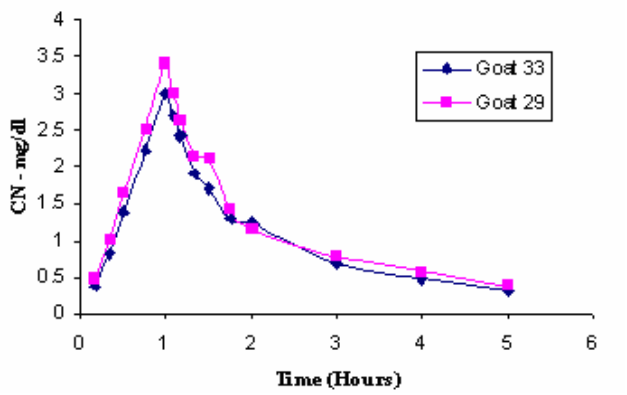

Fig. 2: Blood cyanide (CN^-) concentrations in goats during and after intravenous KCN infusion at 0.6 mg/min for 60 min.

The results of experiments on blood samples drawn from goats during and after infusion at various reaction intervals are shown in fig. 3. In all blood samples, 0.1 mL of 1.9 M H_2SO_4/mL of blood gave clear positive results. Short reaction times (8 or 16 h) were found to give weak results for CN^-, whereas a 24 h reaction interval always produced a clear positive reaction. Thus the standard conditions for the reaction were 1 mL of blood + 0.1 mL of 1.9 M H_2SO_4 reacted for 24 h at 27°C in a closed 25 x 50 mm flat-bottomed polystyrene vial with a polyethylene screw cap using 10 x 30 mm picrate paper attached to a plastic strip.

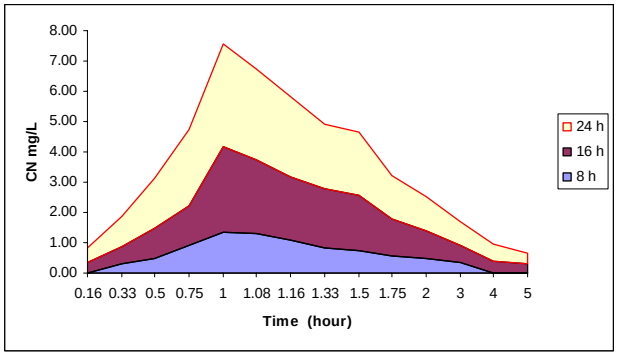

Fig. 3: Blood cyanide (CN^-) levels in goats during and after KCN infusion at reaction times of 8, 16 or 24 h.

To confirm the validity of the method, blood CN^- concentration in rabbits was also estimated at intervals

following oral exposure to KCN, as CN^- exposure occurs primarily via the oral route. Blood CN^- levels showed a time-dependent increase in all experimental rabbits with the maximum concentration (1.34 mg/L) at day 40 (fig. 4).

DISCUSSION

According to Kamalu (1995) and Poulton (1983), long-term CN^- ingestion in the form of cyanogenic plants has been associated with hypothyroidism, pancreatic diabetes, and several neuropathies in both humans and animals.

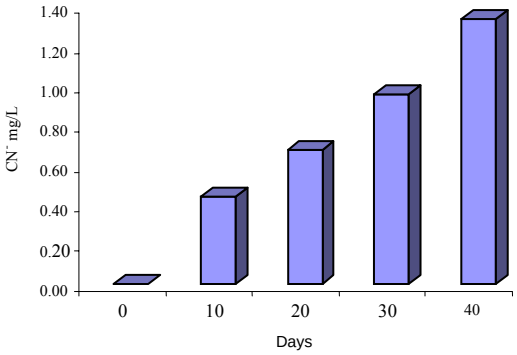

Fig. 4: Blood cyanide (CN^-) concentration in rabbits orally dosed with KCN at 3 mg/kg/day over the period of the experiment.

Commonly used methods of CN^- estimation involve the evolution and trapping of CN^- gas, utilizing apparatus such as Conway microdiffusion cells, Warburg flasks, or disposable plastic cups (Feldstein and Klendshoj, 1954; Gangeloo *et al*., 1980; Laforge *et al*., 1994; Hughes *et al*., 2003). This assay is a modification of methods using the picrate paper kits used to detect CN^- in cassava (Egan *et al*., 1998; Bradbury *et al*., 1999; Bradbury, 2009).and those using diffusion apparatus to detect CN^- in blood (Gangeloo *et al*., 1980; Laforge *et al*., 1994; Hughes *et al*., 2003). Egan *et al*., (1998) allowed enzymatic hydrolysis of cyanogenic glycosides in cassava at specific pH to produce CN^- which reacted with picrate paper for color change, while Huges *et al*., (2003) added sulfuric acid to blood samples in plastic cups as diffusion apparatus and trapped CN^- in NaOH solution which was then reacted to pyridine/ barbituric acid reagent system for spectrophotometery. We modified these methods by adding sulfuric acid to standard CN^- solutions or blood samples in 25 x 50 mm flat-bottomed polystyrene vials for the evolution of CN^- instead of plastic cups or convoy diffusion cells, and CN^- was allowed to react with picrate paper for color change instead of trapping in NaOH solution. This simple, sensitive method to quantify CN^- levels associated with subclinical toxicity in ruminants grazing sorghum pasture, fulfills the requirement for a procedure to analyze blood CN^- that demands minimum technical expertise and low labor and equipment costs. It can also be used as a qualitative test under field conditions of high concentrations of CN^- ($\geq$ 3mg/L of blood) that show color changes with picrate paper and should be suitable for use with other species.

ACKNOWLEDGEMENTS

We acknowledge the assistance of Dr. J. H. Bradbury, Australian National University, Australia for providing guideline to prepare picrate papers and relevant literature. We also thank Drs. Adnan, Imtiaz Ahmad, and Muhammad Saeed; and Mssrs. Muhammad Shahid, Liaqat Ali, and Muhammad Anwar for technical help. We thank the Higher Education Commission (HEC) of Pakistan for financial support.

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