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A Simple and Highly Sensitive Spectrophotometric Method for the Determination of Cyanide in Equine Blood

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An epidemiological association among black cherry trees *(Prunus serotina)*, eastern tent caterpillars *(Malacosoma americana)*, and the spring 2001 episode of mare reproductive loss syndrome in central Kentucky focused attention on the potential role of environmental cyanogens in the causes of this syndrome. To evaluate the role of cyanide (CN^-) in this syndrome, a simple, rapid, and highly sensitive method for determination of low parts per billion concentrations of CN^- in equine blood and other biological fluids was developed. The analytical method is an adaptation of methods commonly in use and involves the evolution and trapping of gaseous hydrogen cyanide followed by spectrophotometric determination by autoanalyzer. The limit of quantitation of this method is 2 ng/mL in equine blood, and the standard curve shows a linear relationship between CN^- concentration and absorbance ($r > .99$). The method throughput is high, up to 100 samples per day.

Normal blood CN^- concentrations in horses at pasture in Kentucky in October 2001 ranged from 3–18 ng/mL, whereas hay-fed horses showed blood CN^- levels of 2–7 ng/mL in January 2002. Blood samples from a small number of cattle at pasture showed broadly similar blood CN^- concentrations. Intravenous administration of sodium cyanide and oral administration of mandelonitrile and amygdalin yielded readily detectable increases in blood CN^- concentrations.

This method is sufficiently sensitive and specific to allow the determination of normal blood CN^- levels in horses, as well as the seasonal and pasture-dependent variations. The method should also be suitable for investigation of the toxicokinetics and disposition of subacutely toxic doses of CN^- and its precursor cyanogens in the horse as well as in other species.

Keywords Blood, Cyanide, Equine, Horse, Kentucky, MRLS

Cyanide (CN^-) at parts per million (ppm) concentrations is a rapid-acting poison in humans and most animals. Its low molecular weight (26 atomic mass units) and high diffusibility lead to its rapid absorption following administration by most routes. Absorption is followed by equally rapid distribution and entry into cells and mitochondria, where CN^- combines with the trivalent iron of cytochrome oxidase to inhibit cellular respiration. The end result is acute cytotoxic hypoxia, severe metabolic acidosis, lactic acidemia, and death if exposure is maintained (Delaney 2001).

CN^- is found in many biological systems, including plants, where it may serve as a protective toxin (Conn 1978). In plants, cyanide is commonly incorporated in glucosides, the specific glucoside or glucoside polymer determined by the plant species. Black cherry trees *(Prunus serotina)*, which grow wild in central Kentucky, contain high concentrations of the cyanogenic glucoside prunasin, a monoglucoside that contains mandelonitrile. Damage to black cherry leaves from any cause initiates an enzymatic cascade whereby the mandelonitrile is released and then decays to yield free CN^- (Morse and Howard 1898; Smeathers et al. 1975). This reaction (Fig. 1) is capable of producing a

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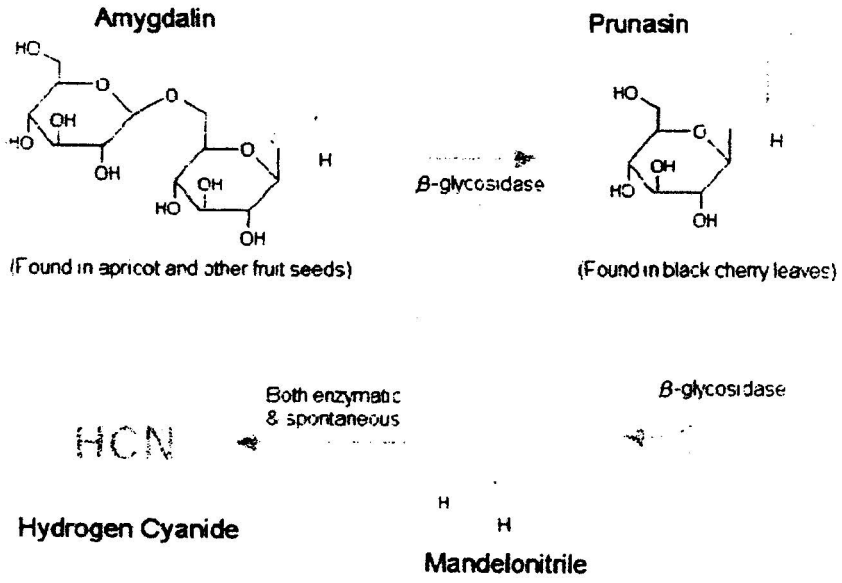


FIG. 1. Hydrolysis of amygdalin and prunasin to produce cyanide.

toxicologically significant quantity of CN^-, as has been demonstrated by the death of livestock that are known to have consumed the leaves of downed cherry trees or limbs (Smeathers et al. 1975).

An epidemiological association among black cherry trees, eastern tent caterpillars, and mare reproductive loss syndrome (MRLS) in the spring of 2001 focused attention on the potential role of environmental cyanogens in the etiology of MRLS (Smeathers et al. 1975; Fitzgerald et al. 2002). To evaluate the role of CN^- in this syndrome, a simple, rapid, and highly sensitive method was needed for the determination of CN^- concentrations in biological fluids. Such a method required greater sensitivity than standard forensic methods generally used for determination of the presence of CN^-. Lethal concentrations of CN^- in blood are in the low ppm range, while normal "background" CN^- concentrations in nonsmoking humans (Lundquist et al. 1987; Tsuge et al. 2000), and presumably in horses at pasture, were expected to be in the low parts per billion (ppb) range.

Among the many analytical techniques reported for the quantification of CN^- are fluorimetry (Felscher and Wulfmeyer 1998; Lundquist et al. 1987), electrochemical detection (Bond et al. 1982), gas chromatography (Cardeal et al. 1995; Lundquist and Sorbo 1989; McAuley and Reive 1983), visible spectrophotometry (Dunn and Siek 1990; Houeto et al. 1994; Laforge et al. 1994; Lundquist et al. 1985), and mass spectrometry (Tracqui et al. 2002). The multitude and variety of analytical methods reported for CN^- may very well indicate the difficulty of its analysis in that there is no generally preferred method. Our early work in adapting gas chromatography and mass spectrometry methods utilizing CN^- derivatization (Kage et al. 1996; Meiser et al. 2000) proved less than satisfactory. The derivatization could be performed only with serum or aqueous samples, was extremely sensitive to interferences, and gave inconsistent results that made little analytical or toxicological sense. Therefore, a more traditional analytical approach based on diffusion and spectrophotometric detection was investigated and adopted.

The adaptation of a common analytical approach to analysis of CN^- detailed in this article appears to provide major advantages in terms of sensitivity, precision, ease of operation, economy, and throughput. This assay is an adaptation of methods using the pyridine/barbituric acid reagent system after isolation of CN^- from blood by diffusion or distillation of hydrogen cyanide (HCN), methods that are widely used in forensic and government regulatory laboratories (Feldstein and Klendshoj 1954; Gangeloo et al. 1980; Laforge et al. 1994; O'Dell 1993). Using a simple, disposable diffusion apparatus and a Technicon Autoanalyzer, a rapid, economical, and highly sensitive analytical method of detecting CN^- in biological samples was developed. This article describes and validates the method and shows that it is sufficiently sensitive to detect the normal concentrations of CN^- in the blood of horses and cattle at pasture. Furthermore, the experiments suggest that this method is sufficiently fast, sensitive, and specific enough to allow for characterization of the distribution, metabolism, and termination of the action of CN^- in the horse after administration of subtoxic doses of CN^- and many of its common biological precursors. Also, the method should be applicable with equal facility to the determination of the presence of CN^- in other species.

MATERIALS AND METHODS

Horses and Sample Collection

Mature thoroughbred mares weighing 428–504 kg were used for this study. The animals were maintained on grass hay and feed (12% protein), which was a 50:50 mixture of oats and an

alfalfa-based protein pellet. The horses were fed twice a day. The animals were vaccinated annually for tetanus and were dewormed quarterly with ivermectin (MSD Agvet, Rahway, NJ). A routine clinical examination was performed before each experiment to ensure that the animals were healthy and sound. Additionally, the heart and eyes were examined to ensure that those organs had no evidence of previous disease. All animals used in these experiments were managed according to the rules and regulations of the University of Kentucky Institutional Animal Care and Use Committee, which also approved the experimental protocol.

Blood was also collected from 10 Holstein-Friesen and two Jersey adult milking cows and stored in heparin-coated tubes at 4°C until analysis for CN^- was performed on the same day. The animals were maintained on corn silage mixed with grains (18% protein) and alfalfa hay (50% of both on a dry-matter basis). The cows were vaccinated annually with 5 WAY Lepto and *Haemophilus somnus* (Grand Laboratories, Larchwood, IA). The animals were examined by a veterinarian to ensure that they were in good health and had proper nutritional status.

Cyanide Infusion

Mature thoroughbred mares weighing 558 and 589 kg were used for the iv infusion of sodium cyanide (NaCN) study. The skin over the jugular vein was washed with Betadine Scrub and rinsed with methanol. An intravenous catheter (14 g × 52 mm. Abbocath-T, North Chicago, IL) was inserted into the jugular vein and sutured in place. NaCN solutions were prepared by dissolving NaCN (J.T. Baker, Philipsburg, NJ) in saline and were infused at a rate of 9 mg NaCN/min for 60 min, using an ambulatory withdrawal pump (Dakmed, Buffalo, NY). During infusion, the horses were monitored closely for signs of toxicity, which include restlessness, anxiety, flared nostrils, rapid respiration, sweating, and increased heart rate. Blood samples were obtained for analysis before infusion (0 h), during infusion (0.17, 0.33, 0.67, and 1 h), and after the infusion was stopped (0.08, 0.17, 0.5, 0.75, 1, 2, 3, 4, 5, and 6 h). The samples were placed into Vacutainer serum tubes (Becton Dickinson, Rutherford, NJ) and heparinized Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) and refrigerated at 4°C until analysis within 24 h.

Mandelonitrile Administration

Mature thoroughbred mares weighing 594 and 612 kg were administered mandelonitrile orally at a dose of 3 mg/kg. Blood samples were obtained for analysis before dosing (0 h) and after dosing (at 0.5, 1, 2, 4, 6, 8, 12, and 24 h) and placed into Vacutainer serum tubes and plasma tubes. They were stored at 4°C until analyzed within 24 h.

Amygdalin Administration

Mature thoroughbred mares were dosed with 10 g of amygdalin (D-mandelonitrile 6-O-B-D-glucoside, approximately 99% isolated from apricot kernels; Sigma Chemicals, St. Louis, MO) which was mixed with 3 lb of sweet feed. The feed was consumed in a period of 2–4 minutes. Blood was collected at 0.83, 0.5, 0.75, 1, 2, 3.5, 5, and 8 h after dosing. Horses were stabled during the entire experiment and were fed hay ad libitum.

Safety Precautions

Two antidotes were prepared to counter possible adverse effects resulting from the administration of cyanide. A 3% solution of sodium nitrite was prepared by adding 1.8 g of sodium nitrite (J.T. Baker, Philipsburg, NJ) to 60 mL of sterile saline. If necessary, this mixture was to be administered intravenously at a rate of 10–20 mL/min. A 25% solution of sodium thiosulfate (Baker), was prepared by adding 100 g sodium thiosulfate to 400 mL of saline. This mixture was to be administered at a rate of 200 mL/min immediately after the administration of the 3% sodium nitrite.

Reagents

All analytical reagents were prepared and stored in sealed Pyrex containers at room temperature, with the exception of the pyridine-barbituric acid reagent, which was refrigerated until use. The chloramine-T reagent was prepared by dissolving 2.0 g chloramine-T hydrate (98%; Sigma-Aldrich, St. Louis, MO) in 500 mL water. The pyridine-barbituric acid reagent was prepared by placing 15 g barbituric acid (98%, Sigma-Aldrich) into a 1-L beaker and washing the sides of the beaker with approximately 200 mL of water, adding 75 mL of pyridine (99% Sigma-Aldrich), and mixing, followed by the addition of 15 mL of concentrated hydrochloric acid and further mixing with a magnetic stirring bar until all particles were dissolved. The reagent was then rinsed into a 1-L volumetric flask, brought to volume with water, and stored at 4°C for a maximum of 1 week. The 1.0 M sodium dihydrogenphosphate (NaH_2PO_4) buffer was prepared by dissolving 138 g of NaH_2PO_4-H_2O (ACS grade, EM Science, Gibbsrille, NJ) in 1 L of reagent water. The 0.25 N sodium hydroxide (NaOH) solution was prepared by dissolving 10.0 g NaOH in 1 L of water.

The Diffusion Apparatus

The diffusion apparatus (Fig. 2) was made by suspending a 10-mL plastic cup (Fisher Scientific, Pittsburgh, PA) inside a 120-mL plastic cup (Fisher) using Scotch tape. Then 10 mL of 1 M sulfuric acid (H_2SO_4) was pipetted into the larger cup, and exactly 2.5 mL of 0.25 N NaOH was pipetted into the smaller cup. The cyanide-containing sample (0.1–2.0 mL blood) was pipetted into the H_2SO_4, and the cup was immediately sealed with its lid and allowed to sit overnight at room temperature. During this period, HCN gas was evolved from the acid solution and trapped in the NaOH solution. The small cup was then removed, and the NaOH solution was decanted into an autoanalyzer sample cup.

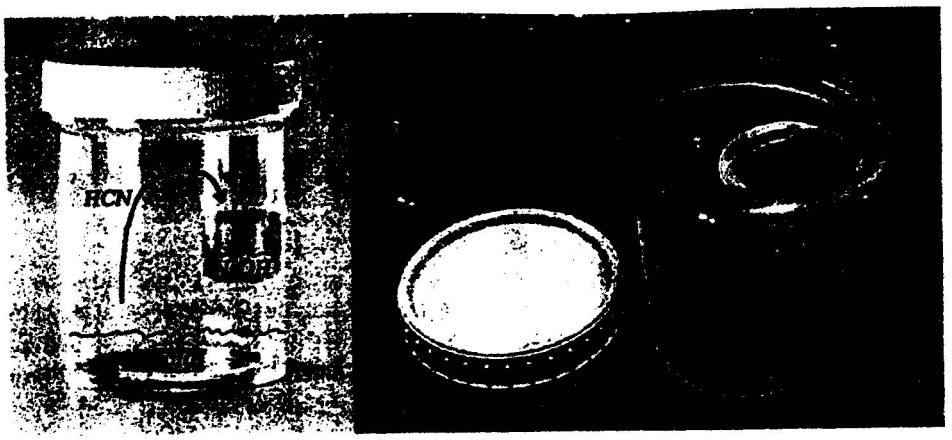
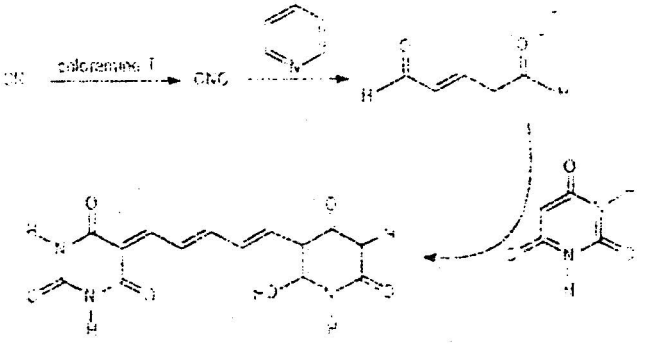


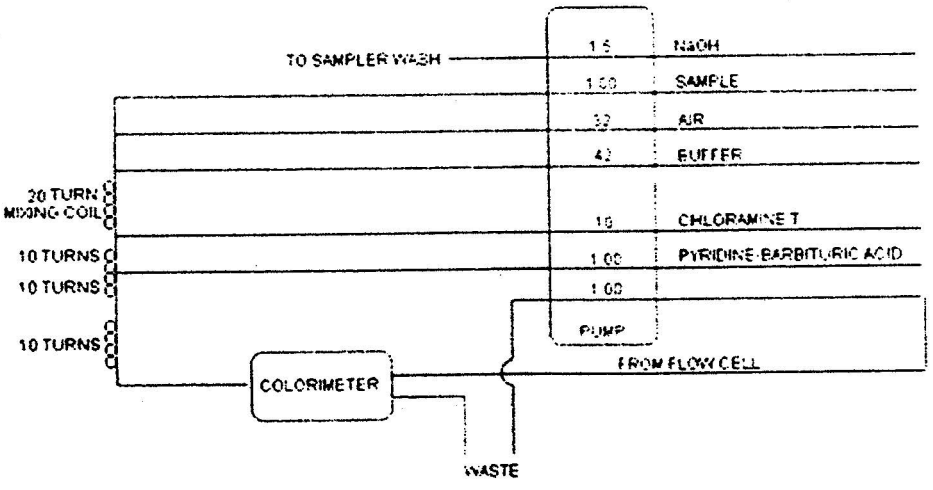
FIG. 2. Cyanide diffusion cup.

The Colorimetric Reaction

The colorimetric analysis was performed as follows. In the presence of chloramine-T, the CN^- ion was converted to cyanogen chloride, which reacted with pyridine-barbituric acid to form a red-blue–colored complex, the intensity of which was measured spectrophotometrically at 570 nm (Fig. 3a). Because the development of color by these reagents does not have a definite endpoint but reaches a maximum intensity and then fades, both the accuracy and the precision of the method depend on a reproducible interval between the mixing of the reagents and the reading of the developed color. Use of the autoanalyzer ensured that the color development of each sample occurred under identical conditions. Therefore, using the automated method meant that the detection limit was much lower than it would have been if a method in which the reagents and spectrophotometer required manual manipulation had been used. Also, the autoanalyzer facilitated the handling of large numbers of samples (Fig. 3b). The intensity of the colored complex developed with this system was



(a)



(b)

FIG. 3. (a) A Konig reaction produces a chromophoric product. (b) Schematic of autoanalyzer flow with high sensitivity 50 mm light path flow cell.

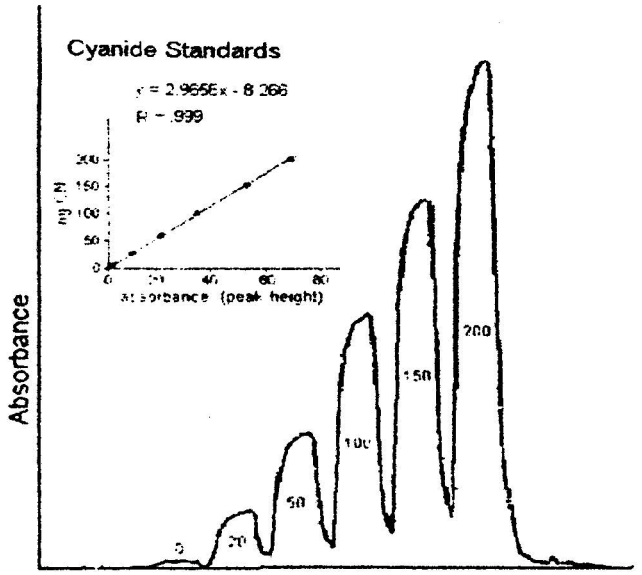


FIG. 4. Typical autoanalyzer chart and standard curve having a linear response between 3 and 300 ng CN. with a sample size of 0.1–2 mL blood.

sensitive to the concentration of salt or NaOH in the solution. Therefore, the same concentration of NaOH solution (0.25 N) used to trap the evolved HCN was also used to establish the baseline autoanalyzer readings.

Method Specifications

This method has been used to quantitate CN^- concentrations as low as 2 ng/mL in a 2-mL blood sample. Standard curves were linear in the range of 2 to 300 ng/mL, with a regression coefficient $r > .99$ (Fig. 4). Recovery of CN^- from 200 ng/mL spiked blood samples was 99.3 ± 1.2% after overnight (~16 h) evolution of HCN.

The 0.25 N NaOH was sufficiently basic to trap HCN and keep it in solution. Exposure of NaOH solutions to air for extended periods may lead to changes in pH due to reaction with atmospheric carbon dioxide. However, no loss of CN^- was observed when the autoanalyzer sample cups were left open to the atmosphere for as long as 4 h.

RESULTS AND DISCUSSION

Numerous methods of CN^- analysis involve the evolution and trapping of HCN gas utilizing apparatus such as Conway microdiffusion cells or Warburg flasks (Feldstein and Klendshoj 1954; Gangeloo et al. 1980; Laforge et al. 1994; O'Dell 1993). The simple and inexpensive disposable plastic alternative to the costly Warburg flask has allowed for the analysis of large numbers of samples with a significant savings of both time and equipment costs. Besides cost, a second consideration was method sensitivity. Traditional forensic methods of CN^- analysis often may not require sensitivity below the ppm level because they are structured to detect CN^- in samples that have high concentrations. In MRLS, none of the pregnant mares involved showed clinical signs of illness. If CN^- was a predisposing or causative factor in MRLS, then the concentrations of CN^- present were subclinical with regard to toxicity. Therefore, a useful method had to be versatile and sensitive enough to quantify CN^- levels associated with subclinical toxicity, as well as normal background concentrations in horses at pasture. This assay fulfilled these sensitivity requirements and was also proven to be a method of elegant simplicity, requiring minimal technician training and having extremely stable interassay reproducibility.

Simple adjustments of the volume of the sample allowed for adjustment of the sensitivity. As shown in Figure 4, the method was sufficiently sensitive to measure very low background concentrations in horses at pasture, which can be as low as 2 ppb CN^-. Standard curves generated by this method were linear between 2 and 300 ng CN^-, with a regression coefficient r value greater than 0.99. Day-to-day reliability of the analysis was demonstrated by analyzing two different spiked serum concentrations of CN^- (20 and 200 ng/ml) on five randomly selected days. Means and coefficients of variation were, respectively, 19.8 ± 10% and 198.5 ± 2.4%.

A standard concern with any assay for CN^- is the stability of the biological sample. Several factors may possibly contribute to changes in CN^- concentration during storage. CN^- in the form of hydrogen cyanide is a highly volatile molecule that is easily lost from an analytical system. In addition, the possibility exists of cyanogenic bacteria proliferating in a stored sample and yielding spuriously high CN^- values (Knowles 1976). The CN^- ion is known to react readily with a number of substrates. CN^- may react with oxygen to form the cyanate ion. CN^- may react with several metallic ions as well as sulfur-containing compounds (cystine); it also reacts with aldehydes or ketones to form cyanohydrins. The reliability of analytical results is best ensured by refrigerated sample storage in the absence of light and analysis as soon as possible (Cassinelli 1986; Egekeze and Oehme 1979). CN^- has a strong affinity for hemoglobin and most of the CN^- in blood is trapped in the red blood cells (McMillan and Svoboda 1982), which may tend to stabilize the sample. As shown in Figure 5, storage of blood samples for 5 days at 4 C resulted in insignificant change in the final CN^- concentrations when compared to samples that had been analyzed within 4 h of collection. While the collection of blood samples in heparinized (green-top) tubes and refrigerated storage appeared to protect the integrity of blood CN^- samples for at least 5 days, we analyzed all samples within 24 h of collection.

This method was sufficiently sensitive to quantitate the subtoxic concentrations of CN^- found in equine blood after administration of compounds containing CN^-. Figure 6 shows the concentrations of blood CN^- during and after intravenous infusion of sodium cyanide (9 mg/min) for 60 min. Blood CN^- increased from background to between 1000 and 1400 ng/mL during the period of infusion and then decayed exponentially toward control values once infusion stopped. No clinical signs of CN^- toxicity or other adverse responses were seen in these infusion experiments.

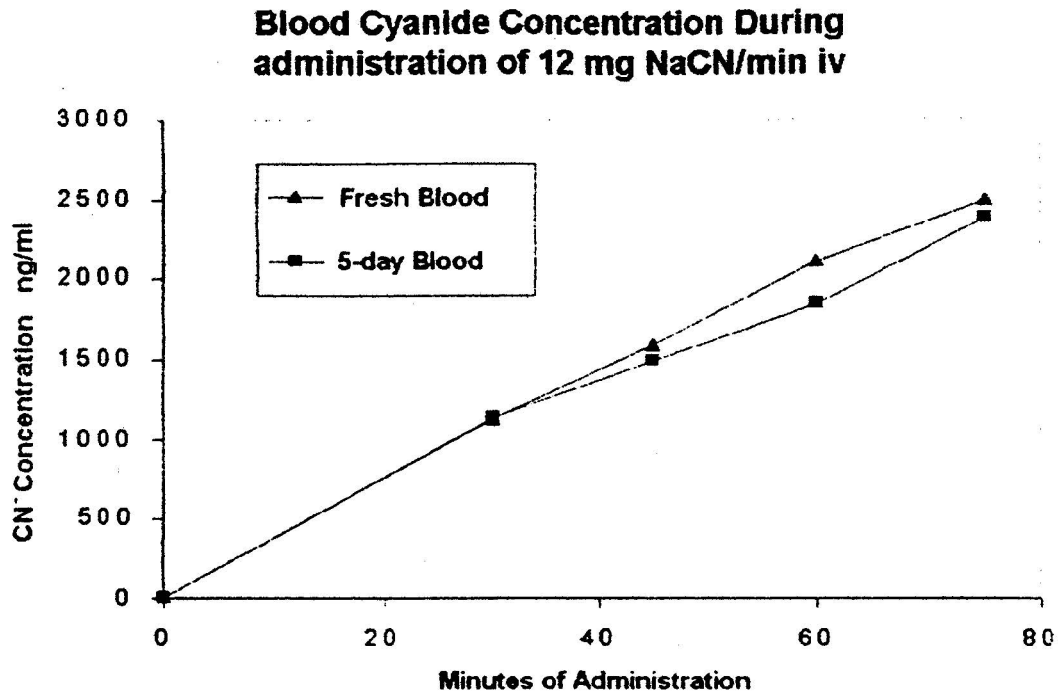


FIG. 5. The effect of refrigerated storage on blood CN^- content. Duplicate heparinized samples were collected during an iv administration of NaCN at 12 mg/min. One set of samples was analyzed within 4 h; the other was stored at 4 C for 5 days before being analyzed.

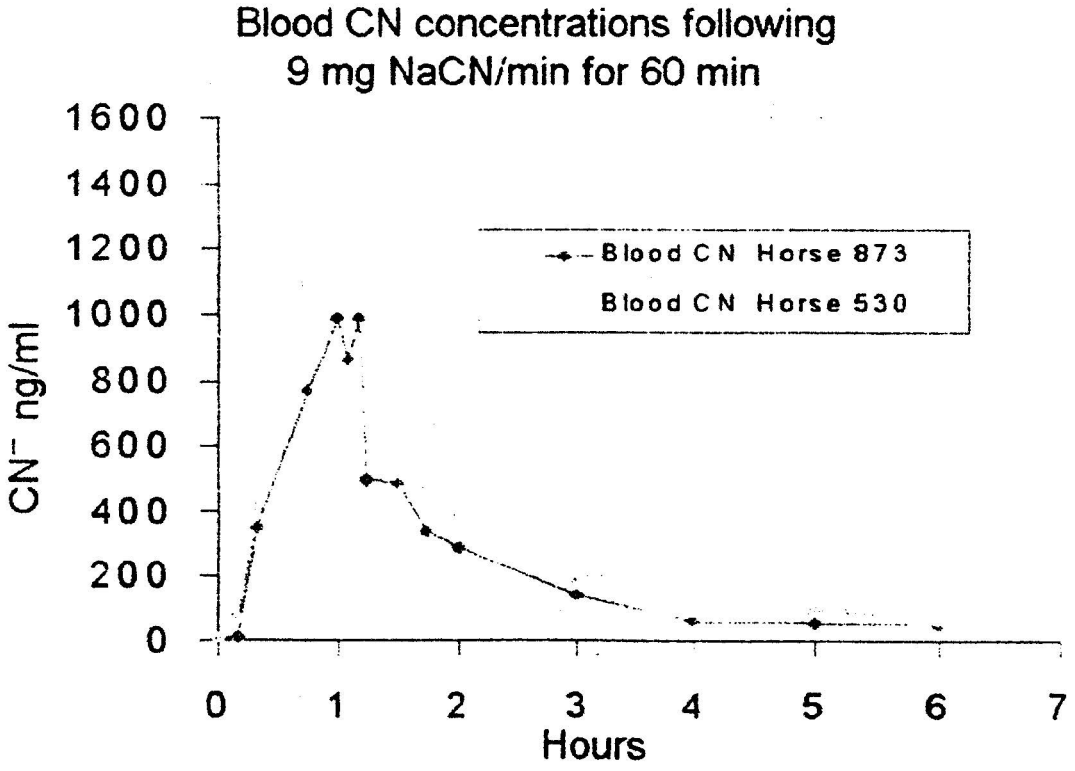


FIG. 6. Blood CN^- concentrations during and after iv administration of NaCN at 9 mg/min for 60 min.

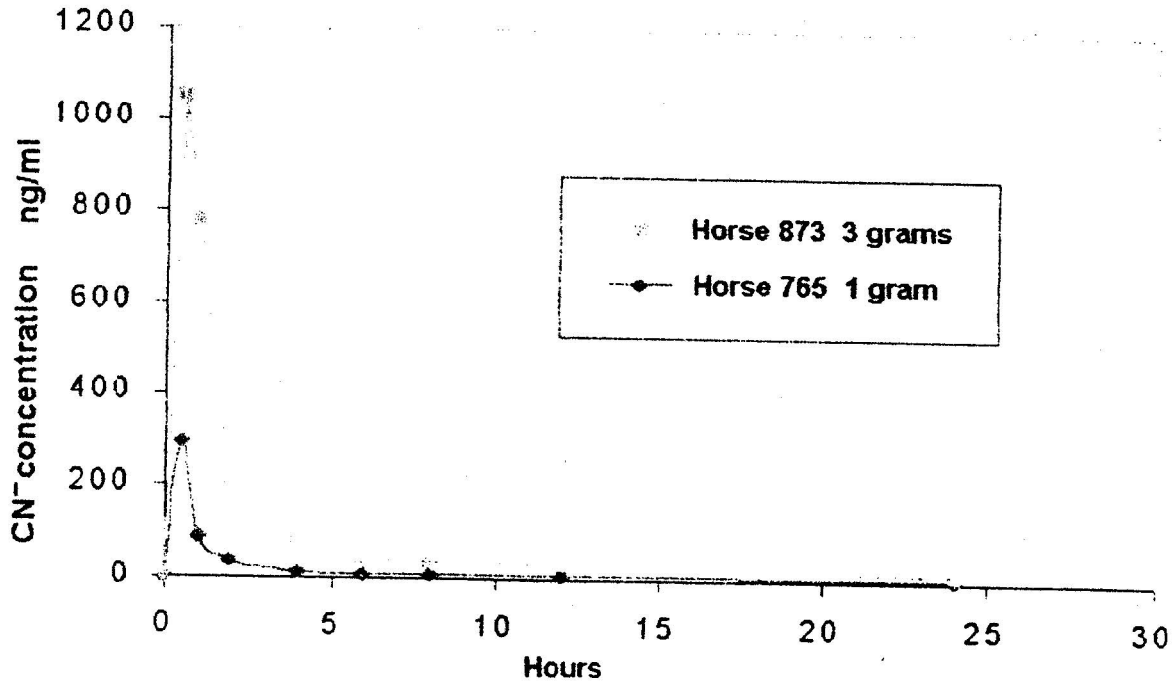


FIG. 7. Blood CN^- concentration following oral administration of 1 and 3 g of mandelonitrile.

The proximal CN^- donor in the black cherry tree's cyanogenic glycoside cascade is mandelonitrile. Figure 7 shows blood CN^- concentrations after oral administration of 1 and 3 g of mandelonitrile to two horses. Mandelonitrile contains 19.53% CN^- by weight; therefore, if 100% of the bound CN^- is released or is bioavailable, the doses are equivalent to the administration of 195 mg and 586 mg doses of CN^-, respectively. These were range-finding doses because we were unable to find information in the literature concerning the potential bioavailability of CN^- contained in mandelonitrile. Administration of the 1-g dose (~1.7 mg/kg) of mandelonitrile was clinically uneventful; however, the horse given the 3-g dose (~5 mg/kg) became uncoordinated within 2 min and stumbled while trying to move around the stall. This episode of lack of coordination was transient, lasting not more than 3 min; the horse settled down and ate hay within a few minutes. However, these signs of CN^- toxicity suggest that oral administration of mandelonitrile rapidly and effectively delivers CN^- into the bloodstream of a horse.

Blood CN^- concentrations peaked at about 250 and 1050 ng/mL 30 min after administration of the 1-g and 3-g doses, respectively, and were declining 30 min after the administration of these ranging doses of mandelonitrile. The data suggest that the CN^- content of mandelonitrile is highly bioavailable and is delivered rapidly.

The ability of amygdalin to increase blood CN^- concentrations in horses was also demonstrated. Prunasin, the principal natural cyanogenic glycoside found in black cherry trees was not available in sufficient quantity to administer to horses, so amygdalin, a glucoside of prunasin readily available from biochemical supply houses, was administered instead. As shown in Figure 8, oral administration of 10 g of amygdalin, which contains roughly 500 mg CN^-, resulted in sharply increasing blood concentrations of CN^- that peak at between 2 and 4 h after administration. The data show that oral administration of a single dose of amygdalin can transiently increase blood concentrations of CN^- at least tenfold. No clinical signs of CN^- toxicity or other adverse responses were seen as a result of these amygdalin administrations.

Another objective of this study was to establish the "normal" background concentrations of blood CN^- likely to be found in horses at pasture in Kentucky. Heparinized blood samples were collected from thoroughbred horses maintained in various fields at the University of Kentucky farm in October 2001 and also from hay-fed horses in January 2002. As set forth in Figure 9a, the blood CN^- concentrations of horses at pasture during the autumn of 2001 ranged from 3–18 ng/mL, with apparent differences occurring according to the particular field in question. In contrast, blood samples drawn from the horses in January 2002 (Fig. 9b) showed much lower blood concentrations of CN^-, with the modal concentration of CN^- being approximately 2 ng/mL, or about the limit of detection of this method, and with no individual concentrations above 6 ng/mL. These data are in good general agreement with other researchers' data (McGorum and Kirk 2001), and suggest that normal blood CN^- concentrations in horses at pasture in Kentucky are generally low, at least during the fall months, and can be particularly low in hay-fed horses in the winter. Similar blood concentrations of CN^- were seen in samples drawn from 12 cattle (Fig. 10).

In summary, this article describes an inexpensive, rapid, highly sensitive, high-throughput, diffusion-based assay for CN^- in blood samples from horses and other animals, including humans. It is capable of quantifying blood CN^- concentrations

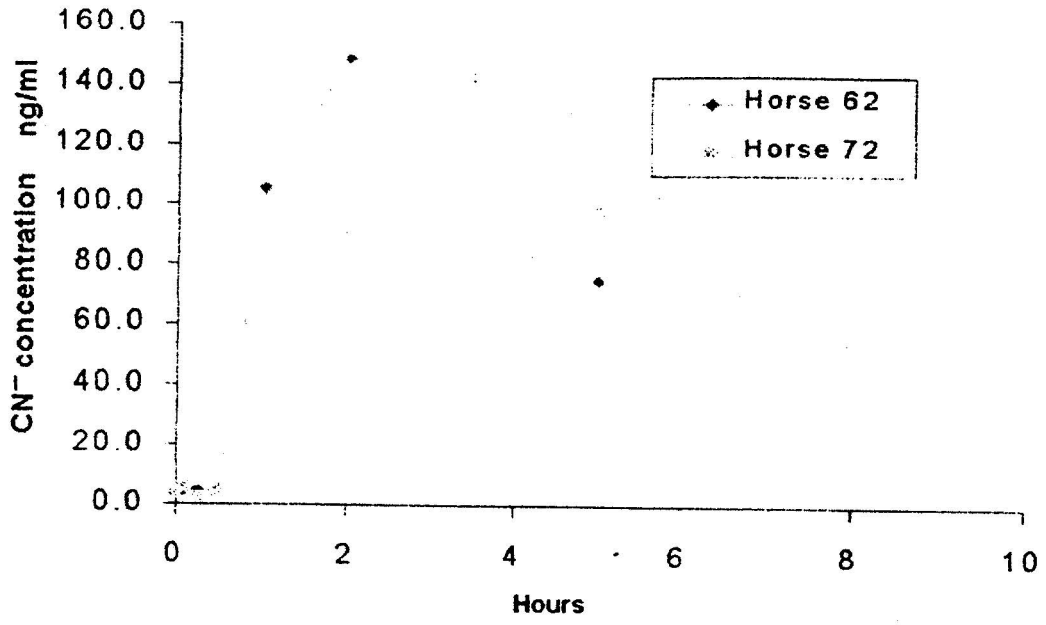
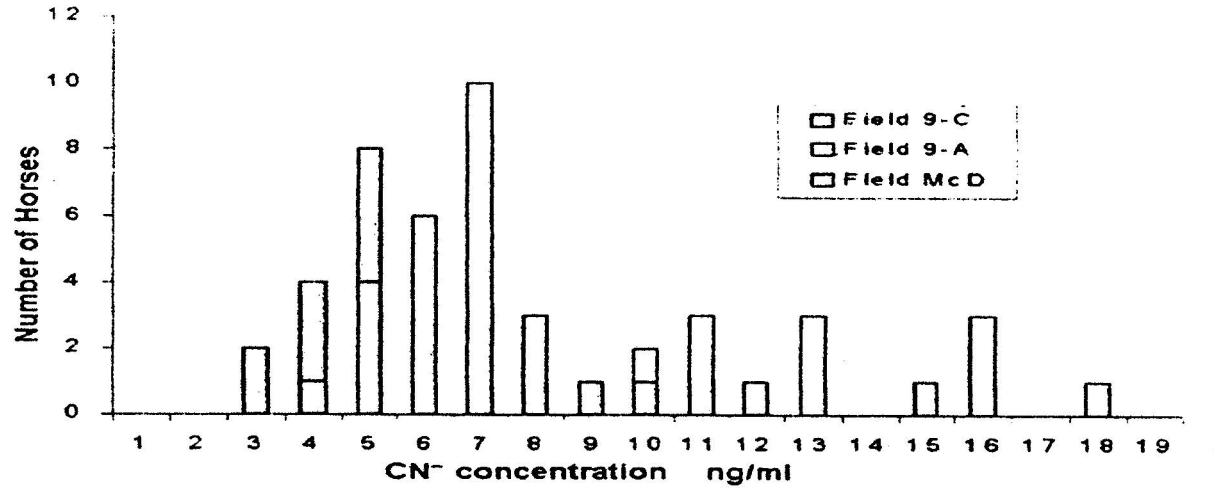
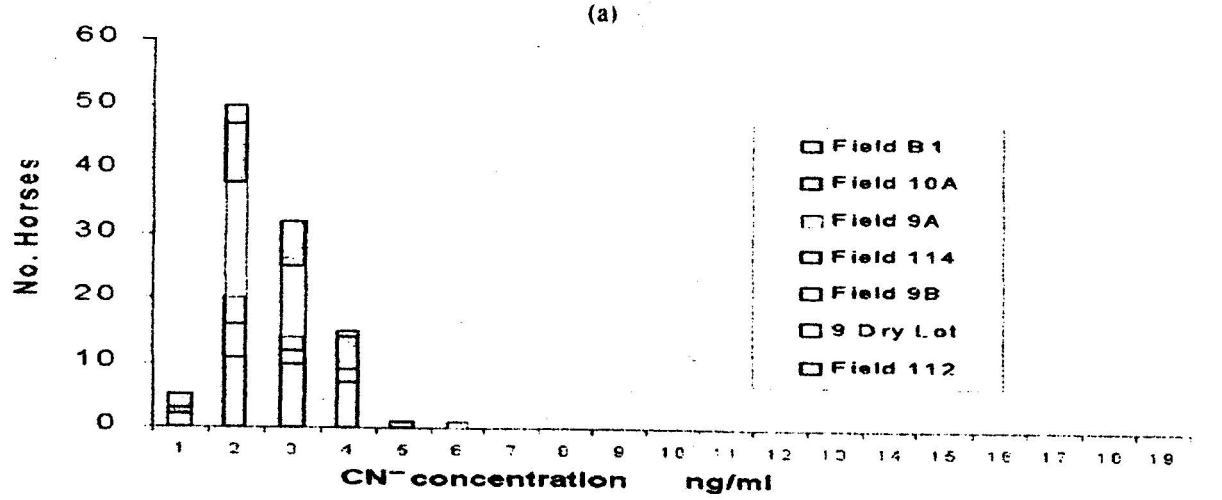


FIG. 8. Blood CN^- concentration following oral administration of 10 g of amygdalin.



(a)



(b)

FIG. 9. (a) Distribution of normal blood CN^- concentrations of horses in different fields, October 2001; n = 48. (b) Distribution of normal blood CN^- concentrations in horses in different fields, January 2002; n = 100.

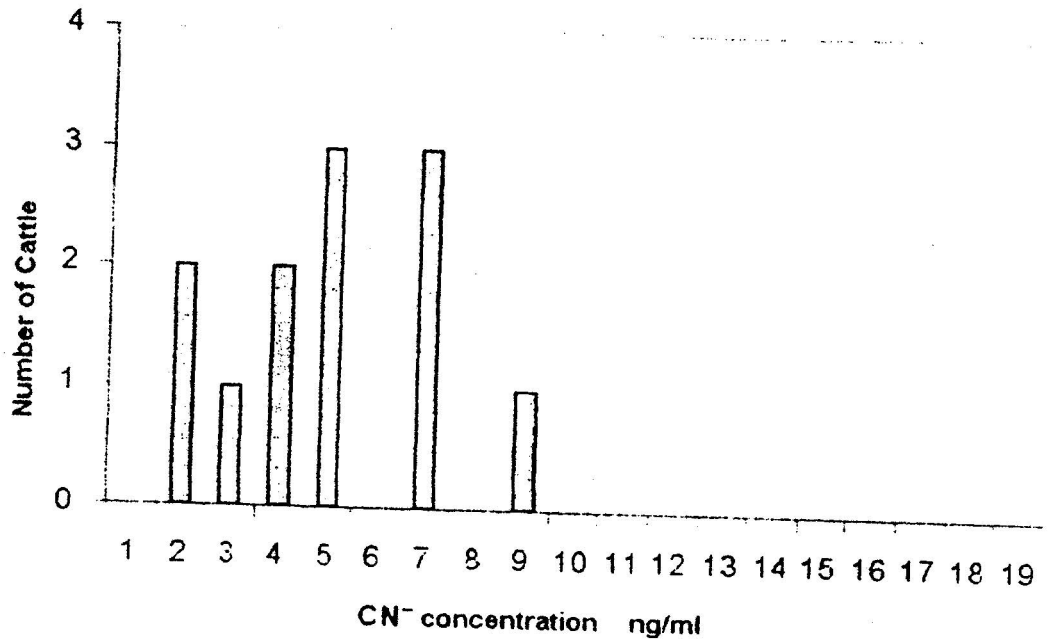


FIG. 10. Distribution of "normal" blood CN^- concentrations in adult milking cows, March 28, 2002; n = 12.

down to 2 ppb in blood. The diffusion apparatus was constructed of simple, disposable plastic components, leading to substantial savings in instrumentation and supplies costs. When drawn into heparinized tubes and stored refrigerated, blood CN^- samples were stable for at least 5 days. This method readily detected and quantified blood concentrations of CN^- after the administration of sodium cyanide, mandelonitrile, and amygdalin to horses. Horses at fall pasture in Kentucky show blood CN^- concentrations between 3 and 18 ppb, with significant differences in blood CN^- concentrations according to the individual pasture. Consistent with this finding, hay-fed horses at winter pasture showed much lower blood concentrations of CN^-.

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