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Research Article

Exogenous Methemoglobin as a Cyanide Antidote in Rats[1]

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The effects of the administration of methemoglobin (MetHb) prepared *in vitro* were evaluated in Sprague–Dawley rats given increasing doses of potassium cyanide (KCN). Median lethal dose (LD50) studies were conducted by giving intraperitoneal injections of KCN (in 0.3- to 0.5-ml volumes), then 2 min later administering intravenous (iv) doses of 1000, 1500, or 2500 mg/kg of MetHb through the tail vein. Control rats received an equivalent volume of saline. The resulting LD50 values for KCN were 7.4 ± 1.1, 11.7 ± 1.1, 13.9 ± 1.0, and 14.2 ± 1.0 mg/kg (mean ± SD) for the control (no MetHb) and 1000-, 1500-, and 2500-mg/kg dose groups, respectively. Additional groups of rats were given 1000, 1500, or 2500 mg/kg MetHb and submitted for necropsy. The gross finding of darkened kidneys was present in both dose groups, but became consistent and more prominent in the 2500-mg/kg dose group. Evidence of pathologic changes was not present in other organs. Single-dose pharmacokinetic studies were conducted using iv doses of 1600 and 2500 mg/kg MetHb. The elimination half-life was similar in both doses (62.6 min), but the volume of distribution (95.3 ± 7.2 and 126.3 ± 5.2 ml/kg, mean ± SE) and clearance (1.1 ± 0.1 and 1.5 ± 0.1 ml/min/kg) were significantly different ($P < 0.05$) for the 1600- and 2500-mg/kg dose groups, respectively. From these data we conclude that although MetHb is cleared from the vascular system rapidly, it may be an effective and nontoxic antidote for doses of cyanide up to twice that of the control LD50.

KEY WORDS: methemoglobin; cyanide antidote; cyanide poisoning; pharmacokinetics; rats.

INTRODUCTION

Many cyanide compounds are very toxic, and people may be exposed to these compounds from both natural and manmade sources. Many foods, some drugs, and even tobacco smoke produce enough cyanide to cause concern (1,2). People also may be exposed to hazardous cyanide compounds through the industrial use of cyanide-containing products for metal ore refining, metal cleaning, paint manufacturing, hide tanning, and the production of various plastics and synthetic materials. Another important source of cyanide exposure is through accidental fires (3). One study showed that 36 fire survivors who had clinical evidence of smoke inhalation all had elevated levels of carboxyhemoglobin and cyanide (4).

In the United States, the current standard of practice for treating cyanide intoxication includes the use of intravenous sodium nitrite and sodium thiosulfate solutions (1). The rationale for this treatment regimen is based on the work of Chen and Rose (5) and others (6), who proposed the mechanism that nitrites convert hemoglobin to methemoglobin (MetHb), which then binds the cyanide ion and reduces toxic effects, and that thiosulfate serves as a source of sulfur for conversion of the cyanide to less toxic thiocyanate. This proposed mechanism for nitrite detoxification has recently been questioned, primarily because of the slow rate at which MetHb forms and the resulting length of time required for effective levels of MetHb to be produced (6). However, when nitrites are administered before exposure to cyanide, MetHb formation does play a role in the detoxification mechanism (7).

The effectiveness of MetHb in the treatment of cyanide poisoning can be used to advantage by preparing the MetHb *in vitro*. Exogenously administered methemoglobin has been shown to be an effective antidote for cyanide intoxication in rats, as shown by a 62% increase in the survival rate of rats given oral sodium cyanide (8). However, the dose of cyanide used in this study was below the median lethal dose (LD50) for cyanide. In many cases of cyanide intoxication, the dose

[1] The views expressed in this article are those of the authors and do not reflect the official policy or position of the Department of the Army, the Department of Defense, or the U.S. Government.

In conducting the research described in this report, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals*, as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care, Institute of Laboratory Animal Resources, National Research Council.

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of cyanide is unknown but possibly is greater than the LD50 dose. The present study was undertaken to examine the application of exogenously administered MetHb in rats given doses of cyanide that exceed the LD50 dose and to determine the pharmacokinetic profile of the exogenously administered MetHb.

MATERIALS AND METHODS

Potassium cyanide solutions were prepared fresh in 0.9% saline for each experiment from reagent-grade chemicals (J. T. Baker Chemical Co., Phillipsberg, N.J.) and maintained in sealed containers. Other reagents used were reagent grade (USP) or better.

Methemoglobin solutions were prepared from stroma-free hemoglobin (SFH) solutions obtained either from outdated human blood by the method of Rabiner *et al.* (9) or from Travenol Laboratories, Inc. The SFH was combined with a 10% solution of potassium ferrocyanide (5 ml/450 ml of SFH), and the mixture was allowed to stand overnight at 4°C. This mixture was dialyzed with 0.9% saline to remove any residual potassium ferrocyanide or reaction products, concentrated to a MetHb concentration of 11 to 23 g/dl, filtered through a 0.22-μm filter (Millistat, Millipore Corp., Bedford, Mass.) into 150-ml plasma transfer packs, stored at −80°C until needed, then thawed and stored at 4°C between uses. The MetHb concentration was determined by the method of Evelyn and Malloy (10).

Male Sprague–Dawley rats (average weight, approximately 450 g) were maintained on a 12-hr light–dark cycle and were allowed food and water *ad libitum*. The median lethal dose studies were conducted using four groups of four rats injected intraperitoneally (ip) with increasing doses of potassium cyanide (in 0.3- to 0.5-ml volumes). Two minutes later, the test dose of MetHb was given by rapid (<60-sec) intravenous (iv) injection into a tail vein. The rats were returned to their cages and observed for 24 hr. Deaths within the first 24 hr were considered to be related directly to cyanide. At the end of 24 hr, surviving rats were euthanized by ip injection of sodium pentobarbital. MetHb doses of 1000, 1500, and 2500 mg/kg were studied. Median lethal dose values were calculated using the method of moving averages (11).

Control groups of rats received injections of either 1500 mg/kg of MetHb, 2500 mg/kg of MetHb, or 2500 mg/kg of human serum albumin (Armour Pharmaceutical Co., Kankakee, Ill.). At the end of 24 hr, these rats were euthanized and submitted for necropsy. Immediately upon death, blood samples were taken and gross necropsies performed. Brain, lungs, liver, heart, urinary bladder, gastrointestinal tract, kidneys, adrenals, spleen, stifle (femur/tibia), and sternum were collected and fixed with 10% buffered formalin. After routine imbedding of samples in paraffin, representative sections were cut at approximately 7 μm and stained with hematoxylin and eosin.

For pharmacokinetic studies, a carotid artery catheter was surgically implanted, and the rats were allowed to recover 3 days before the MetHb was injected. The dose of MetHb (1600 or 2500 mg/kg) was injected through the tail vein in less than 1 min and timed 0.3-ml blood samples were collected from the carotid artery catheter. The blood samples were stored at 4°C until analyzed. No more than 10 samples were withdrawn from any single animal. Catheter patency was maintained by flushing between sample collections with 0.3 ml heparinized saline (10 U/ml). Twenty-four hours after the injection of MetHb, the rats were euthanized and submitted for necropsy. The time versus MetHb concentration data obtained were analyzed by nonlinear regression analysis. The resulting pharmacokinetic parameters for both doses were compared using Student's *t* test. All statistical procedures were performed using the BMDP statistical programs (12).

MetHb concentrations were measured in whole-blood samples spectrophotometrically at 620 nm in 1-cm quartz cuvettes. A 40-μl aliquot of whole blood was added to 2 ml of 10 m*M* sodium borate buffer (pH approximately 9.1) containing a 0.1% Flaminox solution (Fisher Scientific Co., Springfield, N.J.). During the course of this study, Triton X-100 (J. T. Baker Chemical Co., Phillipsberg, N.J.) was substituted for Flaminox in the buffer solution because Flaminox was discontinued by the manufacturer. The sample was mixed by inversion and its absorbance measured on a model 8451A Diode Array spectrophotometer (Hewlett–Packard, Santa Clara, Calif.). Standard curve solutions were prepared by the addition of 0.0, 0.025, 0.050, 0.075, 0.1, 0.2, 0.3, 0.4, and 0.5 ml of the previously prepared MetHb solution to the borate buffer for a final volume of 1 ml. A 40-μl aliquot of each concentration was added to 2 ml of borate buffer and the absorbance determined as previously stated. Blood standards were prepared from pooled rat blood by adding MetHb to give concentrations as previously stated.

RESULTS AND DISCUSSION

Methemoglobin Preparation

Three lots of MetHb were prepared for this study. The first lot was obtained using SFH prepared from outdated human packed red blood cells, and the other two lots were obtained from SFH prepared under contract. The method of preparation of the MetHb was the same for each lot and produced essentially identical products containing 120 mEq/liter Na^+, 0.05 mEq/liter K^+, pH 7.25–7.3, an osmolality of 282–305 mOsm/liter, and concentrations of 11, 20, and 23 g/dl. The final MetHb solution contained no detectable nonheme protein (measured by isoelectric focusing techniques) or cyanide ions. The higher concentrations were prepared to allow higher doses of MetHb to be administered without exceeding 25% of the estimated rat blood volume. MetHb solutions containing greater than about 25 g/dl were difficult to sterilize by filtration; thus, this concentration became the upper limit for usable preparations.

Median Lethal Dose Studies

As shown in Fig. 1, the LD50 increased as the doses of MetHb increased: from 7.4 ± 1.1 mg/kg (mean ± SD) in the control animals (no MetHb) to 11.7 ± 1.1, 13.8 ± 1.0, and 14.2 ± 1.0 with doses of 1000, 1500, and 2500 mg/kg, respectively. The LD50 values for all MetHb doses were significantly different ($P < 0.05$) from the LD50 values for the controls. The LD50 for the 1500-mg/kg group was signifi-

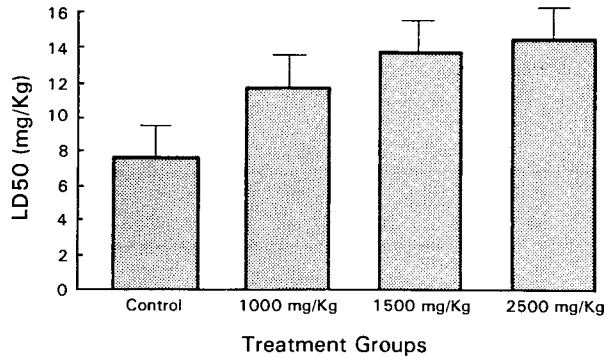

Fig. 1. Increase in the median lethal dose in the treatment groups compared with the control group.

cantly different from that for the 1000-mg/kg group but not from that for the 2500-mg/kg group ($P < 0.05$). A clear asymptotic trend was apparent in the LD50 as the MetHb doses increased, suggesting that 2500 mg/kg approaches a maximally effective dose. At the LD50 doses of KCN, based on the molar ratio of MetHb binding sites to cyanide ions, a maximum of 34, 43, and 71% of the total available sites for cyanide could have been bound for the 1000-, 1500-, and 2500-mg/kg MetHb doses, respectively. Notably, doubling the relative amount of MetHb gave only a 21% improvement in the effectiveness of the antidote. Studies in this laboratory using modified methemoglobin products have shown that changing the affinity of MetHb for cyanide can have large effects on the KCN LD50 doses (unpublished data). Factors other than cyanide–MetHb binding, such as the rate and extent of KCN absorption from the peritoneal cavity as well as the rate of distribution of cyanide ion into tissue, may also modify the therapeutic effectiveness of MetHb. The present data, however, suggest that doses exceeding 1000 mg/kg provided additional but limited protection against cyanide intoxication.

In a recent study, Ten Eyck *et al.* (13) studied the use of MetHb in cyanide poisoning. Using similarly prepared MetHb, they determined that MetHb was useful as an antidote for up to eight times the LD90 determined for intravenously administered cyanide. This difference, when compared to the present study, may have resulted from two important procedural differences. The study by Ten Eyck *et al.* was based on an LD90 as a toxicity indicator, and this criterion is inherently less accurate and less reproducible as a measure of toxicity than the more widely accepted LD50 value (14), particularly when using compounds that have a steep slope to the log dose versus response curve, such as cyanide. More importantly, in the study by Ten Eyck *et al.*, the cyanide was administered centrally into the carotid artery and 30 sec later the MetHb was given through the same catheter. Administering the MetHb so quickly after the cyanide dose permits greater binding of intravascular free cyanide to the MetHb, thus reducing the potential for the cyanide toxic effect—but may overestimate the true physiologic value of the MetHb administration. However, as in the present study, Ten Eyck *et al.* also noted an apparent dose-dependent decrease in the effectiveness of using MetHb as an antidote.

Pathology

Three groups of nonsurgically treated rats and three groups of rats that had surgically implanted catheters were submitted for pathology studies. The nonsurgically treated rats received 1500 mg/kg (eight rats, Group 1) or 2500 mg/kg (five rats, Group 2) of MetHb. Rats that had surgically implanted catheters received 1000 mg/kg MetHb (5 rats, Group 3), 1500 mg/kg MetHb (13 rats, Group 4), 2500 mg/kg MetHb (8 rats, Group 5), or 2500 mg/kg human serum albumin (5 rats, Group 6). Gross and microscopic examinations were performed on all animals, and blood for analysis was obtained from four rats in Group 2. Four rats in Group 1 and five rats in Group 5 showed gross mild, diffuse, bilateral kidney discoloration, which was not detectable microscopically. All rats in Groups 1, 2, and 5 had grossly discolored kidneys and three rats in Group 1 had black speckling of the kidneys. Microscopically, rats in Groups 2 and 5 had protein droplets in the cytosol of the proximal convoluted tubules consisting of 1- to 2-μm, round, eosinophilic structures, which were tinctorially consistent with MetHb. Two rats in Group 2 and all rats in Group 5 had protein globules in the cortical tubular lumina. These globules consisted of occasional 5- to 7-μm, amorphous, amphophilic, irregularly round structures. One rat had a mild nephrosis that consisted of variable numbers of eosinophilic, hypertrophied cortical tubular cells, with a few necrotic cells that were sloughing into the tubular lumina. This rat had a significantly elevated blood urea nitrogen (BUN) level. All rats tested had a slight elevation of the BUN level, which was not clinically significant. Other changes seen in other organs were mild, incidental, and unrelated to the dosage with MetHb.

Analytical Development

Standard curves based on MetHb absorbance at 620 nm were linear over the range 0 to 110 mg/dl. Regression of the absorbance versus MetHb concentration data for 30 separate days gave a mean regression equation: absorbance (mAU) =

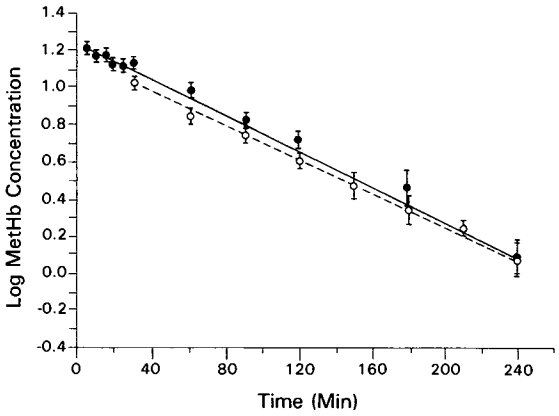

Fig. 2. Whole-blood concentrations of methemoglobin as a function of the time following intravenous bolus doses of (○) 1600 mg/kg and (●) 2500 mg/kg in rats. Each point represents the mean ± SE for 6 and 12 rats for the 1600- and 2500-mg/kg doses, respectively. The solid line represents the least-squares best-fit curve for a one-compartment model.

Table I. Pharmacokinetic Parameters for Methemoglobin in Rats

Group	Initial concentration (C_o) (mg/ml)	Volume of distribution (V_d) (ml/kg)	Elimination rate constant (K_{el})	Terminal-phase half-life ($T_{1/2}$) (min)	Total-body clearance (ml/min/kg)	Weight (kg)
1600 mg/kg[a]	17.5 ± 1.2	95.3 ± 7.2*	0.0113 ± 0.0007	62.6 ± 4.1	1.06 ± 0.05*	0.586 ± 0.025*
2500 mg/kg[b]	20.3 ± 0.8	126.3 ± 5.2*	0.0119 ± 0.0009	62.6 ± 5.3	1.49 ± 0.12*	0.421 ± 0.027*

[a] Mean ± SE for six rats.
[b] Mean ± SE for 12 rats.
* Significantly different ($P < 0.05$).

0.00140 ± 0.00038 + 0.00325 ± 0.00002 × concentration (mg/ml), mean ± SE.

Although many methods exist for determining MetHb concentrations in whole blood or plasma, these methods either were designed for clinical application and were not applicable for this study or were too complex and time-consuming for processing large numbers of samples quickly (15). We, therefore, modified an existing method (16) for the current application. And while the Rossi-Bernardi *et al.* (16) method was fast and simple, using this multiple-wavelength method to calculate the MetHb concentration yielded levels that were 10–30% below the actual prepared concentrations. However, minimal interferences from other heme moieties at 620 nm allowed us to see this single wavelength to quantitate the MetHb accurately, precisely, and quickly.

Pharmacokinetics

As Fig. 2 shows, the MetHb was essentially completely cleared from the blood by 4 hr in both the 1600-mg/kg and the 2500-mg/kg doses. The data from each dose fit a one-compartment open model with bolus input well and produced the pharmacokinetic parameters shown in Table I. The elimination rates for both doses were essentially the same. The volume of distribution (V_d; calculated as dose/C_o) for MetHb aproximates the extracellular water volume (17). The greater total-body clearance of MetHb at the higher dose reflects primarily the greater calculated V_d. Although quantitative urine collection was not done, MetHb appeared in the urine in less than 15 min after the dose was given. The elimination mechanism for MetHb is presumed to be similar to that for unbound hemoglobin. This mechanism involves the reticuloendothelial system, liver, and kidneys and is dose dependent (18). However, the present data suggest that with MetHb apparent first-order elimination occurs in rats at doses of 2500 mg/kg or less.

In summary, the present study demonstrated the value of using *in vitro* prepared MetHb as an antidote to cyanide intoxication when the cyanide doses are twice the LD50 determined in control animals. There appeared to be minimal acute pathological effects resulting from intravenous MetHb administration but the long-term toxicities have not yet been evaluated. Spectrophotometric measurements of MetHb at 620 nm provided a fast and accurate method of determining whole-blood levels of MetHb. Following single iv doses of 1600 and 2500 mg/kg, the elimination of MetHb appeared to be first order, with a $T_{1/2}$ of 62.6 min, and the V_d at these doses approximated that of extracellular water.

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