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Lieutenant Colonel, MS
Deputy Chief of Staff for Information Management
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Task 89-12: Characterization of Soman Toxicity in Atropine and Oxime (HI-6 and MMB-4) Treated Rhesus Monkeys

To
U.S. Army Medical Research and Development Command
Institute of Chemical Defense

March, 1991

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5 March 1991. Other requests for this document to be referred to Commander, U.S. Army Medical Research and Development Command, ATTN: SIRD-RMI-1, Fort Detrick, Frederick, Maryland 21702-5012.
**Task 30-11: Characterization of Suran Toxicity in Atropine and
Atropine (HI-6 and MB-4) Treated Rhesus Monkeys**

A study was instituted to characterize in rhesus monkeys the toxicity, based on lethality, morbidity, and pathology, of GD when exposure was followed by treatment with atropine and either of two test oximes, HI-6, and MB-4. The 48-hour LD-50 was determined for untreated monkeys and monkeys treated with suran (HI-6 and MB-4). HI-6 was significantly more effective than MB-4 in treating GD intoxication. The times to death were significantly longer for animals treated with atropine/HI-6. Duration of treatment, convulsions, and prostration were determined to not be significantly related to either GD dose or treatment group. Neither atropine/MB-4 or atropine/HI-6 appeared to alter the gross appearance of organs or tissues from those seen in untreated monkeys.
FINAL REPORT

Contract DAMD17-89-C-9050
A Medical Research and Evaluation Facility (MREF) and Studies
Supporting the Medical Chemical Defense Program

on

TASK 89-12:
CHARACTERIZATION OF SCMN TOXICITY IN
ATROPINE AND OXIME (HI-6 AND MMB-4)
TREATED RHESUS MONKEYS

to

U.S. ARMY MEDICAL RESEARCH
AND DEVELOPMENT COMMAND

March, 1991

Dr. Carl T. Olson
Dr. Ronald G. Menton
Ms. Robyn C. Kiser
Ms. M. Claire Matthews
Mr. Timothy L. Hayes
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Dr. Garrett S. Dill

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Columbus, OH 43201-2693

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21702-5012.
FINAL REPORT

on

TASK 89-12:
CHARACTERIZATION OF SCXAN TOXICITY IN ATROPINE AND OXIME (HI-6 AND MMB-4) TREATED RHESUS MONKEYS

to

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

March, 1991

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Study Veterinarian
QUALITY ASSURANCE STATEMENT

This study was inspected by the Quality Assurance Unit and reports were submitted to management and the study director as follows:

<table>
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Report to study director and management: 6/8, 7/26, 10/11, and 12/14/90

To the best of my knowledge the methods described were the methods followed and the data presented accurately represent data generated during the study.

Quality Assurance Unit
Date
Health and Environment Group
GOOD LABORATORY PRACTICES COMPLIANCE STATEMENT

To the best of my knowledge, all aspects of this study were conducted in compliance with the U.S. Food and Drug Administration's Good Laboratory Practices regulations (21 CFR Par. 53). This report was reviewed by Battelle's Quality Assurance Unit to verify that the information contained herein accurately depicts the data collected in the study.

Carl T. Olson, D.V.M., Ph.D.  Date
Study Director
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1.0 INTRODUCTION

There is a need for improved antidotes, specifically oximes, for treatment of human beings exposed to organophosphorus (OP) chemical warfare agents. Although pralidoxime chloride (2-PAM), the standard therapeutic oxime, is reportedly effective for treating exposure to the nerve agents sarin (GB) and VX, it is only a marginally effective treatment for exposure to soman (GD) and tabun (GA). HI-6 and MMB-4 are two oximes most likely to be developed over the next year as replacements for 2-PAM. The decision whether to use either of these oximes as a replacement for 2-PAM is critically dependent on the demonstration of improved efficacy against GD intoxication in studies with nonhuman primates. The objective of this task was to characterize in rhesus monkeys the toxicity, based on lethality, morbidity, and pathology, of GD when exposure was followed by treatment with atropine and either of the two test oximes, HI-6 or MMB-4. Treatments were administered at 1 min after GD injection using a demonstrated efficacious dose of atropine of 0.4 mg atropine free base per kilogram of body weight(1) and 100 μmol of either candidate oxime per kilogram of body weight. The study was performed at Battelle's Medical Research and Evaluation Facility (MREF).

2.0 EXPERIMENTAL DESIGN

2.1 Test Animals

Male rhesus monkeys, Macaca mulatta, of Chinese origin and supplied by the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) were used in this study because there is considerable scientific evidence that the responses of monkeys are predictive of responses in man. Monkeys weighed between 4 and 7 kg and were housed individually in stainless-steel cages approximately 24 inches wide, 34 inches high, and 26 inches deep. Room temperatures were maintained at 63-80 °F and relative humidity at 30-70 percent. (A small number of excursions outside these ranges occurred. Records are on file at the MREF.) Fluorescent lighting on a light/dark cycle
of 12 hr each per day was used. Purina Certified Monkey Chow biscuits were fed twice daily and supplemented with locally purchased fresh fruit twice weekly. Chemical analyses of certified feeds are available from Purina. Water was supplied from the Battelle West Jefferson water system and given as lithium through automatic watering systems. Water is analyzed for chemical impurities annually and for potability quarterly. No contaminants that would interfere with the results of the study are known to be present in the feed or water.

All animals arrived with tattoos so that positive identification could be maintained. Monkeys were maintained in quarantine for a minimum of 1 month, during which time they were examined by the study veterinarian and blood samples were taken for hematology and serum chemistry analyses. Fecal samples were taken for gastrointestinal parasite infestation evaluation, and three tuberculin tests were performed at approximately 2 week intervals. Based upon results of physical examinations and clinical laboratory findings, all monkeys were found to be acceptable for study. Monkeys were randomized, based on body weight, into a group of five animals for a GD 48-hr LD₅₀ determination and 40 animals for efficacy testing (20 in each oxime treatment group) to obtain group homogeneity of weight, as possible, across phases and stages of the experiment. Monkeys were placed on slotted, V-shaped platforms to obtain body weights and blood samples (femoral venipuncture), and for injection of GD and treatment compounds.

2.2 Materials and Methods

2.2.1 Chemistry

Atropine in citrate buffer (RU7144, BL07753) was supplied by USAMRICD. Verification of identity and analysis of concentration of this lot of atropine had been accomplished previously by SRI International.\(^2\) Chemical verification and concentration analysis of atropine was also accomplished at the MREF using high performance liquid chromatography (HPLC, MREF SCP-89-55). GD was supplied by USAMRICD. Purity of GD stored at Battelle is periodically confirmed by Battelle chemists. For animal testing in
1.4 mg of GD per mL of physiologic saline solution was
mixed and aliquots were stored in 10-mL and 20-mL serum vials at
approximately 70°C. After preparation, gas chromatographic analyses of
the stock solution were accomplished, and after each dosing day
aliquots of the dosing aliquots were analyzed (MREF SOP-88-31).
The test oximes, MMB-4 (1,1′-methylene-bis[4-(hydroximinomethyl)]
propan-1-amine) monohydrochloride; EM03376, WR249, 943AH) and HI-6 (1-2-
methylene-1-pyridino-3-(4-carbamoyl-1-pyridino)-2-oxapropine
in-water; EM03372, WR249, 653AH) were supplied in solid form by USAMRICD.
Identity and purity confirmations of test oximes were not performed at
the time. On each day of efficacy testing, a HI-6 (MW 373) in water solution
was prepared at a concentration of 378 mg/mL, and a MMB-4 (MW 329) in water
solution was prepared at a concentration of 329 mg/mL so that treatments could
be administered at 0.1 mL/kg to deliver 100 μmol/kg of either test oxime.

2.2.2 GD LD₅₀ Studies

An estimate of the GD 48-hr LD₅₀ in this population of monkeys given
treatments was established. This was accomplished in an up-down type
experiment (31), challenging one monkey a day with a dose dependent upon
results from previous days. Doses of GD used were selected by the study
statistician in order to estimate the 48-hr LD₅₀ most efficiently. The
estimate of the GD LD₅₀ was used to predict protective ratios (PRs; LD₅₀ of
treated animals/LD₅₀ of untreated animals) for each treatment regimen.

2.2.3 Oxime Efficacy Studies

To test treatment efficacy of HI-6 and MMB-4 given in conjunction
with 0.4 mg/kg atropine free base, two weight-homogenized groups of monkeys
were established for dosing purposes. A stagewise strategy was used to
deliver GD doses in the experimental design. Using doses of GD recommended
by the study statistician, groups of equal numbers of monkeys were challenged
with GD and treated with atropine and 100 μmol of either HI-6 or MMB-4 per kg
body weight at 1 min after GD injection. Monkey body weights taken within
24 hr of GD injection were used to calculate volumes of GD and treatment compounds to be administered. Hair over the calf of the right leg and over the anterior lateral aspect of the left leg was clipped and injection sites delineated with a permanent-ink marker. Monkeys were given GD intramuscularly (IM) in the gastrocnemius muscle of the right leg. GD dosing was accomplished with monkeys restrained on a platform and within a hood approved for the use of highly hazardous material. After decontamination of the skin at the IM dosing site using ethanol followed by a hypochlorite solution and then water, monkeys were removed from the hood and at 1 min following GD injection were given atropine and either HI-6 or MMB-4 in succession in the anterior lateral area of the left thigh in the region of the vastus lateralis muscle. Treatment injection sites were separated by 2-3 cm. Twenty-five gauge, 5.8-inch needles were used for all IM injections. Syringes used for dosing were Hamilton (Reno, NV) microliter syringes of the smallest compatible volume (Syringes are filled to no more than 95 percent of labeled volume.) to obtain maximum accuracy in delivered dose. Individual, labeled syringes were loaded with the calculated volumes of GD prior to the start of dosing, weighed, and placed on ice until used. After dosing was completed, syringes were weighed again to determine weight losses and calculate the amount actually delivered. Syringes used for injection of atropine, HI-6, and MMB-4 were also weighed both before and after treatments were injected. On every day of dosing, solutions of HI-6 and MMB-4 made up that morning and used to treat monkeys were sampled for chemical analysis to confirm desired concentrations (MREF SOP-89-64).

Monkeys were returned to individual cages after treatments had been completed, and were closely and continuously observed for the first 4 hr following dosing and at intervals thereafter with observations annotated at 6, 8, 12, 24, 36, and 48 hr and daily thereafter to the seventh day. Signs specifically monitored included muscle fasciculations, tremors, convulsions, prostration, salivation/bronchial discharge, miosis/mydriasis, uncoordinated movements, and death.
2.2.4 Pathology

Monkeys that died prior to 48 hr in both the GD LD_{50} and oxime treatment efficacy phases of the study underwent necropsies performed by an experienced veterinary pathologist and gross lesions were recorded. Monkeys which survived for 48 hr but were in moribund condition were anesthetized, tissues perfusion-fixed, necropsies performed, and tissue samples taken for histopathologic evaluation. Following necropsy, all animal remains were incinerated.

2.2.5 Statistical Analyses

An up-down approach was used to estimate the 48-hr GD LD_{50} in untreated monkeys. Based on historic information on the slope of the GD dose-lethal response curve and probit analyses of data as they were obtained, the best doses for challenging succeeding animals were selected in order to estimate most efficiently the 48-hr GD LD_{50} in the present population of monkeys. The experiment was designed to use a maximum of five monkeys to estimate the 48-hr GD LD_{50}, but, if, after two or more monkeys had been challenged, the estimated LD_{50} fell within the 95 percent confidence limits of Battelle's historic rhesus monkey 48-hr GD LD_{50}, that historic LD_{50} value would be accepted as the approximate LD_{50} in the present population of monkeys and further experimentation for determination of the LD_{50} would not be accomplished.

Based upon required sample size analyses to estimate the minimum number of animals needed to provide definitive information on PRs, 20 monkeys were allocated to each of the two treatment oxime/atropine therapies. If fewer animals were needed to demonstrate a PR significantly greater (z of 0.05) than the historic atropine/2-PAM PR (approximately 1.7) and a survival rate of at least 84 percent at 2 x 48-hr GD LD_{50}, experimentation would cease. If it became apparent after at least five monkeys had been challenged and treated with each regimen that a PR of at least 2.5 was not being approximated, further guidance from the USAMRICD Task Area Manager (TAM) would be sought.
Doses of GD were selected by the study statistician to best estimate the LD₃₀ and slope of the dose-response curve in treated monkeys. Animals were to be dosed in groups with equal numbers of monkeys in each treatment group per dosing day unless sufficient information was obtained for one treatment regimen but not the other. From the information obtained, probit analyses were performed and estimates made of the 48-hr LD₃₀, LD₅₀, and LD₇₀ values in treated monkeys. PRs were also calculated. Signs of GD intoxication were statistically analyzed, as feasible, to determine any differences in treatment efficacy.

3.0 RESULTS

3.1 Chemistry

The stock solution of GD in physiologic saline prepared for dosing monkeys in Task 89-12 was analyzed and concentration was determined to be 1.24 mg/mL. The average analyzed concentration of daily dosing aliquots used in LD₅₀ and efficacy experiments was within 10 percent of this 1.24 mg/mL concentration (1.14 mg/mL with a standard deviation of 0.04). This slight decrease in concentration may be due to degradation of the dosing solution during the time it was held on ice while dosing of animals was being accomplished or during rethawing prior to chemical analysis.

Concentration of atropine free base, as determined by SRI(²), was 2.5 mg/mL, and this concentration was used to calculate volumes of atropine solution for injection in monkeys. The average of two analyses for atropine free base concentration performed at the MREF was 2.33 mg/mL. If the true concentration of atropine free base was 2.33 mg/mL rather than the 2.5 mg/mL used to calculate injection volumes, the result would be treatments with 0.37 mg/kg rather than the 0.4 mg/kg target dose of atropine free base.

Analyses of HI-6 and MMB-4 solutions used for treating monkeys demonstrated concentrations within 5 percent of target concentrations. HI-6 at a concentration of 378 mg per mL of water went into solution rather slowly and required vortex mixing or sonication to speed the dissolution process. Once in solution, HI-6 remained in solution even if refrigerated.
MMB-4 readily went into solution at 329 mg per mL of water, but would precipitate if solutions were refrigerated or were left standing at room temperature for more than a few hours.

3.2 GO LD₃₀ Studies

An up-down type approach was conducted to estimate the 48-hr GO LD₃₀ in untreated monkeys of this population. On separate days, five monkeys were injected IM with various doses of GO based on estimates of the GO LD₃₀ and slope of GO dose-lethal response curves of previous experiments and based on results, as obtained, in this population of monkeys. Because of results obtained in efficacy phases of this study, four additional monkeys were injected with GO and given no treatment. Results of GO LD₃₀ testing are presented in Section 3.5, Statistics.

3.3 Oxime Efficacy Studies

On the first day of oxime efficacy testing, eight monkeys were given various doses of GO and four were treated with atropine and MMB-4 and four were treated with atropine and HI-6. A week later, eight additional monkeys were treated similarly using GO doses selected by the study statistician and based on results from the first day of testing. Analyses of the data at this time demonstrated that neither treatment was achieving a PR of 2.5, and, as stated in the protocol, the USAMRICO TAM was notified of these results. The decision was made to perform one more day of similar testing to confirm results, and this was accomplished approximately 1 month following the second day of testing. On the fourth and final day of efficacy testing, four monkeys were challenged with various doses of GO and treated with atropine/HI-6, and, because MMB-4 had proven ineffectual, four additional monkeys were injected with GO and given no therapy in an effort to more accurately define the GO LD₃₀ in untreated animals. The results of oxime efficacy testing are presented and discussed in Section 3.5, Statistics.
25 rhesus monkeys were necropsied: seven had been given GD with no therapy; seven were given HMB-4 as part of the therapy regimen; and 11 were given HI-6. Twenty-two of these monkeys received gross necropsies only, with no tissues saved for microscopic examination. Three monkeys which received no treatment but survived more than 48 hr after GD challenge were euthanatized in moribund condition. For these three animals, pentobarbital sodium was given intravenously to induce a deep plane of anesthesia. All tissues were then perfused via cannula with heparinized saline followed by 10 percent neutral buffered formalin. Samples of the following tissues were collected and post-fixed by immersion in 10 percent neutral buffered formalin: brain, spinal cord, heart, kidneys, liver, lungs, ileum, stomach, diaphragm with phrenic nerve, biceps muscle, (forearm) common digital extensor muscle, ulnar nerve, radial nerve, brachial plexus, distal sciatic nerve, pituitary, adrenal glands, and gross lesions (when present). Left eyes were also collected, but post-fixed by immersion in Bouin's solution.

Results of the gross and, when applicable, microscopic examinations are presented in Appendix C. In all cases, necropsy results were compatible with death or moribund condition due to GD intoxication. Usually no gross morphologic changes were detected. Neither HI-6 nor HMB-4 therapy appeared to alter the gross appearance of organs or tissues from that seen in monkeys exposed to GD alone.

Gross lesions which could be attributed to GD included epicardial and thymic hemorrhage seen in a few monkeys. Brain/meningeal congestion and/or hemorrhage was present in several monkeys; whether this is a primary effect of GD intoxication is uncertain since cranial trauma caused by GD-induced convulsions could easily have caused these lesions. A number of monkeys had acute hemorrhage of cranial (temporalis) muscles, presumably caused by trauma suffered during convulsions. One monkey had yellow/green foam in the bronchi which was suggestive of terminal aspiration of stomach contents. All other lesions found at necropsy were incidental to GD (or therapy) injection.
Tissues saved from the three monkeys (89-1582, 89-1600, and 89-1633) which received no therapy were trimmed for targeted sites, cut at 5 microns, stained with hematoxylin and eosin, and examined microscopically. Targeted tissue sections included the following organs, tissues, and/or sites:

- **Eye**
- **Brain**
  - frontal, entorhinal, parietal/occipital cortex
  - amygdaloid and caudate nuclei
  - hippocampus
  - thalamus
  - midbrain
  - pons
  - medulla
  - cerebellum
  - olfactory bulb (when present)
- **Pituitary**
- **Spinal cord** (three sections)
- **Peripheral nerve** (sciatic, brachial plexus, ulnar, radial, phrenic)
- **Adrenal gland**
- **Liver**
- **Kidney**
- **Lung**
- **Ileum**
- **Stomach**
- **Diaphragm**
- **Muscle** (biceps, common digital extensor)
- **Heart** (SA node, left ventricle, ventricular septum, left atrium, bundle of His).

Significant neuropathology was found in all three monkeys. This consisted of minimal to severe (depending upon animal and brain region) neuronal necrosis in most sections of the cerebrum. Cerebellum, medulla, spinal cord, and peripheral nerves were essentially normal. Neuropathology, identical to the so-called "ischemic cell change," was interpreted to have been caused by GD and resulted in the moribund state prior to euthanasia.

All three monkeys also had scattered areas of skeletal muscle necrosis. These lesions consisted of degeneration and frank mineralization of isolated myofibrils. The presence of the mineralization suggests that the skeletal muscle injury had occurred a couple of days prior to death (presumably during or associated with convulsive activity). One monkey (89-1600) had minimal cardiac myocyte degeneration as well.

Two monkeys (89-1582 and 89-1638) had evidence of hepatic fatty changes suggestive of starvation (excessive release of free fatty acids from adipose tissue). This has been seen previously in moribund animals, and appears to be more an indication of altered metabolic state and moribundity.
than a GD-specific toxic effect. One monkey (89-1638) had evidence of mild renal tubular necrosis, presumably related to its moribund state and poor tissue perfusion prior to euthanasia. All other lesions found microscopically appeared incidental in nature and unrelated to GD exposure.

3.5 Statistics

The study was conducted in two phases. In phase I, a modified up-down type experiment, challenging one monkey per day, was conducted to provide an estimate of the 48-hr GD LD50 in untreated monkeys. In phase II, experiments were carried out to estimate GD dose-lethality response relations for animals treated with atropine (0.4 mg/kg body weight) and either HI-6 or MMB-4 (100 μmol/kg body weight). Since no more than 20 monkeys were to be tested in either treatment group, it was essential that animals be tested at doses representing appropriate regions of the unknown dose-response curve. Therefore, a stagewise adaptive dose allocation strategy was used to select GD doses.

In addition to investigating the effect of GD dose on lethality, each animal was monitored for clinical signs of GD intoxication following injection of the GD dose. The objective of the analyses of clinical signs data was to determine if there were statistically significant differences in onset and/or duration of sublethal responses for GD intoxicated monkeys treated with atropine and either HI-6 or MMB-4.

The results of statistical analyses used in this study rely on a number of assumptions. It was assumed that the probit model, with no background lethality, provided a reasonable approximation to the GD dose-lethality response relationship, at least between the 10th and 90th percentiles. It was also assumed that the slopes of the dose-response relationships for untreated, HI-6 treated, and MMB-4 treated monkeys from Task 89-12 could be approximated by a single slope. It was further assumed that the monkeys were drawn from a single population with no drift in response over time. The absence of a time drift in response permits the use of a stagewise dose allocation strategy, as opposed to testing all the animals simultaneously.

All oxime treatments were given in conjunction with 0.4 mg atropine free base per kg of animal body weight. For the sake of brevity and ease in presenting results, symbols are used for the various treatments.
Atropine/2-PAM therapy is abbreviated 2-PAM, atropine/MMB-4 is MMB-4, and atropine/HI-6 is HI-6.

3.5.1 Analysis of 48-Hr Lethality Data

The combined data from Task 89-12, from untreated monkeys of Task 85-18 of a previous contract, from monkeys treated with atropine and 2-PAM in Task 85-18, and from untreated monkeys of Task 87-34 of a previous contract were used to estimate 48-hr LD_{50} s for untreated monkeys and monkeys treated with either MMB-4, HI-6, or 2-PAM. Lethality results for each group of monkeys are presented in Table 1. A probit dose-response model in log GD dose was fitted to the 48-hr lethality results for six groups of monkeys: 85-18/untreated, 87-34/untreated, 89-12/untreated, 89-12/MMB-4, 89-12/HI-6, and 85-18/2-PAM. This model assumed that the GD dose-lethal response relations for the six groups of monkeys had a common slope but different intercepts. The historical data from Task 87-34 was included to increase the precision of the slope estimate, thereby increasing the precision of the 48-hr GD LD_{50} estimates for MMB-4, HI-6, and 2-PAM. Parameter estimates and results from fitting the common slope model to the 48-hr lethality data are summarized in Table 2. The estimate of the common slope is 11.42 with a standard deviation of 2.20. Based on the common slope model, the predicted lethality rate at 48 hr versus GD dose was plotted for untreated monkeys and for monkeys treated with MMB-4 or HI-6 in Task 89-12 and are presented in Figure 1.

In addition to fitting a common slope model to the combined 48-hr lethality data from the six groups of monkeys, separate probit models were fitted to each group. A hypothesis test of the adequacy of the common slope assumption was performed by comparing the fits from the common slope and separate slopes models. Based on the results of a log-likelihood ratio test, the null hypothesis of a common slope for the six groups of monkeys was not rejected at the 5 percent significance level. While this does not prove that the common slope assumption is correct, it does show that there is no evidence in the data contrary to the common slope assumption.

The estimated parameters from the common slope model were used to calculate the 48-hr GD LD_{15}, LD_{25}, and LD_{50} for each group of monkeys. Table 3 displays the estimated percentiles of the dose-response models for each group of monkeys. There are slight differences from previously reported LD_{50}s for
TABLE 1. 10-HR AND 48-HR LETHALITY, AND TIMES TO DEATH FOR SIX GROUPS OF MONKEYS: 85-18/UNTREATED, 87-34/UNTREATED, 89-12/UNTREATED, 89-12/MMB-4, 89-12/HI-6, 85-13/2-PAM

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<td>Lived</td>
<td>2,700</td>
</tr>
<tr>
<td>89-12</td>
<td>HI-6</td>
<td>1544</td>
<td>5.60</td>
<td>16.60</td>
<td>Lived</td>
<td>Lived</td>
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</tr>
<tr>
<td>89-12</td>
<td>HI-6</td>
<td>1591</td>
<td>5.20</td>
<td>18.30</td>
<td>Died</td>
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<td>18</td>
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<tr>
<td>89-12</td>
<td>HI-6</td>
<td>1900</td>
<td>4.40</td>
<td>21.50</td>
<td>Died</td>
<td>Lived</td>
<td>1,290</td>
</tr>
<tr>
<td>89-12</td>
<td>HI-6</td>
<td>1602</td>
<td>6.20</td>
<td>25.90</td>
<td>Died</td>
<td>Died</td>
<td>38</td>
</tr>
<tr>
<td>89-12</td>
<td>HI-6</td>
<td>1588</td>
<td>4.70</td>
<td>28.40</td>
<td>Died</td>
<td>Died</td>
<td>480</td>
</tr>
<tr>
<td>89-12</td>
<td>HI-6</td>
<td>1604</td>
<td>5.20</td>
<td>30.10</td>
<td>Died</td>
<td>Lived</td>
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</tr>
<tr>
<td>89-12</td>
<td>HI-6</td>
<td>1568</td>
<td>4.90</td>
<td>32.00</td>
<td>Died</td>
<td>Died</td>
<td>92</td>
</tr>
<tr>
<td>89-12</td>
<td>HI-6</td>
<td>1586</td>
<td>5.80</td>
<td>34.70</td>
<td>Died</td>
<td>Died</td>
<td>135</td>
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<tr>
<td>89-12</td>
<td>HI-6</td>
<td>1572</td>
<td>4.50</td>
<td>42.10</td>
<td>Died</td>
<td>Died</td>
<td>570</td>
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<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>4E9</td>
<td>3.40</td>
<td>19.90</td>
<td>Lived</td>
<td>Lived</td>
<td></td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>46R</td>
<td>2.70</td>
<td>20.00</td>
<td>Lived</td>
<td>Lived</td>
<td></td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>333D</td>
<td>4.40</td>
<td>20.10</td>
<td>Lived</td>
<td>Lived</td>
<td></td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>9E355</td>
<td>5.40</td>
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<td>Lived</td>
<td></td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>827C</td>
<td>4.30</td>
<td>21.80</td>
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<td>Lived</td>
<td></td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>405D</td>
<td>3.50</td>
<td>21.90</td>
<td>Lived</td>
<td>Lived</td>
<td></td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>673T</td>
<td>4.50</td>
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<td>Died</td>
<td>37</td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>964</td>
<td>3.20</td>
<td>21.90</td>
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<td>Died</td>
<td>60</td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>47A</td>
<td>4.20</td>
<td>22.50</td>
<td>Lived</td>
<td>Lived</td>
<td></td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>150T</td>
<td>4.30</td>
<td>22.80</td>
<td>Died</td>
<td>Died</td>
<td>206</td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>33X</td>
<td>4.0</td>
<td>23.5</td>
<td>Died</td>
<td>Died</td>
<td>33</td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>215R</td>
<td>5.0</td>
<td>23.9</td>
<td>Died</td>
<td>Died</td>
<td>60</td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>4E1</td>
<td>3.1</td>
<td>24.0</td>
<td>Died</td>
<td>Lived</td>
<td></td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>227B</td>
<td>4.9</td>
<td>24.1</td>
<td>Died</td>
<td>Died</td>
<td>113</td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>395D</td>
<td>4.3</td>
<td>24.3</td>
<td>Died</td>
<td>Died</td>
<td>201</td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>027D</td>
<td>4.4</td>
<td>24.4</td>
<td>Died</td>
<td>Died</td>
<td>78</td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>46V</td>
<td>3.2</td>
<td>24.5</td>
<td>Lived</td>
<td>Lived</td>
<td></td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>315D</td>
<td>3.8</td>
<td>25.4</td>
<td>Lived</td>
<td>Lived</td>
<td></td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>C603</td>
<td>4.6</td>
<td>25.9</td>
<td>Died</td>
<td>Died</td>
<td>142</td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>023B</td>
<td>3.8</td>
<td>26.2</td>
<td>Lived</td>
<td>Lived</td>
<td></td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>45S</td>
<td>2.7</td>
<td>26.2</td>
<td>Lived</td>
<td>Lived</td>
<td></td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>003D</td>
<td>4.6</td>
<td>26.4</td>
<td>Lived</td>
<td>Lived</td>
<td></td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>E42</td>
<td>3.1</td>
<td>26.6</td>
<td>Lived</td>
<td>Lived</td>
<td></td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>23J</td>
<td>3.2</td>
<td>28.4</td>
<td>Died</td>
<td>Died</td>
<td>55</td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>904C</td>
<td>3.6</td>
<td>29.2</td>
<td>Died</td>
<td>Died</td>
<td>355</td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>1200</td>
<td>4.7</td>
<td>30.0</td>
<td>Died</td>
<td>Died</td>
<td>480</td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>070D</td>
<td>4.0</td>
<td>30.0</td>
<td>Lived</td>
<td>Lived</td>
<td></td>
</tr>
<tr>
<td>85-18</td>
<td>2-PAM</td>
<td>830T</td>
<td>3.5</td>
<td>34.7</td>
<td>Died</td>
<td>Died</td>
<td>325</td>
</tr>
</tbody>
</table>

Times to death are approximated for some animals dying at later times when observations were not being made continuously.
### TABLE 2. PARAMETER ESTIMATES AND MODEL RESULTS FROM FITTING COMMON SLOPE PROBIT DOSE-RESPONSE MODEL TO THE 48-HR LETHALITY RESULTS FROM SIX GROUPS OF MONKEYS

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Weighted SS</th>
<th>Weighted MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>7</td>
<td>370,399.89</td>
<td>52,914.27</td>
</tr>
<tr>
<td>Residual</td>
<td>113</td>
<td>90.97</td>
<td>0.81</td>
</tr>
<tr>
<td>Uncorrected Total</td>
<td>120</td>
<td>370,490.86</td>
<td></td>
</tr>
<tr>
<td>(Corrected Total)</td>
<td>119</td>
<td>160,103.31</td>
<td></td>
</tr>
<tr>
<td>Sum of Loss</td>
<td></td>
<td>101.57</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter(a)</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>Asymptotic 95 Percent Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>11.42</td>
<td>2.20</td>
<td>7.07 - 15.77</td>
</tr>
<tr>
<td>B01</td>
<td>-8.48</td>
<td>2.62</td>
<td>-13.67 - -3.28</td>
</tr>
<tr>
<td>B02</td>
<td>-4.90</td>
<td>1.89</td>
<td>-8.64 - -1.17</td>
</tr>
<tr>
<td>B03</td>
<td>-3.88</td>
<td>1.74</td>
<td>-7.32 - 0.44</td>
</tr>
<tr>
<td>B04</td>
<td>-5.19</td>
<td>2.00</td>
<td>-9.16 - -1.22</td>
</tr>
<tr>
<td>B05</td>
<td>-7.98</td>
<td>2.61</td>
<td>-13.15 - -2.81</td>
</tr>
<tr>
<td>B06</td>
<td>-10.95</td>
<td>3.06</td>
<td>-17.01 - -4.90</td>
</tr>
</tbody>
</table>

(a) B1 is the estimate of the common slope, B01 is the estimate of the intercept for the untreated monkeys in Task 85-18, B02 is the estimate of the intercept for the untreated monkeys in Task 87-34, B03 is the estimate of the intercept for the untreated monkeys in Task 89-12, B04 is the estimate of the intercept for HMB-4 treated monkeys in Task 89-12, B05 is the estimate of the intercept for HI-6 treated monkeys in Task 89-12, and B06 is the estimate of the intercept for 2-PAM treated monkeys in Task 85-18.
FIGURE 1. COMPARISON OF PROBIT DOSE-RESPONSE MODELS ESTIMATED FOR 48 HR LETHALITY FOR MMB-4, HI-6, AND UNTREATED MONKEYS BASED ON COMMON SLOPE MODEL.
### Table 3. Estimated Percentiles for the Dose-Response Relationship Between GD Dose and 48-Hr Lethality Based on Common Slope Probit Model Fitted to Six Groups of Monkeys

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Task</th>
<th>n</th>
<th>48-Hr GD LD₅₀ Estimate (µg/kg)</th>
<th>95% C.I. (µg/kg)</th>
<th>48-Hr GD LD₅₀ Estimate (µg/kg)</th>
<th>95% C.I. (µg/kg)</th>
<th>48-Hr GD LD₅₀ Estimate (µg/kg)</th>
<th>95% C.I. (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>85-18</td>
<td>36</td>
<td>12.4</td>
<td>(10.3, 13.9)</td>
<td>15.1</td>
<td>(13.5, 16.9)</td>
<td>18.5</td>
<td>(16.6, 21.9)</td>
</tr>
<tr>
<td>Untreated</td>
<td>87-34</td>
<td>19</td>
<td>6.0</td>
<td>(5.0, 6.9)</td>
<td>7.4</td>
<td>(6.4, 8.6)</td>
<td>9.0</td>
<td>(7.8, 11.3)</td>
</tr>
<tr>
<td>Untreated</td>
<td>89-12</td>
<td>9</td>
<td>4.9</td>
<td>(3.8, 6.0)</td>
<td>6.0</td>
<td>(4.9, 7.4)</td>
<td>7.3</td>
<td>(6.0, 9.6)</td>
</tr>
<tr>
<td>HMB-4</td>
<td>89-12</td>
<td>12</td>
<td>6.4</td>
<td>(4.9, 7.8)</td>
<td>7.8</td>
<td>(6.3, 9.8)</td>
<td>9.5</td>
<td>(7.8, 12.6)</td>
</tr>
<tr>
<td>H1-6</td>
<td>89-12</td>
<td>16</td>
<td>11.2</td>
<td>(8.1, 14.3)</td>
<td>13.7</td>
<td>(10.4, 17.8)</td>
<td>16.7</td>
<td>(13.0, 22.6)</td>
</tr>
<tr>
<td>2-PAM</td>
<td>85-18</td>
<td>28</td>
<td>20.4</td>
<td>(17.3, 22.6)</td>
<td>24.9</td>
<td>(22.4, 27.9)</td>
<td>30.5</td>
<td>(27.3, 36.6)</td>
</tr>
</tbody>
</table>
some monkeys, since statistical analyses of LD\textsubscript{50}s in this report are based on GD doses calculated on weight differentials of syringes pre- and post-dosing, whereas some previous results have been based on target doses. The 48-hr GD LD\textsubscript{50} estimated for MMB-4 was determined to be statistically less than the 43-hr GD LD\textsubscript{50} estimated for HI-6 at the 5 percent significance level. Also, the 48-hr GD LD\textsubscript{50} estimated for untreated monkeys in Task 85-18 was statistically greater than the 48-hr GD LD\textsubscript{50} for untreated monkeys in Task 89-12.

Because the 48-hr GD LD\textsubscript{50} for untreated monkeys in Task 85-18 was determined to be statistically greater than the 48-hr GD LD\textsubscript{50} for untreated monkeys in Task 89-12, comparisons between monkeys treated with MMB-4, HI-6, or 2-PAM are best made from PRs. The PR for 2-PAM was calculated as the ratio of the 48-hr GD LD\textsubscript{50} estimated for 2-PAM treated monkeys in Task 85-18 to the 48-hr GD LD\textsubscript{50} estimated for untreated monkeys in Task 85-18. PRs for MMB-4 and HI-6 were calculated as the ratios of the 48-hr GD LD\textsubscript{50} estimated for MMB-4 and HI-6, respectively, in Task 89-12 to the 48-hr GD LD\textsubscript{50} estimated for untreated monkeys in Task 89-12. PRs and 95 percent confidence intervals are presented in Table 4 for MMB-4, HI-6, and 2-PAM. While the PRs estimated for HI-6 and 2-PAM were determined to be statistically greater than one (at the 5 percent significance level), the PR estimated for MMB-4 was determined to not be significantly different from one. Hypothesis tests were conducted to determine if the PRs determined for MMB-4, 2-PAM, and HI-6 based on 48-hr lethality were statistically different. Based on pairwise comparisons of the logarithms (ln) of the PRs, the results of the hypothesis tests may be summarized as follows:

1. The PR calculated for HI-6 was determined to be statistically (at the 5 percent significance level) greater than the PR calculated for MMB-4.

2. The PR calculated for HI-6 was determined to be statistically greater than the PR calculated for 2-PAM at the 10 percent significance level but not at the 5 percent significance level.

3. The PR calculated for MMB-4 was determined to not be statistically different from the PR calculated for 2-PAM.
TABLE 4. ESTIMATED PROTECTIVE RATIOS FOR 48-HR LETHALITY FOR MMB-4, HI-6, AND 2-PAM

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protective Ratio for 48-Hr Lethality</th>
<th>95 Percent C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMB-4</td>
<td>1.30</td>
<td>(0.98, 1.73)</td>
</tr>
<tr>
<td>HI-6</td>
<td>2.28</td>
<td>(1.67, 3.13)</td>
</tr>
<tr>
<td>2-PAM</td>
<td>1.65</td>
<td>(1.42, 1.91)</td>
</tr>
</tbody>
</table>

The dose-response relationships estimated between GD dose and 48-hr lethality for MMB-4, HI-6, or 2-PAM treated monkeys were used to calculate the lethality rate at two times the 48-hr GD LD₅₀ of untreated monkeys for each treatment. As shown in Table 5, the lethality rate calculated for HI-6 at two times the untreated 48-hr GD LD₅₀ was determined to be statistically less, at the 5 percent significance level, than the lethality rate calculated for MMB-4.

TABLE 5. PREDICTED 48-HR LETHALITY RATE AT TWO TIMES UNTREATED 48-HR GD LD₅₀ FOR MMB-4, HI-6, AND 2-PAM

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Task</th>
<th>Untreated 48-Hr GD LD₅₀ for Task</th>
<th>GD Dose</th>
<th>Estimated Lethality Rate at GD Dose</th>
<th>95 Percent C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMB-4</td>
<td>89-12</td>
<td>6.0</td>
<td>12.0</td>
<td>0.98</td>
<td>(0.79, 1.00)</td>
</tr>
<tr>
<td>HI-6</td>
<td>89-12</td>
<td>6.0</td>
<td>12.0</td>
<td>0.25</td>
<td>(0.03, 0.73)</td>
</tr>
<tr>
<td>2-PAM</td>
<td>85-18</td>
<td>15.1</td>
<td>30.3</td>
<td>0.83</td>
<td>(0.63, 0.95)</td>
</tr>
</tbody>
</table>
3.5.2 Analysis of 90-Min, 4-Hr, and 10-Hr Lethality Data

While most of the lethality observed in untreated animals or animals treated with MMB-4 in Task 89-12 occurred shortly after GD exposure, only four of the 11 lethality observed in animals treated with HI-6 occurred within 2 hr after GD exposure. This led to the conjecture on the ability of the oxime HI-6 to delay death. Consequently, 48-hr lethality may not be the endpoint best suited for discriminating between the efficacies of MMB-4, HI-6, and 2-PAM, and, therefore, lethality at other times was also investigated.

Probit dose-response models in log GD dose were fitted to the data from six groups of monkeys (85-18/untreated, 87-34/untreated, 89-12/untreated, 89-12/MMB-4, 89-12/HI-6, and 83-18/2-PAM) using both 90 min lethality and 4-hr lethality as the measured response. These models assumed that the dose-response relations for the six groups of monkeys had a common slope but different intercepts. There were strong indications, however, of a lack of fit of the common slope model for both the model fitted to the 90 min lethality data and the model fitted to the 4-hr lethality data, i.e., the hypothesis of a common slope for the dose-response relationships of the six groups of animals was rejected at the 5 percent significance level. Further analysis indicated that the lack of fit for both the 90-min and the 4-hr lethality data was due to the absence of a dose-response relationship between lethality at these times and GD dose for animals treated with HI-6 or 2-PAM.

Because it was not possible to estimate adequately the dose-response relationships for HI-6 and 2-PAM from either the 90-min or the 4-hr lethality data, the incidence of death was examined at alternative timepoints. Figure 2 displays a plot of the times to death of nonsurvivors in each group of monkeys. Results displayed in Figure 2 suggested that lethality at 10 hr might be better suited for discriminating among treatment efficacies of MMB-4, HI-6, and 2-PAM. Therefore, a probit dose-response model in log GD dose was fitted to 10-hr lethality data from the six groups of monkeys. This model assumed that the dose-response relations for the six groups of monkeys had a common slope but different intercepts. Ten-hr lethality data are displayed in the seventh column of Table 1, and parameter estimates and results from fitting the common slope model to these data are summarized in Table 5. The
FIGURE 2. TIMES TO DEATH OF NON-SURVIVORS FOR EACH GROUP OF MONKEYS

Time to Death (Minutes)
### Table 6. Parameter Estimates and Model Results from Fitting Common Slope Probit Dose-Response Model to the 10-Hr Lethality Results from Six Groups of Monkeys

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Weighted SS</th>
<th>Weighted MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>7</td>
<td>64,435.55</td>
<td>9,205.08</td>
</tr>
<tr>
<td>Residual</td>
<td>113</td>
<td>109.51</td>
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<tr>
<td>Uncorrected Total</td>
<td>120</td>
<td>64,545.06</td>
<td></td>
</tr>
<tr>
<td>(Corrected Total)</td>
<td>119</td>
<td>43,829.83</td>
<td></td>
</tr>
<tr>
<td>Sum of Loss</td>
<td></td>
<td>115.26</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter(s)</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>Asymptotic 95 Percent Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>8.24</td>
<td>1.64</td>
<td>4.99 to 11.49</td>
</tr>
<tr>
<td>B01</td>
<td>-4.90</td>
<td>1.97</td>
<td>-8.81 to -0.99</td>
</tr>
<tr>
<td>B02</td>
<td>-2.38</td>
<td>1.43</td>
<td>-5.22 to -0.46</td>
</tr>
<tr>
<td>B03</td>
<td>-1.84</td>
<td>1.36</td>
<td>-4.54 to -0.86</td>
</tr>
<tr>
<td>B04</td>
<td>-2.76</td>
<td>1.58</td>
<td>-5.89 to -0.37</td>
</tr>
<tr>
<td>B05</td>
<td>-5.64</td>
<td>2.21</td>
<td>-10.02 to 1.26</td>
</tr>
<tr>
<td>B06</td>
<td>-6.53</td>
<td>2.29</td>
<td>-11.07 to 2.00</td>
</tr>
</tbody>
</table>

(s) B1 is the estimate of the common slope, B01 is the estimate of the intercept for the untreated monkeys in Task 85-18, B02 is the estimate of the intercept for the untreated monkeys in Task 87-34, B03 is the estimate of the intercept for the untreated monkeys in Task 89-12, B04 is the estimate of the intercept for MMB-4 treated monkeys from Task 89-12, B05 is the estimate of the intercept for HI-6 treated monkeys in Task 89-12, and B06 is the estimate of the intercept for 2-PAM treated monkeys in Task 85-18.
estimate of the common slope is 8.24 with a standard deviation of 1.64. Based on the common slope model, predicted lethality rate at 10 hr versus GO dose was plotted for untreated monkeys and for monkeys treated with MMB-4 or HI-6, and are presented in Figure 3.

In addition to fitting a common slope model to the combined 10-hr lethality data from the six groups of monkeys, separate probit models were fitted to each group. A hypothesis test of the adequacy of the common slope assumption was performed by comparing the fits from the common slope and separate slopes models. Based on the results of a log-likelihood ratio test, the null hypothesis of a common slope for the six groups of monkeys was not rejected at the 5 percent significance level.

The estimated parameters from the common slope model were used to calculate the 10-hr GO LD_{50}, LD_{70}, and LD_{90} for each group of monkeys. Table 7 displays the estimated percentiles of the dose-response models for each group of monkeys. The 10-hr GO LD_{50} estimated for MMB-4 was determined to be statistically less than the 10-hr GO LD_{50} estimated for HI-6 at the 5 percent significance level. Because the 10-hr GO LD_{50} for untreated monkeys in Task 85-18 was determined to be statistically greater than the 10-hr GO LD_{50} for untreated monkeys in Task 89-12, comparisons between MMB-4, HI-6, and 2-PAM are best made from PRs. The PR for 2-PAM was calculated as the ratio of the 10-hr GO LD_{50} estimated for 2-PAM in Task 85-18 to the 10-hr GO LD_{50} estimated for untreated monkeys in Task 85-18. PRs for MMB-4 and HI-6 were calculated as the ratio of the 10-hr GO LD_{50} estimated for MMB-4 and HI-6, respectively, in Task 89-12 to the 10-hr GO LD_{50} estimated for untreated monkeys in Task 89-12. PRs and 95 percent confidence intervals are presented in Table 8. While the PRs estimated for HI-6 and 2-PAM were determined to be statistically greater than one (at the 5 percent significance level), the PR estimated for MMB-4 was determined to not be significantly different from one. Hypothesis tests were conducted to determine if the PRs determined for MMB-4, 2-PAM, and HI-6 based on 10-hr lethality were statistically different. Based
FIGURE 3. COMPARISON OF PROBIT DOSE-RESPONSE MODELS ESTIMATED FOR 10 HR LEATHALITY FOR MBB-4, HI-6, AND UNTREATED MONKEYS BASED ON COMMON SLOPE MODEL.
TABLE 7. ESTIMATED PERCENTILES FOR THE DOSE-RESPONSE RELATIONSHIP BETWEEN GD DOSE AND 10-HR LETHALITY BASED ON COMMON SLOPE PROBIT MODEL FITTED TO SIX GROUPS OF MONKEYS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Task</th>
<th>n</th>
<th>10-Hr GD LD_{10} Estimate (µg/kg)</th>
<th>95% C.I. (µg/kg)</th>
<th>10-Hr GD LD_{50} Estimate (µg/kg)</th>
<th>95% C.I. (µg/kg)</th>
<th>10-Hr GD LD_{95} Estimate (µg/kg)</th>
<th>95% C.I. (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>85-18</td>
<td>36</td>
<td>12.0</td>
<td>(9.5, 13.9)</td>
<td>15.9</td>
<td>(13.7, 18.5)</td>
<td>21.0</td>
<td>(18.1, 24.1)</td>
</tr>
<tr>
<td>Untreated</td>
<td>87-34</td>
<td>19</td>
<td>6.0</td>
<td>(4.7, 7.1)</td>
<td>7.9</td>
<td>(6.6, 9.8)</td>
<td>10.4</td>
<td>(8.6, 14.4)</td>
</tr>
<tr>
<td>Untreated</td>
<td>89-12</td>
<td>9</td>
<td>5.1</td>
<td>(3.7, 6.7)</td>
<td>6.8</td>
<td>(5.2, 9.2)</td>
<td>8.9</td>
<td>(6.8, 13.5)</td>
</tr>
<tr>
<td>MMB-4</td>
<td>89-12</td>
<td>12</td>
<td>6.6</td>
<td>(4.7, 8.6)</td>
<td>8.7</td>
<td>(6.7, 11.6)</td>
<td>11.5</td>
<td>(8.9, 16.8)</td>
</tr>
<tr>
<td>III-6</td>
<td>89-12</td>
<td>16</td>
<td>14.8</td>
<td>(10.1, 19.2)</td>
<td>19.5</td>
<td>(14.6, 25.7)</td>
<td>25.8</td>
<td>(19.9, 36.2)</td>
</tr>
<tr>
<td>2-PAM</td>
<td>85-18</td>
<td>28</td>
<td>19.0</td>
<td>(15.1, 21.9)</td>
<td>25.1</td>
<td>(21.8, 29.2)</td>
<td>33.1</td>
<td>(28.6, 42.9)</td>
</tr>
</tbody>
</table>
TABLE 8. ESTIMATED PROTECTIVE RATIOS FOR 10-HR LETHALITY FOR MMB-4, HI-6, AND 2-PAM

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protective Ratio for 10-Hr Lethality</th>
<th>95 Percent C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMB-4</td>
<td>1.30</td>
<td>(0.90, 1.87)</td>
</tr>
<tr>
<td>HI-6</td>
<td>2.89</td>
<td>(1.98, 4.21)</td>
</tr>
<tr>
<td>2-PAM</td>
<td>1.58</td>
<td>(1.30, 1.91)</td>
</tr>
</tbody>
</table>

On pairwise comparisons of the ln of the PRs, the results of the hypothesis tests conducted at the 5 percent significance level may be summarized as follows:

(1) The PR calculated for HI-6 was determined to be statistically greater than the PRs calculated for MMB-4 and 2-PAM.

(2) The PR calculated for MMB-4 was determined to not be statistically different from the PR calculated for 2-PAM.

The dose-response relationships estimated between GD dose and 10-hr lethality for MMB-4, HI-6, and 2-PAM treated monkeys were used to calculate the lethality rate at two times the untreated 10-hr GD LD₅₀ for each treatment. As shown in Table 9, the lethality rate calculated for HI-6 at two times the untreated 10-hr GD LD₅₀ was determined to be statistically less, at the 5 percent significance level, than the lethality rates calculated for MMB-4 and 2-PAM.
3.5.3 Analysis of the Times to Death

While the analysis of the 48-hr lethality data provided only indications that HI-6 was more efficacious than 2-PAM, the PR estimated for HI-6 was determined to be statistically greater than the PR estimated for 2-PAM based on 10-hr lethality data. It is of interest, therefore, to compare the times to death for MMB-4, HI-6, and 2-PAM treated monkeys. Times to death for the animals studied in these tasks are displayed in the last column of Table 1. Times to death were treated as right censored at 48 hr for animals that survived the 48-hr observation period. This means that the time to death was not known but would have been greater than or equal to the assigned value if the observation period had been longer. Natural ln of the times to death were modeled as a linear function of the ln of normalized GD doses for MMB-4, HI-6, and 2-PAM. Because the 48-hr GD LD$_{50}$ for untreated monkeys in Task 85-18 was statistically greater than the 48-hr GD LD$_{50}$ for untreated monkeys in Task 89-12, GD doses were normalized by the 48-hr GD LD$_{50}$: GD doses were divided by 15.1 μg/kg for animals treated with 2-PAM in Task 85-18, and GD doses were divided by 6.0 μg/kg for animals treated with MMB-4 or HI-6 in Task 89-12. This model assumed that the relationships between ln time to death and ln normalized GD dose had a common slope but different intercepts.
Parameter estimates and results from fitting the common slope model to this data are summarized in Table 10.

### Table 10. Parameter Estimates and Model Results for LN-LN Regression Model Between Times to Death and GD Dose

<table>
<thead>
<tr>
<th>Variable</th>
<th>DF</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>Chi Square</th>
<th>P&gt;Chi</th>
<th>Label/Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>301</td>
<td>1</td>
<td>8.70</td>
<td>1.07</td>
<td>66.4</td>
<td>0.0001</td>
<td>MMB-4 Intercept</td>
</tr>
<tr>
<td>802</td>
<td>1</td>
<td>12.11</td>
<td>1.69</td>
<td>51.5</td>
<td>0.0001</td>
<td>HI-6 Intercept</td>
</tr>
<tr>
<td>803</td>
<td>1</td>
<td>9.64</td>
<td>0.851</td>
<td>128.3</td>
<td>0.0001</td>
<td>2-PAM Intercept</td>
</tr>
<tr>
<td>81</td>
<td>1</td>
<td>-4.38</td>
<td>1.17</td>
<td>14.0</td>
<td>0.0002</td>
<td>Slope Coefficient</td>
</tr>
<tr>
<td>σ</td>
<td>1</td>
<td>2.58</td>
<td>0.363</td>
<td></td>
<td></td>
<td>Standard deviation of times to death</td>
</tr>
</tbody>
</table>

The estimated parameters of the ln-ln regression model were used to predict the time to death at a normalized GD dose of 2 for each treatment, and these values are displayed in Table 11 for MMB-4, HI-6, and 2-PAM. Statistical hypothesis tests were conducted to determine if the predicted times to death at 2 normalized GD doses were significantly different for the three treatments. Based on pairwise comparisons of the ln of the predicted times to death, the results of the hypothesis tests may be summarized as follows:
(1) The predicted time to death at a normalized GD dose of 2 for HI-6 was determined to be statistically greater than the predicted time for MMB-4 at the 5 percent significance level.

(2) The statistical significance of the difference between predicted times to death at 2 normalized GD doses for HI-6 and 2-PAM was marginal: the observed significance level was calculated to be 0.052.

(3) The difference between predicted times to death at 2 normalized GD doses for MMB-4 and 2-PAM was determined to not be statistically significant.

TABLE 11. PREDICTED TIMES TO DEATH AT TWO TIMES UNTREATED 48-HR GD LD₅₀ FOR MMB-4, HI-6, AND 2-PAM

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GD Dose</th>
<th>Normalized GD Dose</th>
<th>Predicted Time to Death (min)</th>
<th>95 Percent C.I. (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atr/MMB-4</td>
<td>12.0</td>
<td>2</td>
<td>290</td>
<td>(55, 1,500)</td>
</tr>
<tr>
<td>Atr/Hi-6</td>
<td>12.0</td>
<td>2</td>
<td>8,700</td>
<td>(1,200, 65,000)</td>
</tr>
<tr>
<td>Atr/2-PAM</td>
<td>30.3</td>
<td>2</td>
<td>730</td>
<td>(230, 2,400)</td>
</tr>
</tbody>
</table>

3.5.4 Analyses of Clinical Signs Data

The procedures used for monitoring clinical signs were modified from those used in Task 85-18, and therefore only the 37 monkeys tested in Task 89-12 were included in the analysis of clinical signs. The objective of the analysis of the clinical signs was to determine if statistically significant differences in onset and/or duration of sublethal responses in monkeys treated with MMB-4 and HI-6 existed. Each animal was continuously monitored for clinical signs of GD intoxication during the first 4 hr following GD challenge, and at decreasing frequencies during the following intervals thereafter: 4-6 hr, 6-8 hr, 8-12 hr, 12-24 hr, 24-36 hr, and 36-48 hr.
Clinical signs compiled for statistical analyses were onset and cessation times of tremors, convulsions, and prostration. Annotated clock times from the clinical signs observation sheets, and letter codes designating the observation interval in which events were observed are given in Table 12 for time to death, and onset and cessation of tremors, convulsions, and prostration. Because observations were not made continuously after the first 4 hr, the length of the observation period used in statistical analyses for cessation of tremors and convulsions was restricted to the first 4 hr following GD injection. Because of similarities in response across treatment groups, onset and cessation times of prostration for animals that died within 48 hr were not used in the analyses and are not shown in Table 12. Table 13 displays numbers of animals in various time categories for times to death and times to onset of tremors, convulsions, and prostration. For those animals that exhibited tremors or convulsions, the majority of onset times occurred between 0 and 15 min, and because of the large number of animals that died prior to 48 hr, the amount of data for onset of prostration was limited. Frequency counts did not reveal any potential treatment group effects for these data, and, therefore, no further statistical analyses were done for the onset times.

The clinical signs chosen for statistical modeling and analyses were durations of tremors, convulsions, and prostration. When both onset and cessation times were noted, duration was calculated as the difference between the cessation and onset times using the annotated clock times. Clock times were not recorded for cessation of tremors, so midpoints of the time interval in which tremors were last observed were used to calculate duration of tremors. If a clinical sign was not exhibited during the observation period and the animal lived throughout the observation period, then the duration was set equal to zero. Durations were treated as right censored, i.e., durations were set equal to the length of the observation period minus the onset time for animals that exhibited the sign throughout the observation period (animal lived) and were set equal to the end of the observation period in which animals died minus the onset time for nonsurvivors.
<table>
<thead>
<tr>
<th>Table 12. Clinical Observations for Onset and Cessation of Tremors, Convulsions, and Prostration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
</tr>
<tr>
<td>00-1060</td>
</tr>
<tr>
<td>00-1045</td>
</tr>
<tr>
<td>00-1039</td>
</tr>
<tr>
<td>00-1043</td>
</tr>
<tr>
<td>00-1020</td>
</tr>
<tr>
<td>00-1078</td>
</tr>
<tr>
<td>00-1044</td>
</tr>
<tr>
<td>00-1070</td>
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<tr>
<td>00-1033</td>
</tr>
<tr>
<td>00-1030</td>
</tr>
<tr>
<td>00-1046</td>
</tr>
<tr>
<td>00-1010</td>
</tr>
<tr>
<td>00-1054</td>
</tr>
<tr>
<td>00-1051</td>
</tr>
<tr>
<td>00-1060</td>
</tr>
<tr>
<td>00-1032</td>
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<td>00-1064</td>
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<td>00-1079</td>
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<td>00-1054</td>
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<td>00-1006</td>
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<td>00-1072</td>
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<td>00-1026</td>
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<tr>
<td>00-1039</td>
</tr>
<tr>
<td>Monkey ID Number</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>09-1574</td>
</tr>
<tr>
<td>09-1572</td>
</tr>
<tr>
<td>09-1520</td>
</tr>
</tbody>
</table>

**LETTER CODE DESIGNATION** (event occurred within this time period after dosing)

A: 0-15 min  F: 15-30 min  E: 30-45 min  P: 60-90 min  L: 120-180 min  Q: 24 hr (24-30 hr)
B: 15-30 min  G: 30-60 min  J: 180-210 min  M: 6 hr (6-8 hr)  Z: 48 hr (48-60 hr)
C: 30-45 min  H: 60-120 min  K: 6 hr (6-8 hr)  Z: 48 hr (48-60 hr)

*Animal remained prostrate beyond 48 hr.*
### TABLE 13. SUMMARY OF ONSET TIMES FOR TREMORS, CONVULSIONS, AND PROSTRATION

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Number Survived &gt;4 hr</th>
<th>Number Survived &gt;48 hr</th>
<th>Time to Tremors 0-15 min</th>
<th>Time to Tremors 15 min-4 hr</th>
<th>Tremors Not Observed in 4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>9</td>
<td>6</td>
<td>5</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>MMB-4</td>
<td>12</td>
<td>6</td>
<td>5</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>HI-6(1)</td>
<td>16</td>
<td>10</td>
<td>5</td>
<td>16</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Time to Convulsions 0-15 min</th>
<th>Time to Convulsions 15 min-4 hr</th>
<th>Convulsions Not Observed in 4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>MMB-4</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>HI-6(1)</td>
<td>13</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Time to Prostratin 0-15 min</th>
<th>Time to Prostratin 15 min-48 hr</th>
<th>Prostratin Not Observed in 48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>MMB-4</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>HI-6</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

(1) Animal #89-1632 died prior to 4 hr without exhibiting convulsions.
Regression models were fitted to the duration data for tremors, convulsions, and prostration using duration as the response variable and ln GD dose and treatment group as the independent variables. For each sign, regression models were fitted both to the data from all the animals and to the data from survivors only. Table 14 summarizes the results for statistical models fitted to the duration of clinical signs data. For each fitted model, the estimated slope of the regression model and its standard error are displayed in the third column of the table. Slopes determined to be statistically different from zero at the 5 percent significance level are noted by asterisks. A hypothesis test was conducted for each fitted model to determine if any statistically significant differences existed between the duration times for the three groups of monkeys based on the fitted model. Outcomes of the hypothesis tests are shown in the fourth column of the table. The estimated parameters of the regression models were used to predict the average duration times of signs for a GD dose of 12.0 µg/kg (two times the 43-hr LD₅₀ for untreated monkeys of Task 89-12) for each treatment. The predicted group means together with 95 percent confidence intervals are shown in the last three columns of Table 14.

When models were fitted to the data from all the animals, the estimated regression slope was significantly greater than zero for all three signs. That is, durations of tremors, convulsions, and prostration were predicted to increase with increasing GD dose. Also, the durations of convulsions and prostration showed significant differences among the three groups of monkeys. Mean duration times predicted for animals given 12.0 µg/kg GD exhibited the same relative ordering among the treatment groups for each clinical sign: mean duration time predicted for HL-6 treated animals was less than the mean duration time predicted for HMB-4 treated animals which was less than the mean duration time predicted for untreated animals.

When the models were fitted to the data from survivors only, estimated regression slopes were not statistically significantly different from zero for any of the three signs. In fact, estimated slopes were negative for durations of tremors and convulsions, suggesting that duration times decrease with increasing GD dose. The large standard errors associated with these negative slopes, however, imply that this conclusion may not be...
<table>
<thead>
<tr>
<th>Clinical Sign</th>
<th>Analyzed</th>
<th>Slope (SE)</th>
<th>Group Effect&lt;sup&gt;(b)&lt;/sup&gt; (p-value)</th>
<th>Predicted Mean Durations with 95 Percent Confidence Limits at 12.0&lt;sup&gt;(a)&lt;/sup&gt; μg/kg GD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Untreated</td>
</tr>
<tr>
<td>Duration of Tremors</td>
<td>All</td>
<td>181&lt;sup&gt;*&lt;/sup&gt; (58.6)</td>
<td>No (0.187)</td>
<td>257 (125, 390)</td>
</tr>
<tr>
<td>Duration of Convulsions</td>
<td>All</td>
<td>204&lt;sup&gt;*&lt;/sup&gt; (57)</td>
<td>Yes (0.036)</td>
<td>267 (139, 396)</td>
</tr>
<tr>
<td>Duration of Prostration</td>
<td>All</td>
<td>5,960&lt;sup&gt;*&lt;/sup&gt; (1,410)</td>
<td>Yes (0.002)</td>
<td>6,840 (4,190, 9,500)</td>
</tr>
<tr>
<td>Duration of Tremors</td>
<td>4 hr Survivors Only</td>
<td>-4.06 (22.1)</td>
<td>No (0.103)</td>
<td>21.4 (0, 71.8)</td>
</tr>
<tr>
<td>Duration of Convulsions</td>
<td>4 hr Survivors Only</td>
<td>26.3 (20.3)</td>
<td>No (0.992)</td>
<td>38.5 (0, 84.9)</td>
</tr>
<tr>
<td>Duration of Prostration</td>
<td>Survivors Only</td>
<td>-521 (825)</td>
<td>No (0.31)</td>
<td>330 (0, 1,860)</td>
</tr>
</tbody>
</table>

<sup>(a)</sup> 12.0 μg/kg = 2 * 6.0 μg/kg, where 6.0 μg/kg is the 48-hr GD LD<sub>50</sub> for the Task 89-12 untreated monkeys as estimated by the common-slope probit model for six groups of monkeys.

<sup>(b)</sup> Yes indicates that there were statistically significant differences between the duration times for the three groups of monkeys.

* The slope was determined to be statistically different from 0 at the 5 percent significance level.
warranted. Treatment group effects were determined to be statistically insignificant for any of the three signs. Confidence intervals for the mean duration times predicted for each treatment group were very wide, with considerable overlap among the treatment groups.

In summary, when the regression models were fitted to the data from all animals, the trends estimated between duration times and GD dose and treatment group were similar for all three clinical signs. Furthermore, the patterns of association between duration times and GD dose and treatment group strongly resembled those estimated from the 48-hr lethality data. Specifically, these results are:

1. Durations were significantly related to GD dose with durations predicted to increase with higher GD doses.
2. Durations differed significantly among treatment groups; mean durations were shorter for HI-6 treated animals than for MMB-4 treated animals, which were in turn shorter than for untreated animals.

When the regression models were fitted to the data from the survivors only, durations were not statistically significantly associated with either GD dose or treatment group. The dissimilarity between the results based on all the data and those based on survivors only indicate that the data from nonsurvivors strongly influenced the outcome of models fitted to both survivors and nonsurvivors combined.

4.0 CONCLUSIONS

Dose-response experiments were carried out in Task 89-12 to determine the 48-hr GD LD₅₀ for untreated rhesus monkeys and monkeys treated with atropine/HI-6 or atropine/MMB-4. The 48-hr GD LD₅₀ was estimated to be 6.0 μg/kg in untreated monkeys, 7.8 μg/kg in atropine/MMB-4 treated monkeys, and 13.7 μg/kg in animals treated with atropine/HI-6. Based on the 48-hr lethality data, HI-6 was determined to be statistically significantly more effective than MMB-4 in treating GD intoxication. PRs estimated from 48-hr lethality data for atropine/MMB-4 and atropine/HI-6 treated monkeys were...
statistically compared to the PR in monkeys for atropine/2-PAM estimated from the 48-hr lethality data of Task 85-18. While the PR calculated for HI-6 was larger than the PR calculated for 2-PAM, the difference was determined to not be statistically significant at the 5 percent significance level.

Times to death observed for animals treated with atropine/HI-6 were substantially longer than the times to death observed in untreated animals or animals treated with atropine/MMB-4 in Task 89-12. Therefore, dose-response models were fitted to the lethality data using a range of timepoints. Based on lethality at 10-hr data, atropine/HI-6 was determined to be statistically (5 percent significance level) more effective than atropine/MMB-4 in treating GD intoxication. PRs estimated from the 10-hr lethality data for MMB-4 and HI-6 were statistically compared to the PR estimated from the 10-hr lethality data in Task 85-18 for atropine/2-PAM. The PR calculated from the 10-hr lethality data for atropine/HI-6 was statistically greater (at the 5 percent significance level) than the PRs calculated from 10-hr lethality data for atropine/MMB-4 and atropine/2-PAM.

Durations of tremors, convulsions, and prostration were determined to be statistically significantly related to GD dose only when the analysis utilized results from both survivors and nonsurvivors. Trends and comparisons derived from models fitted to the duration data mirrored results from analysis of the time to death data. When the models were refitted to data using only the survivors, duration of tremors, convulsions, and prostration were determined to not be statistically significantly related to either GD dose or treatment group.

Twenty-five monkeys were necropsied: seven injected with GD and given no treatment, seven injected with GD and treated with atropine/MMB-4, and 11 treated with atropine/HI-6. Only three monkeys which survived more than 48 hr following GD injection were sacrificed in moribund condition, and none of these three monkeys had received any therapy. No gross lesions were usually detected, which is compatible with death or moribundity due to GD intoxication. Neither atropine/MMB-4 nor atropine/HI-6 therapy appeared to alter the gross appearance of organs or tissues from that seen in monkeys exposed to GD and given no treatment.
5.0 RECORD ARCHIVES

Forty-seven monkeys for use in Task 89-12 arrived at Battelle on February 13, 1990. LD_{50} studies were run between March 20 and April 4, 1990. Oxime efficacy study dosing occurred from April 17 to June 12, 1990. Records pertaining to the conduct of this study are contained in Battelle laboratory record books which are specific for this task. These record books are clearly labeled as to contents of each volume and include pre-study animal quarantine and observation records as well as all study data. These records and the final report will be maintained at the MREF until acceptance of the final report by the U.S. Army. At that time, records will be forwarded to the U.S. Army or archived at Battelle. Agent and oxime dosing solutions are unstable under prolonged storage and have been destroyed. Slides of tissue samples taken at necropsies will be sent to the Army or maintained at Battelle.

6.0 ACKNOWLEDGMENTS

The names, titles, and degrees of the principal contributors to this study are listed below:

<table>
<thead>
<tr>
<th>Name</th>
<th>Title</th>
<th>Degree</th>
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</thead>
<tbody>
<tr>
<td>Dr. Garrett S. Dill</td>
<td>Principal Investigator</td>
<td>D.V.M.</td>
</tr>
<tr>
<td>Dr. Carl T. Olson</td>
<td>Study Director</td>
<td>D.V.M., Ph.D.</td>
</tr>
<tr>
<td>Dr. Ronald G. Menton</td>
<td>Study Statistician</td>
<td>Ph.D.</td>
</tr>
<tr>
<td>Ms. Robyn C. Aizer</td>
<td>Study Supervisor</td>
<td>B.S.</td>
</tr>
<tr>
<td>Ms. M. Claire Matthews</td>
<td>Statistician</td>
<td>M.A.</td>
</tr>
<tr>
<td>Mr. Timothy L. Hayes</td>
<td>Study Chemist</td>
<td>B.A.</td>
</tr>
<tr>
<td>Dr. Allen W. Singer</td>
<td>Study Pathologist</td>
<td>D.V.M.</td>
</tr>
<tr>
<td>Dr. Peter L. Jepsen</td>
<td>Study Veterinarian</td>
<td>D.V.M.</td>
</tr>
</tbody>
</table>

There are a number of people who made performance of this task possible. Their invaluable assistance is gratefully acknowledged by the authors. Among the many are: James Arp and Sheri Moore for chemical analyses; Michael Hingson for supervision and daily preparations; Kandy Audet,

7.0 REFERENCES


APPENDIX A

MREF PROTOCOL 56
Characterization of Soman Toxicity in Atropine and Oxime (HI-6 and MM8-4) Treated Rhesus Monkeys

Study Performed by Battelle Memorial Institute
505 King Avenue, Columbus, Ohio 43201-2693

1. Study Director: Carl T. Olson, D.V.M., Ph.D.
2. Program Director: Garrett S. Dill, D.V.M.
3. Statistician: Ronald G. Menton, Ph.D.
4. Pathologist: Allen W. Singer, D.V.M.
5. Study Veterinarian: Peter L. Jepsen, D.V.M.
6. Sponsor: U.S. Army Medical Research and Development Command (USAMRDC)

8. Introduction:

There is a need for improved antidotes, specifically oximes, for treatment of exposure to organophosphate (OP) chemical warfare agents. Although pralidoxime chloride (2-PAM), the standard therapeutic oxime, is reportedly effective against exposure to the nerve agents Sarin (GB) and VX, it is only marginally effective against Soman (GD) and Tabun (GA) exposure. HI-6 and MM8-4 are two oximes most likely to be developed over the next year as replacements for 2-PAM. The decision as to whether to use either of these oximes as a replacement for 2-PAM is critically dependent on the demonstration of improved efficacy against GD intoxication in studies with nonhuman primates.

9. Objective:

The objective of this study is to characterize in Rhesus monkeys the toxicity of GD when exposure is followed by treatment with atropine and either of the two candidate oximes, HI-6 or MM8-4, on the basis of lethality, morbidity, and pathology. Treatment will be administered at a fixed time after GD injection using a dose of atropine (as the citrate salt) of 0.2 mg (or as used in Task 89-08) atropine free base and 100 μmol of candidate oxime per kilogram of body weight. This study is conducted under the Good Laboratory Practice (GLP) guidelines of the Food and Drug Administration (FDA).
10. Experimental Design:

A. Test System

(1) Animals - Male Rhesus monkeys, *Macaca mulatta*, of Indian origin were specified for this study because there is considerable scientific evidence that the monkey is predictive of responses in man. Rhesus monkeys of Indian origin were selected because the majority of work in this area has been done with monkeys of Indian origin and because there is evidence that Rhesus monkeys of Chinese origin respond somewhat differently to these study conditions than those of Indian origin. Monkeys for use in this study will be provided by USAMRICD. Experiments are conducted in a stage-wise fashion to limit the number of animals used to the minimum necessary to achieve statistically valid results. Monkeys are observed for 7 days following exposure. Discomfort and injury of animals are limited to that which is unavoidable in the conduct of scientifically valuable research. If, in the opinion of the Study Veterinarian or the Study Director, a monkey appears to be in a moribund state and in pain, that animal will be euthanatized with sodium pentobarbital or as specified in Section 10.E. Anesthetics, analgesics, or tranquilizers cannot be used for the relief of pain or anxiety in these studies because they would interfere with the biological effects of the challenge agent or test compounds. External stimuli and manipulation are minimized to decrease any associated anxiety. Protocols of all experiments using animals are reviewed and approved by Battelle's Institutional Animal Care and Use Committee (IACUC) prior to initiation of the study. The Program Director accepts responsibility for the proper care and use of animals in the conduct of research described in protocols.

(2) Initial Weight - Monkeys placed on study weigh between approximately 2 and 5 kg.

(3) Quarantine - All primates received at Battelle undergo at least a 1 month quarantine period. All animals are examined by the Study Veterinarian within one week of arrival at Battelle. Blood samples are taken for hematology and serum chemistries. Fecal samples are taken for parasite infestation evaluation. Three tests for the presence of tuberculosis are performed by injecting tuberculin intradermally in the palpebral skin at 2 week or longer intervals.
(4) Animal Selection - Based on physical examinations and clinical laboratory findings, acceptable animals are identified by the Study Director and Study Veterinarian. These animals are randomized, based on body weight, to obtain homogeneity of weight across treatment groups.

(5) Animal Identification - Animals are received with tattoos. If a monkey arrives without a tattoo or with an identification number that duplicates another animal's, a new tattoo will be applied.

(6) Housing - Monkeys are housed individually in stainless-steel cages, approximately 24 inches wide, 34 inches high, and 26 inches deep, with automatic watering systems.

(7) Acclimation - Prior to the start of each exposure, monkeys are acclimated to placement on a slotted, V-shaped platform where arms and legs can be restrained by means of lanyards. This is used when obtaining body weights and blood samples (femoral venipuncture) and for restraint when injections are made.

(8) Lighting - Fluorescent lighting is used with a light/dark cycle of 12 hr each per day.

(9) Temperature - Monkey room temperatures are maintained at 77 ± 5 F.

(10) Humidity - Relative humidity of monkey rooms is maintained at 50 ± 10 percent.

(11) Diet - Purina certified monkey chow biscuits are fed twice daily and are periodically supplemented with fresh fruit. No contaminants that would interfere with the results of the study are known to be present in the feed. Analyses of the feed can be obtained from Purina.

(12) Water - Water is supplied from the Battelle water system and given ad libitum through automatic watering systems. No contaminants that would interfere with the results of the study are known to be present in the water. Water is analyzed quarterly for potability and annually for contaminants.

(13) Battelle's Animal Resources Facilities have been registered with the U.S. Department of Agriculture (USDA) as a Research Facility (Num: J1-21) since August 14, 1967, and are periodically inspected in accordance with the provisions of the Federal Animal Welfare Act. In addition, animals for use in research are obtained only from laboratory animal suppliers duly licensed.
by the USDA. Battelle's statement of assurance regarding the
Department of Health and Human Services (DHHS) policy on humane
care of laboratory animals was accepted by the Office of
Protection from Research Risks, National Institutes of Health on
August 27, 1973. Animals at Battelle are cared for in
accordance with the guidelines set forth in the "Guide for the
Care and Use of Laboratory Animals" (DHHS Publication No. (NIH)
85-23) and/or in the regulations and standards as promulgated by
the Agricultural Research Service, USDA, pursuant to the
Laboratory Animal Welfare Act of August 24, 1966 as amended

(14) On January 31, 1978, Battelle received full accreditation of its
animal care programs and facilities from the American
Association for Accreditation of Laboratory Animal Care
(AAALAC). Battelle's full accreditation status has been renewed
after every inspection since the original accreditation. The
MREF is a part of the facilities granted full accreditation.

B. Test Material

(1) Treatment Compounds - Treatment compounds, atropine, HI-6, and
MK-44-4, are provided by USAMRICD. Identity and determination of
concentration of atropine solutions are performed at Battelle.
No identity or purity confirmations will be accomplished on HI-6
or MK-44-4 at Battelle.

(2) Chemical Agent - GD is supplied by USAMRICD. Purity,
appropriate identification (batch number, lot number, state),
and stability data are provided by USAMRICD. Purity and
stability of agent stored at Battelle is periodically confirmed
by Battelle personnel.

(3) Surety, security, and safety procedures for the use of chemical
agents are thoroughly outlined in facility plans, in personnel
requirements for qualification to work with chemical surety
material (CSM), and in standard operating procedures for storage
use of CSM.

C. Test Groups

(1) Initial Tests to Confirm LD_{50}

No more than 5 monkeys are used to approximate the 48 hr GD LD_{50}
in animals given no treatment. This is accomplished in a
modified up-down type experiment, challenging 1 or 2 monkeys per
day. If after 1 or more monkeys have been challenged, the
estimated GD LD₅₀ in this study falls within the 95 percent confidence limits of the historic Battelle GD LD₅₀ in Indian Rhesus monkeys, the historic LD₅₀ will be accepted for this group of animals.

(2) Treatment Efficiency of HI-6 or MMG-4 Given in Conjunction with Atropine

Using varying doses of GD, as recommended by the study statistician, groups of equal numbers of monkeys will be challenged with GD and treated with atropine and 100 μmol of either HI-6 or MMG-4 per kilogram body weight at a time after exposure provided by the Sponsor Monitor. GD will be injected in the right leg in the posterior tibial area in the region of the gastrocnemius muscle at a site clipped of hair and pre-marked. Atropine and oximes will be injected intramuscularly in succession at separate sites 2 to 3 cm distant from each other in the Quadriceps femoris muscle of the left leg. Atropine injections will be made with a solution containing approximately 2.3 mg free base/mL. HI-6 is injected at a volume of 0.1 mL/kg using a water solution containing 378 mg of HI-6/mL. MMG-4 is injected at 0.1 mL/kg using a water solution containing 329 mg of MMG-4/mL. Syringes used for dosing will be Hamilton microliter syringes of the smallest compatible volume (Syringes are filled to no more than 95 percent of labeled volume.) to obtain maximum accuracy in the measurement of delivered dose. Individual, labeled syringes are loaded with the calculated volume of GD prior to the start of dosing, weighed and placed on ice until used. After dosing is accomplished, syringes are weighed again to determine the weight loss and calculate the volume delivered. Pre- and post-weighing of syringes will also be accomplished with those used for dosing atropine, HI-6, and MMG-4. On every day of dosing, samples of the GD stock used are taken and chemically analyzed by gas chromatography to confirm expected GD concentration of the dosing solution.

Monkeys are returned to their individual cages after treatments have been completed and are closely and continuously observed for the first 4 hr following dosing and at intervals thereafter with observations annotated at 6, 8, 12, 24, 36, and 48 hr and daily thereafter to the 7th day. Signs specifically monitored include muscle fasciculations, tremors, convulsions, prostration, salivation/bronchial discharge, miosis/mydriasis, uncoordinated movements, and death.
Brain specimens to be embedded separately for histopathology are the frontal cortex, entorhinal cortex, basal ganglia, hippocampus, thalamus, midbrain, pons, medulla, and cerebellum. Trimmed specimens are processed using routine paraffin embedding methods. Sections 5-8 μm thick are stained with hematoxylin and eosin (H&E) and examined by an experienced veterinary pathologist. Pathologic findings are tabulated. Selected special stains may be requested subsequent to examination of H&E stained sections.

After necropsy, animal remains are incinerated. Surviving animals are returned to USAFRIRO.

11. Statistical Approach:

A modified up-down approach is used to estimate the untreated 48 hr GD LD₅₀ in this group of monkeys. Monkeys will be dosed with GD one or two at a time, starting at doses approximating 20 to 80 percent of the historic LD₅₀. If an animal dies at a given dose, the dose the next monkey receives, on a mg/kg body weight basis, is reduced, and conversely, if the first monkey lives, the next animal receives a higher dose. Based on historic information on the slope of the GD dose-lethal response curve and probit analysis of data as they are obtained, the best doses for challenging succeeding animals will be selected by a statistician in order to most efficiently estimate the 48 hr GD LD₅₀ in the present population of monkeys. If, after a minimum of 2 monkeys have been challenged, the estimated LD₅₀ falls within the 95 percent confidence limits of the Battelle historic Indian Rhesus monkey 48 hr GD LD₅₀, that historic LD₅₀ value will be accepted as the approximate LD₅₀ value for the present population of monkeys and is used to estimate protective ratios (LD₅₀ of treated animals/LD₅₀ of untreated monkeys).

Based upon required sample size analyses to estimate the minimum number of animals needed to provide definitive information on protective ratios, a sample size of 20 monkeys will be allocated for each of the two treatment oxime/atropine therapies. If fewer animals are needed to demonstrate a protective ratio significantly different (α of 0.05) from the historic atropine/2-PAM protective ratio and a survival rate of at least 84 percent at 2 x LD₅₀ of GD, experimentation will cease at that time. If definitive information is not obtained using 20 monkeys per treatment group, the use of an additional ten monkeys per group may be authorized. If it becomes apparent after at least five animals have been challenged and treated with each regimen that a protective ratio of at least 2.5 is not being approximated, the study will cease until further guidance is obtained from USAFRIRO personnel. Doses of GD will be selected by the study statistician to best estimate the LD₅₀ and slope of the dose-response curve in treated monkeys. Animals will be dosed in groups, with equal numbers of animals in each treatment group per dosing.
day, at the start of the study and the study will continue in this manner unless sufficient information is obtained for one treatment regimen but not the other. From the information obtained, probit analyses assuming both common slopes and separate slopes will be performed and estimates made of 48 hr LD₅₀, LD₇₀ and LD₉₀ values. Protective ratios are also calculated. Signs of QD intoxication will be statistically analyzed, as feasible, to determine any differences in treatment efficacy.

12. Records to be Maintained:

A. CSM accountability log and inventory
B. Preparation of reagents, dose analyses and dosage administration
C. Animal data
D. Mortality data
E. Clinical observations and results of neurologic examinations
F. Necropsy and histopathology records
G. Decontamination, monitoring, and disposal records.

13. Reports:

A. A draft final report is prepared within 30 days after completion of the exposures and analyses of the data. The draft final report includes:

(1) Signature page of key study personnel
(2) Experimental design
(3) Animal selection criteria and husbandry
(4) Test material description, analyses, preparation, and administration
(5) Clinical observations, and necropsy and histopathology findings
(6) Statistical analyses of data
(7) Discussions and conclusions.

B. Following receipt of draft final report comments from USAMRDC, a final report will be prepared within 30 days.
14. References:


15. Approval Signatures:

Carl T. Olson, D.V.M., Ph.D.
Study Director

Garrett S. Dill, D.V.M.
Principal Investigator

Ronald G. Menton, Ph.D.
Statistician

Affen W. Singer, D.V.M.
Pathologist

Peter L. Jepsen, D.V.M.
Study Veterinarian

Ramona A. Mayer, Manager
Regulatory Compliance

Date

11-22-89

11/30/89

12-1-89

12-1-89

12-4-89
Characterization of Soman Toxicity in Atropine and Oxime (HI-6 and MMB-4) Treated Rhesus Monkeys

Protocol Amendment No. 1


MAJ James R. Stewart, D.V.M. is replaced with LTC Don W. Korte, Ph.D.

Reason: MAJ Stewart was replaced by LTC Korte as Contracting Officer's Representative.

Impact on Study: None.

Changes: Page 2, Section 10.A.(1).

"Male Rhesus monkeys, Macaca mulatta, of Indian origin were specified for this study because there is considerable scientific evidence that the monkey is predictive of responses in man. Rhesus monkeys of Indian origin were selected because the majority of work in this area has been done with monkeys of Indian origin and because there is evidence that Rhesus monkeys of Chinese origin respond somewhat differently to these study conditions than those of Indian origin.(1)" is replaced with "Male Rhesus monkeys, Macaca mulatta, of Chinese origin are used for this study because there is considerable scientific evidence that the monkey is predictive of responses in man."

Page 2, Section 10.A.(2).

"Initial Weight - Monkeys placed on study weigh between approximately 2 and 7 kg." is replaced with "Initial Weight - Monkeys placed on study weigh between approximately 4 and 7 kg."

Page 4-5, Section 10.C.(1).

"If after 2 or more monkeys have been challenged, the estimated LD50 in this study falls within the 95 percent confidence limits of the historic Battelle LD50 in Indian Rhesus monkeys, the historic LD50 will be accepted for this group of animals." is replaced with, "If after 2 or more monkeys have been challenged, the estimated LD50 in this study falls within the 95 percent confidence limits of"
recent Battelle GD LD_{50} estimates in Rhesus monkeys, the historic LD_{50} will be accepted for this group of animals."

Page 6, Section 10.C.(2).

Delete the following: "A basic neurologic examination of each monkey is conducted within 3 weeks prior to GD exposure and again at approximately 24, 48, and 160 hr following exposure. Findings will be documented."

Page 7, Section 11.

In the following sentence, the phrase in quotation marks is replaced with "recent Battelle". If, after a minimum of 2 monkeys has been challenged, the estimated LD_{50} falls within the 95 percent confidence limits of the "Battelle historic Indian" Rhesus monkey 48 hr GD LD_{50}, that historic LD_{50} value will be accepted as the approximate LD_{50} value for the present population of monkeys and is used to estimate protective ratios (LD_{50} of treated animals/LD_{50} of untreated monkeys).

Page 8, Section 12.E.

Replace "Clinical observations and results of neurologic examinations." with "Clinical observations."

Page 9, Section 14.

Delete this References section.

Reason: The monkeys provided by USAMRICO for this study are large Rhesus monkeys of Chinese origin. This change may affect somewhat the way in which these animals are handled and makes the conduct of a meaningful neurologic examination difficult.

Impact on Study: For comparing efficacy of two oximes given in conjunction with atropine to treat intoxication produced by an organophosphate, the impact on the study should be negligible. Comparison of results of this study with results from other studies conducted with Rhesus monkeys of Indian origin may be difficult.

Changes: Page 3, Section 10.A.(9).

"Monkey room temperatures are maintained at 77 ± 5 F." is changed to read "Monkey room temperatures are maintained at 68 - 80 F."
Page 3, Section 10.A.(10).

"Relative humidity of monkey rooms is maintained at 50 ± 10 percent."
reason: This change was made in the Battelle SOP to agree with the requirements of the National Institute of Health's "Guide for the Care and Use of Laboratory Animals".

Impact on Study: This should have no impact on the results of the study.

Changes:
Page 6, Section 10.e.

"If an animal dies spontaneously before being perfused, tissues will be immersion-fixed in 10 percent neutral buffered formalin, pH 7.2." This change will read, "If an animal dies spontaneously 48 hr or more after agent injection but before being perfused, tissues will be immersion-fixed in 10 percent neutral buffered formalin, pH 7.2."

Animals are anesthetized and perfused via aorta cannula with 1 L of heparinized (2,000 units/L) saline solution followed by 2-3 L of 10 percent neutral buffered formalin, pH 7.2 and simultaneously exsanguinated via a slit in the inferior vena cava." is changed to read "Animals are anesthetized and perfused via aorta or left ventricular cannula with 1 L of heparinized (2,000 units/L) saline solution followed by 2-3 L of 10 percent neutral buffered formalin, pH 7.2 and simultaneously exsanguinated via a slit in the inferior vena cava or a femoral vein."

Reason: Wording is changed to clarify the intent and to allow some latitude in the preference of technique of experienced pathologists in the perfusion fixation of tissue.

Impact on Study: Changes are expected to have no impact on the study.
Characterization of Soman Toxicity in Atropine and Oxime (HI-6 and MMB-4) Treated Rhesus Monkeys

Protocol Amendment No. 2


"Using various doses of GO, as recommended by the study statistician, groups of equal numbers of monkeys are challenged with GO and treated with atropine and 100 µmol of either HI-6 or MMB-4 per kilogram body weight at one minute after GO exposure." is substituted for "Using various doses of GO, as recommended by the study statistician, groups of equal numbers of monkeys will be challenged with GO and treated with atropine and 100 µmol of either HI-6 or MMB-4 per kilogram body weight at a time after exposure provided by the Sponsor Monitor."

Reason: A treatment time of one minute after GD injection was specified by MAJ James R. Stewart, USAMRICO.

Impact on Study: None. The time of treatment, as stated in the protocol, was specified by the Sponsor Monitor.

Carl T. Olson, D.V.M., Ph.D. 3-22-90
Study Director

LTC Don W. Korte, Ph.D. 3-22-90
USAMRICD CDR
Characterization of Soman Toxicity in Atropine and Oxime (HI-6 and NM-6) Treated Rhesus Monkeys

Protocol Amendment No. 3


"Treatment will be administered at a fixed time after GD injection using a dose of atropine (as the citrate salt) of 0.2 mg (or as used in Task 89-08) atropine free base and 100 µmol of candidate oxime per kilogram of body weight." is replaced with "Treatment is administered at one min after GD injection using a dose of atropine (as the citrate salt) of 0.4 mg atropine free base and 100 µmol of candidate oxime per kilogram of body weight."

Reason: A treatment time of one minute after GD injection (Amendment 2) and an atropine dose of 0.4 mg/kg was requested by Maj James R. Stewart, USAFRICOM.

Impact on Study: The change of the atropine free base dose from 0.2 to 0.4 mg/kg allows more comparability with previous studies in primates which used 0.4 mg/kg doses of atropine. This should also assure effective levels of atropine.

Carl T. Olson, D.V.M., Ph.D.
Study Director

3-24-90

LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR

Date

Date
Characterization of Soman Toxicity in Atropine and Oxime (HI-6 and MM8-4) Treated Rhesus Monkeys

Deviation: This protocol specifies monkeys will be held in rooms with a temperature range of 68-80 F and a relative humidity of 30-70 percent. Conditions in animal rooms are recorded twice daily using a hand-held combination thermometer/hygrometer to obtain temperature and relative humidity readings. The temperature and relative humidity recorded in rooms in which monkeys were held were very occasionally outside these ranges. Excursions outside temperature and/or relative humidity ranges specified in the protocol were reported to a maintenance engineer and adjustments were made to thermostats/humidistats.

Impact on Study: The short-lived excursions outside temperature or relative humidity specifications stated in the protocol should have no impact on the validity of the study.

Carl T. Olson, D.V.M., Ph.D.  
Study Director  
11-6-90  
Date

LTG Don H. Korte, JFJ., M.S.  
USAMRICD COR  
6-16-90  
Date
Characterization of Soman Toxicity in Atropine and Oxime (HI-6 and MMB-4) Treated Rhesus Monkeys

Deviation: This protocol specifies that no more than five monkeys will be used to approximate the 48 hr GD LD₅₀ in animals given no treatment. Five monkeys were used in the initial phase of this experiment to determine the 48 hr GD LD₅₀ in this population of monkeys. In the second, efficacy of oxime therapy, phase of the experiment, 12 monkeys were treated with atropine/MMB-4 and 12 monkeys were treated with atropine/HI-6 prior to the decision to discontinue testing with MMB-4. At this time, the Task Area Manager requested that additional monkeys be challenged with GD injection and given no treatment to better define the 48 hr GD LD₅₀ in untreated monkeys. As a result of this request, four additional monkeys were injected with GD and given no therapy.

Impact on Study: The improved definition of the 48 hr GD LD₅₀ in untreated animals of this population of monkeys should assist the statistical analysis of efficacy of oxime therapies. No effect on the validity of the study is anticipated.

Carl T. Olson
Carl T. Olson, D.V.M., Ph.D.
Study Director

LTC Don A. Korte, Jr., M.S., COR
USAMRICD COR

Date

Date
APPENDIX B

MREF SOP-88-31, MREF SOP-89-55, MREF SOP-89-64
STANDARD OPERATING PROCEDURE
MREF SOP-88-31

TITLE: Measurement of Chemical Surety Material in Dilute Solutions of GA, GB, GD, IG, HD-L, HD, L, and VX

LABORATORY: MREF

PLACE OF OPERATION OR TEST: Samples throughout MREF; Analyses in Room 17 or Room 37

This Standard Operating Procedure (SOP) has been prepared as prescribed by Contract DAMD17-89-C-9050 and will be effective for one year from date of approval unless sooner rescinded or superseded.

No deviation from this SOP will be permitted. Whenever the approved method is changed, the SOP will be revised.

Supervisory personnel will assure that all personnel involved with this SOP have been properly trained and instructed in its provisions and attest to this requirement by affixing their signatures on page 3.

A copy of this SOP will be posted at the Medical Research and Evaluation Facility (MREF) job site at all times.

Submitted By: Timothy L. Hayes, Principal Research Scientist
Signature/Date 2/19/90

Approved By: Garrett S. Dill, O.V.M., Manager
Signature/Date

Approved By: David L. Stitick, C.M., Safety/Surety Officer
Signature/Date 3/27/90

Revised February 19, 1990
Approved By:

Signature/Date

Quality Assurance Unit
Health and Environment Group
Printed Name/Title

Signature/Date

Charles K. Burdick, Director
Total Quality Program
Health and Environmental Group
Printed Name/Title

Revised February 12, 1983
SIGNATURES

I have read and understand the contents of MREF SOP-88-31.

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<thead>
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Revised: February 12, 1990
STANDARD OPERATING PROCEDURE 88-31

Measurement of Chemical Surety Material in Dilute Solutions of GA, GB, GD, TGD, HD-L, HD, L, and VX

A. Statement of Work: This SOP describes analytical methods for the analyses of dilute solutions of chemical surety material (CSM) to include dose confirmation samples and dosing stock solutions generated at the MREF. These measurements are performed by comparing the analytical results of exempt chemical surety material (XCSM) samples to analytical standards prepared of the same CSM. The analytical standards are prepared and referenced to Standard Analytical Reference Material (SARM) according to MREF SOP-88-30.

The determination of CSM concentration in the diluted samples is performed on a regular basis at the MREF. The analysis must be performed prior to the expiration date established for the particular CSh/solvent combination under the storage conditions described herein. For most program situations, this has been determined to be approximately 2 weeks after sample preparation.

B. Responsibility:

1. Personnel Qualifications: Technical staff will be current with the requirements of the MREF and all applicable MREF SOPs. All technical staff will be familiar with handling hazardous materials within the MREF laboratory. The technical staff must have a fitted SurvivAir respirator in accordance with FSSP SOP-MREF-9. In addition, must know the location of the nerve agent kit, mechanical resuscitator, eye wash fountain, and de-luge shower as well as how to use them. They must maintain either visual or audible contact with each other in order to detect unauthorized actions or be ready to rescue or render first aid to the other in the event of an accident. Personnel working with solutions of CSM that do not exceed XCSM criteria must read and sign this SOP.

2. Leaders: Leaders of each operation will be designated by the Study Director for that operation. Each leader will ensure that the following are observed:
   a. Only authorized personnel meeting requirements set forth in Section 9.1 are allowed in the room during XCSM operations.
   b. XCSM control and accountability are maintained.

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c. Adequate, approved, protective equipment is available at all times to personnel at their work site.

d. All leader and technical staff responsibilities specified in the MREF FSSP are followed.

e. Each employee has been trained in the techniques of administering first aid and self aid.

f. Work under this SOP is performed only in the area(s) or room(s) designated by this SOP.

g. No food, beverage, or tobacco product is consumed, used, or brought into the laboratory. The wearing of contact lenses is prohibited in the laboratory.

h. The safety requirements of this SOP, as well as normal laboratory safety, are maintained.

i. Decontamination solutions are present prior to handling XCSM.

j. All quantities of XCSM that leave the hood or room are properly contained and labeled.

k. All applicable SOPs are read and signed by all technical staff involved in the operation.

3. Technical Staff: Technical staff will be responsible for abiding by requirements set forth in Section 8.2. In addition, they must use personal, protective equipment provided and develop safe work habits to protect themselves and fellow workers from injury and to prevent damage to material, equipment, and facilities. They must not perform XCSM operations without the presence of a qualified second person.

4. Research Organization: The organization involved in this research is the MREF of Battelle Memorial Institute, 505 King Avenue, Columbus, Ohio 43201-2693.

C. Materials to be Used:

1. XCSM: XCSM is also referred to as research, development, test, and evaluation (RDT&E) dilute solutions of CSM. The XCSM solutions that can be used following this SOP are those prepared from the following CSM.


Revised February 12, 1990
b. Sarin (CAS 107-49-8 or 50642-23-4): GB, isopropyl methylphosphonofluoridate.

c. Soman (CAS 96-64-0 or 50642-24-3): GD, pinacolyl methylphosphonofluoridate.

d. VX (CAS 50782-69-9 or 51848-47-6 or 53800-40-1 or 70938-84-0): VX, O-ethyl S-(2-diisopropylaminoethyl)methylphosphonothiolate.

e. Mustard (CAS 505-60-2 or 39472-40-7 or 68157-62-0): HD, bis-dichloroethyl sulfide.

f. Mustard-Lewisite Mixture: HL, a mixture of bis-dichloroethyl sulfide and dichloro(2-chlorovinyl)arsine.

g. Lewisite (CAS 541-25-3): L, dichloro(2-chlorovinyl)arsine.

2. Solvents and Chemicals: Hexane, acetonitrile, or appropriate solvent. Quality of solvent recommended is spectrometric grade, distilled in glass.

3. Decontamination Materials: Sodium hypochlorite (5 percent solution) for XH0, XL, XHOL, and XVX. Sodium hydroxide (10 percent solution) for G agents.

D. Equipment: Safety equipped cart, freezer (locked), refrigerator (locked), latex gloves, labels, first aid kit, plastic-backed, absorbent paper, brown paper, 4-L beakers, squirt bottles, wiping tissues, beakers, bottles, maxi-vials, pipettes, pipette bulbs, tissue paper, laboratory coat, safety shoes, protective eyewear, spatula, stainless-steel pans, bubbler monitors, scissors, solid sorbent traps, glass stir rods, syringes, needles, forceps, GC vials, drierite, 20-mL scintillation vials, an air-supplied respirator with air cylinder, 10-mL volumetric flasks, and a vial support block.

E. Hazards Involved:

1. Anticholinesterase: The hazard from XVX is primarily that of liquid injection, ingestion, or absorption through the skin or eyes. XVX can be lethal if generated in a vapor form in confined or poorly ventilated spaces. Although liquid spills of XVX do not present a vapor hazard, this material is very slow to evaporate so that virtually the entire spill (minus the solvent) may persist as a liquid contact hazard for several days.

   a. Mechanism of Action and Physiological Effects: These XCSM cause inhibition of cholinesterase enzyme in the body. Repeated low level exposures to these XCSM will have cumulative effects or
cholinesterase inhibition. Blood cholinesterase is regenerated slowly and the inhibition effect will last several weeks. Clinical signs and symptoms may suddenly occur following repeated exposures, but is unpredictable in time of onset and severity.

Casualty Producing Route: Entry: Inadvertent skin contact with these XCS is the most common cause of laboratory accidents/incidents. SM absorption rate will likely be accelerated through cutaneous cuts and abrasions.

Signs and Symptoms: The first indication of exposure of anticholinesterase XCS is likely to be a reaction at the point of exposure, e.g., localized sweating and/or twitching. If exposed to vapor from a solvent vapor generating system, pinpointed pupils (miosis), tightness in the chest, and/or a runny nose will likely be the first symptoms. For other than these extreme exposures, no symptoms are likely to be exhibited. However, under these extreme conditions if the exposure is sufficient, symptoms may progress beyond the local reaction to produce systemic poisoning. The following signs and symptoms are typical of systemic poisoning; the number and severity of which will depend upon degree of exposure:

1. Nausea--possible vomiting.
2. Diarrhea.
3. Weakness.
5. Convulsions.
6. Central nervous system depression.
7. Coma.
8. Cessation of breathing.

Exposure Factors: Onset of signs and symptoms from a percutaneous exposure may be delayed by the adsorption time. Onset after a vapor inhalation exposure may be quite rapid, and death may occur within 10 min. Vapor exposure to the eyes results in immediate miosis at very low concentrations.

2. Solvents: The solvents used in preparing the dilute material may have hazards associated with their use. A copy of the Material Safety Data

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Sheet (M5S) is available in the administrative area of the MREF or through Battelle's Safety Office, 505 King Ave.

a. Hexane: Hexane is a flammable liquid that must be handled and stored as a solvent with a dangerous fire risk. The flash point of hexane is -22.7°C, with an autoignition temperature of 260°C. The 1988-1989 American Conference of Governmental Industrial Hygienists (ACGIH) Threshold Limit Values (TLV) for n-hexane is 50 parts per million (ppm) as an 8-hr time weighted average (TWA). For the other hexane isomers, the TLV is 500 ppm as an 8-hr TWA and 1,000 ppm as a 15-min Short Term Exposure Limit (STEL).

b. Acetonitrile: Acetonitrile is a flammable liquid that must be handled as a solvent with a dangerous fire risk. The flash point of acetonitrile is 5.56°C. The 1988-1989 ACGIH TLV for acetonitrile is 40 ppm as an 8-hr TWA and 50 ppm as a 15-min STEL. Also, skin contact may represent a significant route of exposure.

3. Decontamination solutions can cause chemical burns if sodium hydroxide or sodium hypochlorite is left in contact with skin or eyes.

4. Gloves and aprons made of butyl rubber are flammable and have no self-extinguishing capability; therefore, care must be taken to avoid open flame or heat that may ignite them.

F. Safety Requirements:

1. Hoods: Hood face velocity must average 100 L 10 lfm. The average is computed from individual readings taken in approximately each square foot of hood face (usually nine readings). In addition, no individual reading will vary more than 20 percent from the average. No equipment will be within 20 cm of the face of the hood.

2. Protective Equipment: When working with XCSM samples, the following clothing and protective gear are required as a minimum for all personnel.

   - lab coat
   - safety shoes
   - two pairs of latex gloves
   - protective eyewear

   In addition, each worker's individually assigned Survivair combination escape/airline-supplied respirator will be readily available. All provisions of the MREF FSSP apply to the checking and testing of gloves, aprons, respirators, and other protective equipment.

Revised February 19, 1988
3. First Aid: A first-aid kit containing two squirt bottles, one filled with a 5 percent available chlorine sodium hypochlorite solution and one filled with water (labeled, dated, and the contents changed every month), and gauze pads will be located in the room. The location of the nearest eyewash fountain, deluge shower, and fire extinguisher will be known to all workers before work begins.

G. Procedures:

1. Entry: Before entering the secured facility, note the status of the "Agent-in-Use" light at the turnstile. If the "Agent-in-Use" lights are turned on, note the room location and be sure that upon entry to the laboratory area that all safety equipment and procedures described in FSSP SOP MREF-18 are in place. Upon entry of the room, confirm that there are no audible alarms. No operations can be initiated in a room with audible alarms. After entry, personnel will observe the manomastic gauge on the hood. If inspection reveals that the hood has failed, is marginal in flow, or operates outside the guidelines of FSSP SOP MREF-21, the problem is reported to the MREF Manager and the operation does not begin.

2. Hood Set Up: Prior to obtaining XCSM, the operation hood area must be prepared with all materials necessary to perform an XCSM operation. The hood(s) to be used for any operation with XCSM will contain, as a minimum, the appropriate decontaminating solutions, waste containers, forceps, plastic-backed paper, absorbent tissues, primary container holder, and XCSM transfer equipment. All of the above materials will be kept behind the 8-inch line in the hood.

Plastic-backed, absorbent paper must be used to protect the work surface of the hood. Five layers of brown paper will be placed on top of the absorbent paper. Two 4-L beakers containing a minimum of 2 L of decontaminating solution will be placed within the hood.

A vial support block of sufficient size to contain all primary XCSM maxi-vials to be used will be positioned within the hood work area.

3. Handling of XCSM: The handling of XCSM is conducted in accordance with MAREF SOP-83-5. The procedures used within this SOP are described in MAREF SOP-83-5 and shall include the labeling (Section G.3), obtaining (Section G.4), equilibration (Section G.5), transfer (Section G.6), dilution (Section G.7), transport (Section G.8), packaging (Section G.9), transfer for use within the MREF (Section G.10), and securing of XCSM (Section H).

4. Identification of XCSM: All XCSM samples generated must be clearly identified with the following information being regarded as the minimum requirements.

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a. Type of XCSM contained in the sample.

b. Solvent used for CSM dilution.

c. Sample preparation date.

d. Sample identification number (e.g., dose confirmation accountability record book number, page number, and sample number and their identifiers as necessary, 50003-03-02 Somp. Al).

e. Estimated analyte concentration based on measured agent purity and dilution procedure.

f. Project or task number under which the sample was prepared.

5. Sample Preparation and Storage: Preparation of samples must be performed using volumetric glassware, pipettes, and/or microsyringes as required to achieve a resulting concentration below agent surety levels (1.0 mg/mL for VX, 2.0 mg/mL for GA, GB and GD, and 10.0 mg/mL for HD). Preparation and handling of dilute samples is performed in accordance with the operational dosing protocol and/or MREF SOP-83-3. An example of the calculations to determine XCSM concentration of samples prepared in accordance with MREF SOP-83-3 is provided below.

Example Calculations:

a. To determine appropriate dilution procedure, consider the required volume of CSM to be delivered and the final volume of the diluent necessary to achieve a resulting expected concentration not greater than the permissible XCSM concentration, see Section G.4. For example, if 10 μL of neat VX is dosed, confirmation of dosing accuracy and syringe precision and accuracy would require delivering the same volume of agent into appropriate volumetric glassware. To determine the appropriate dilution volume, multiply the volume of the CSM to be delivered, in μL, by the density (d = 1.0083 mg/μL at 20°C) of CSM in mg/μL and multiply the multiple by the purity of the CSM used for dilution. The result of this mathematical step is then divided by the target concentration or the maximum XCSM concentration, for VX the value would be 1.0 mg/mL. The following calculation would hold true for this example if the working VX purity is 95 percent:

\[
\frac{10.0 \times 10^{-3} \text{ mg VX}}{1.0083 \text{ mg/μL} \times 0.95} = 9.58 \text{ mg/mL dilute concentration}
\]

Therefore, a volumetric flask of 10 mL or greater would be appropriate for this dilution.

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b. All dilute solutions should be kept frozen when not in use. All XCSM samples and standard solutions are stored double contained at -70 C in a locked Revco freezer.

6. Standard Preparation: Instrument calibration standards are prepared from standard analytical stock solutions which have been prepared, stored, and referenced to SARM as per MREF SOP-88-30. Calibration standards must be prepared at three concentration levels as a minimum. These concentration levels must extend over the range of expected sample concentration. Appropriate standard concentrations and dilution procedures are to be determined by the chemist at the time of analysis in order to establish precision limits required by sample submitter. The standards will be prepared in the same solvent as the samples unless stability problems in sample preparation solvent have been determined. The recommended solvent for CSM sample preparation for GC analysis is hexane. If standards need to be prepared ahead of time they must be stored at -70 C until analysis.

7. Instrument Set-Up:

a. The GC must be operated with parameters that will yield the best quantitative results for the analytical system. These will vary depending on CSM/solvent combination and concentration levels. The following are recommended as general starting conditions and optimum conditions must be selected by the chemist doing the analyses.

1. Column - Several analytical columns and detectors have been successfully used to analyze the agents listed in this SOP. Several manufacturers of columns and instruments have also been compared and only minor differences have been observed with all systems evaluated being acceptable under their optimal operating conditions. The recommended column is a general purpose column that produces reliable results with all agents tested to date.

   General Analyses:

   Column: 30 m x 0.25-μm I.D. SE-54 with 0.3-μm film thickness
   Carrier Gas: Helium
   Velocity: 30 L/sec for Helium
   Make-up Gas: 30 L/sec
   Detector: Flame Ionization Detector (FID)
   Detector Gases: H₂ = 400 mL/min L 10 mL/min
   Air = 40 mL/min L 5 mL/min
   Injector Temperature: 275 C

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Detector Temperature: 250 ± 10°C

Oven Program:
- Initial temperature: 60°C
- Initial time: 1.0 min
- Level I program rate: 15°C/min
- Final temperature: 250°C
- Final time: 2.0 min
- Post value: 275°C
- Post time: 4.0 min

Injection Mode: Split

Split Flow: 120 L 10 mL/min

Split Liner Packing: 3 percent OV-1 on 80/100 mesh Chromosorb WHP (2-3 mm bed)

Injection Volume: 1 μL

Auto Sampler: Hewlett Packard 7673A or equivalent with cooled sample tray maintained at 5-7°C.

Analysis of Samples of GO in 0.9 percent Biological Saline:

Column: 25 m x 0.32-mm I.D. HP-20 M with .3-μm film thickness or equivalent

Carrier Gas: Helium

Velocity: 30 L 5 cm/sec for Helium

Make-up Gas: 30 L 5 mL/min

Detector Gases: H₂ = 135 mL/min L 10 mL/min

Detector: FPD with 525-nm phosphorous selective filter

Analysis of Samples of GA in Multisol:

Column: 25 m x 0.32-mm I.D. HP-20M with .3-μm film thickness or equivalent

Carrier Gas: Helium

Velocity: 30 L 5 cm/sec for Helium

Make-up Gas: 30 L 5 mL/min

Detector: FPD with 525-nm phosphorous selective filter

Detector Gases: H₂ = 135 mL/min L 10 mL/min

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Injector Temperature: 150 L 5 C (Very important for satisfactory precision and accuracy of results.)

Detector Temperature: 225 L 5 C

Oven Program: Initial temperature = 80 C
Initial time = 1.5 min
Level 1 program rate = 20 C/min
Final temperature = 200 C
Final time = 1.0 min
Post value = 215 C
Post time = 2.0 min

Injection Mode: Split
Split Flow: 120 L 10 mL/min
Split Liner Packing: 10 percent OV-1 on 80/100 mesh Chromosorb WHP (2-3 mm bed)
Injection Volume: 1 mL
Auto Sampler: Hewlett Packard 7673A or equivalent with cooled sample Maintained at 5-7 C.

Note: The viscosity of multisol makes reproducible injections of samples using an autosampler. Therefore, samples in multisol have to be diluted with an appropriate solvent such as tetrahydrofuran (THF). The samples have been shown to be stable for at least 72 hr after dilution at 5-7 C. The recommended dilution is a minimum factor of five for reliable injection results using an autosampler. As previously stated, the standards should also be diluted using THF.

Analysis of TGD Samples in Acetonitrile:

Column: 25 m x 0.32 mm I.D. HP-20M with 0.3-mm film thickness or equivalent
Carrier Gas: Helium
Velocity: 30 L/min for Helium
Make-up Gas: 30 L/min
Detector: FPD with 525-nm phosphorus selective filter
Detector Gases: H2 = 135 mL/min L 10 mL/min
Air = 120 mL/min L 5 mL/min
O2 = 15 mL/min L 2 mL/min

Injector Temperature: 200 L 5 C (Very important for satisfactory precision and accuracy of results.)

Detector Temperature: 225 L 5 C

Oven Program: Initial temperature = 50 C
Initial time = 0.5 min
Level 1 program rate = 20 C/min
Final temperature = 200 C
Final time = 1.0 min

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Post value = 215 C
Post time = 2.0 min

**Injection Mode:** Split for concentrations above 100 μg/mL
Splitless for concentrations below 100 μg/mL

**Split Flow:** 120 L 10 ML/min

**Split Liner Packing:** 10 percent OV-1 on 80/100 mesh Chromosorb WHP (2-3 mm bed)

**Injection Volume:** 1 μL

**Auto Sampler:** Hewlett Packard 7673A or equivalent with cooled sample tray maintained at 5-7 C.

Note: Acetonitrile has been selected for dilution of neat TGD due to increased solubility of thickener and agent stability in this solvent.

b. Install the proper column into the capillary injector and detector ports and leak test the joints. If the column has not been in use, condition at 20 C below manufacturer suggested maximum operation temperature overnight. This must be done by first allowing the column to set at room temperature with carrier gas flow for 20 min and then programming to the final temperature at a slow rate such as 1 C/min. The column can then be left at upper temperature overnight. Initial conditioning is required to insure that oxidants trapped in the column under storage conditions are removed prior to exposure of the column to elevated temperatures. The exposure of the capillary column liquid support to elevated temperatures in the presence of compounds such as oxygen or water will decompose the stationary phase of a capillary column. The exposure of fused silica, the material of which most modern columns are prepared, to water will dissolve the material and also destroy the column by producing chemically bonding active sites within the column.

c. Set the temperatures in the heated zones using the GC terminal using the appropriate example temperatures as listed in Section 2.6.a.(1).

d. Set the gas flow rates as recommended in Section G.6.a.(1) using a soap bubble flow meter of appropriate range and stopwatch. Set the carrier velocity first then turn the detector gases off. The column velocity is measured by injecting a compound under conditions that will yield an insignificant retention of the compound. A typical compound used to set column velocities is methane for FID conditions or acetone for FPD. The column velocity is measured by injecting the methane and timing the time required for the methane or acetone to exit the column. The exit of the methane or acetone is monitored by the detector, and when
the peak is observed, the time is recorded. A sample calculation is:

\[
\begin{align*}
\text{Column Length in cm} & = \text{Carrier Velocity in cm/sec} \\
\text{Retention Time of Methane or Acetone in sec} &
\end{align*}
\]

The column velocity must be set at optimum column temperature (mid-range of the operating conditions) since capillary column velocities change with temperature. The recommended temperature for the recommended conditions is 150°C. Once column velocity has been set, allow the carrier gas to flow continuously through the column during the remainder of the measurements. It should be noted if a carrier gas other than helium is used, a Van Deemter Curve for that gas should be reviewed to establish the optimum velocity.

e. Attach the flow meter to the gas outlet from the detector with the carrier gas on and all other gases off. Measure the column flow rate and record as this will need to be subtracted from all future measurements to get actual flow rates.

Example Calculation for Flow Rate:

(i.e., for a time interval of 15 sec to achieve a volume of 10 mL the following calculation would be performed)

Formula for determining flow rate:

\[
\text{Flow rate in mL/min} = \frac{\text{Volume (mL)}}{\text{Time (min)}}
\]

\[
\begin{align*}
15 \text{ sec} & = .25 \text{ min} \\
60 \text{ sec/min} & = .25 \text{ min} \\
10 \text{ mL} & = 40 \text{ mL/min (flow rate)}
\end{align*}
\]

f. Adjust and repeat measurements until the prescribed value is reached subtracting the carrier flow rate from the observed flow rate to get actual flow rates.

g. Repeat measurement procedure to set hydrogen flow rate.

h. Repeat measurement procedure to set air flow rate.

i. With gases on, ignite the FID or FPD flame by depressing the flame ignition button. Verify flame ignition by checking for continuous

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condensation on a cold surface (e.g., mirror) at the effluent outlet on the FID or FPD.

j. When the flame has been ignited, turn on the FID or FPD electronics and allow 20 min for system equilibration.

k. Key in the following set points on the integrator terminal if available. If a strip chart is used, refer to instrument manual for connections. Typical settings are:

1. Integration Method: Area percent
2. Attenuation: (2)^3
3. Percent Offset: 10
4. Peak Width: 0.04
5. Threshold: 4
6. Run Time: 15.0 STOP
7. Chart Speed: 0.5
8. Detector: on

These set points are only guidelines, but entering values for these parameters is a minimum requirement for integration.

1. Plot the FID or FPD signal on the GC recorder. Zero the plot on the terminal or strip chart recorder so that the baseline is at 10 percent offset.

8. Analysis of Samples: Standards and sample solutions are analyzed using the same procedures.

9. Calculation Procedures:
   a. Identify the CSM peak in the sample and standard chromatograms; record the peak area.
   b. To calculate the concentration of the XCSM samples, construct a calibration curve by doing a linear regression of standard concentration vs. standard peak area for all concentration levels, then fit the sample peak area to the curve to obtain concentration.

10. Quality Control:

Revised February 19, 1970
a. Each step in the analysis of standards and samples must be done reproducibly to achieve good precision and accuracy. This includes preparation of dilute solutions and instrument operation.

b. The samples are to be injected a minimum of three times each with an average response used to determine the purity measurement. The relative standard deviation for any set of injections must not exceed 10 percent. If the relative standard deviation exceeds 10 percent, the experiment must be repeated entirely prior to acceptance of data.

c. The FID is a general purpose GC detector. The detector is linear over an extremely large range which makes it well suited for this type of analysis. In addition, the detector is general purpose in that it detects almost all chromatographable material with two or more carbon atoms. However, due to the non-selective nature of the detector, the detection of interferences may be encountered. Therefore, each new type of testing should be preceded by experimentation to determine whether any interferences are present and if so, to identify and compensate for them.

d. Blanks for solvents must be checked and high purity solvents such as distilled in glass are recommended. An analysis of the system blank must be studied under the same test conditions (first without CSM present and then with CSM spike) and compare the chromatograms. Evaluate the data and make any necessary corrections.

e. If interferences present a problem, then analysis using a mass spectrometer for the detection system is recommended so that interferences can be confirmed and possibly identified as solvent impurities or CSM impurities. A mass spectrometer should also be used when initially establishing GC conditions to insure that the chromatographic peak being measured during purity analyses is the CSM of interest and not an impurity.

II. Instrument Shut-Down:

a. When the instrument is not to be used for extended periods of time, the system must be shut down following manufacturer's instructions to insure column life and instrument stability.

b. Be sure that sufficient gases are supplied for continuous flow of carrier and detector gases for the period of time that the system will be unattended.
H. Decontamination: Proper protective equipment and clothing must be utilized throughout these operations in accordance with FSSP SOPs MREF-23 and/or MREF-25. All absorbent material covering the hood surface will be placed in the decontaminating solution after each operation. All disposable glassware in hoods will be submerged in the appropriate decontaminating solution (5 percent available chlorine in a sodium hypochlorite solution or 10 percent sodium hydroxide solution in water) overnight. All non-disposable glassware in hoods will be filled with the appropriate decontaminating solution (5 percent available chlorine in a sodium hypochlorite solution or 10 percent sodium hydroxide solution in water) overnight. Materials left to soak in decontaminating solution overnight will be removed from the hood on the next work day. The glassware, equipment, or non-expendable materials are rinsed with water and removed from the hood. Expendable items may be placed directly into a primary plastic bag within the hood. The primary plastic bag is then sealed with adhesive tape and placed inside another plastic bag, which is then sealed with adhesive tape to provide double containment of decontaminated materials.

Bags of waste must be labeled "Contaminated Materials" with type of ACSM, date of bagging, bag identification number, and name of person packaging the contaminated materials in accordance with MREF SOP-33-3, Section 4. The double-contained materials can then be incinerated.

I. Emergency Procedures: If an ACSM spill occurs, decontamination solution (containing 5 percent sodium hypochlorite or 10 percent sodium hydroxide) located within the hood is gently poured or swabbed with soaked absorbent paper held with forceps on the area in an amount that is at least tenfold in excess of the spill. This contaminated decontaminating solution is absorbed with diatomaceous earth or other absorbent and deposited into double plastic bags. The cleaning/absorption procedure is repeated again.

In the event of any incident or exposure, the MREF Manager or his designee must be notified immediately.

J. First Aid Procedures: Make sure that you protect yourself from contamination by the casualty. Mask if in doubt. Personnel exposed to a toxic agent will be removed immediately to a shower area where washing and first aid can be administered by co-workers. If there is any question about the source of contamination, place the victim under the emergency shower. Wash the victim down with soap; do not scrub as this may enhance penetration.
1. **Emergency Treatment for Specific Types of XCSM:**

   a. V and G XCSM:

      (1) Decontaminate when the source of contact is certain.

         (a) Transfer the victim to a clean area and thoroughly decontaminate with 5 percent sodium hypochlorite only in the areas below the eyes in the position in which the victim is being held. Wash skin at once with diluted chlorine-type bleach and rinse with copious amounts of water. Rinse eyes with water only; rinsing a minimum of 10 min at the eyewash fountain. Decontaminate with dry tissue or absorbent paper followed by water in areas close to the eyes.

      (2) Decontaminate when source of contact is uncertain.

         (a) Place victim in shower and remove clothing.

         (3) If victim has symptoms of anticholinesterase poisoning beyond miosis, inject him with the contents of the atropine/2-PAM autoinjectors at intervals of 5-10 min up to a maximum of three injections. Note time of each injection on the victim for reference by physician.

         (4) If victim has stopped breathing, employ resuscitation with the ambu-bag immediately. Use the atropine autoinjectors after you have successfully succeeded in restoring respiration.

   b. H and L XCSM:

      (1) Decontamination when the source is certain.

         (a) Transfer the victim to a clean area and thoroughly decontaminate with 5 percent sodium hypochlorite only in the areas below the eyes in the position in which the victim is being held. Wash skin at once with diluted chlorine-type bleach and rinse with copious amounts of water. Rinse eyes with water only; rinsing a minimum of 10 min at the eyewash fountain. Decontaminate with dry tissue or absorbent paper followed by water in areas close to the eyes.

      (2) Place victim in shower and remove clothing.
2. The decontaminated individual is transported by ambulance to University Hospital.

3. In the event of any exposure, the MREF Manager or his designee must be summoned after the immediate emergency is taken care of and informed of the exposure.
STANDARD OPERATING PROCEDURE
MREF SOP-89-55

TITLE: Analysis and Structural Verification of Atropine in Citrate Buffer

LABORATORY: MREF, HML, or King Ave. SOP APPROVAL DATE: 02/26/90

PLACE OF OPERATION OR TEST: Any safety approved laboratory within the
facilities

This Standard Operating Procedure (SOP) has been prepared as prescribed by
Contract DAMD17-89-C-3050 and will be effective for one year from date of
approval unless sooner rescinded or superseded.

No deviation from this SOP will be permitted. Whenever the approved method is
changed, the SOP will be revised.

Supervisory personnel will assure that all personnel involved with this SOP
have been properly trained and instructed in its provisions and attest to this
requirement by affixing their signatures on page 3.

A copy of this SOP will be posted at the job site whenever the operation is
being performed.

Submitted By: Timothy L. Haves 2/26/90
Timothy L. Haves, Research Scientist
Signature/Date Printed Name/Title

Approved By: Garrett S. Dill, D.V.M., Manager
Signature/Date Printed Name/Title

Approved By: David L. Stitcher, CIH, Safety/Safety Officer
Signature/Date Printed Name/Title

Revised February 26, 1990
Approved By:

Signature/Date

Quality Assurance Unit
Health and Environment Group
Printed Name/Title

Signature/Date

Charles K. Burdick, Director
Total Quality Program
Health and Environmental Group
Printed Name/Title

Revised February 20, 1990
I have read and understand the contents of MREF SOP-89-55.

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Revised February 20, 1990
A. Statement of Work: This SOP describes the entire procedures for verification of identity and quantitative measurement of atropine free base by high performance liquid chromatography (HPLC). The procedures for structural verification by nuclear magnetic resonance (NMR) of atropine present in drug formulations are also described. The HPLC effort can be conducted at either the MREF, HML or King Avenue, but the NMR requires the facilities at King Avenue.

B. Responsibility:

1. Personnel Qualifications:

All technical staff will be familiar with handling hazardous materials within the laboratory. Personnel performing the following procedures must read and sign this SOP.

2. Leaders: Leaders of each operation will be designated by the Study Director for that operation. Each leader will insure that the following are observed:

   a. Only authorized personnel meeting requirements set forth in Section 8.1 are allowed in the room during operations.

   b. Adequate, approved, protective equipment is available at all times to personnel at their work site.

   c. All leader and technical staff responsibilities specified in the MREF or HML FSSP are followed when work is conducted at the respective laboratories.

   d. Each MREF and HML employee has been trained in the techniques of administering first aid and self aid.

   e. Work under this SOP is performed only in the area(s) or room(s) designated by this SOP.

Revised February 20, 1990
f. No food, beverage, or tobacco product is consumed, used, or brought into the laboratory. The wearing of contact lenses is prohibited in the laboratory.

g. The safety requirements of this SOP, as well as normal laboratory safety, are maintained.

h. All applicable SOPs are read and signed by all technical staff involved in the operation.

3. Technical Staff: Technical staff will be responsible for abiding by requirements set forth in Section 8.2. In addition, they must use personal, protective equipment provided and develop safe work habits to protect themselves and fellow workers from injury and to prevent damage to material, equipment, and facilities.

4. Research Organization: The organization involved in this research is the MREF of Battelle Memorial Institute, 505 King Avenue, Columbus, Ohio 43201-2593.

C. Materials To Be Used:

1. Solvents and Chemicals: The atropine sulfate solid which will be used on this program for preparation of analytical standards will be provided by the U.S. Army Medical Research and Development Command (USAMRDC) or a source which can provide an established purity.

If the atropine dosing solution is not received in a pre-packaged form upon receipt, the atropine dosing solution in citrate buffer will be stored in subdued lighting at 4°C. If a pre-packaged form has been received, it will be stored as directed by the supplier.

NMR spectra will be obtained on dilute solutions of the drug dissolved in > 99.3 percent deuterium oxide (Stohler Isotope Chemicals or equivalent). NMR tubes will be the Stohler Isotope Chemicals "Ultra Precision" model or the equivalent model from other manufacturers.

Other materials will include acetonitrile (Burck and Jackson HPLC Grade), methanol (Burck and Jackson HPLC Grade), benzene (Burck and Jackson HPLC Grade), deionized water or millipore water, glacial acetic acid (Baker Reagent Grade), tetrabutylammonium chloride (Aldrich 98+ percent), sodium lauryl sulfate (Aldrich 98 percent), sodium heptane sulfonate (1-heptane sulfonic acid, sodium salt) (Aldrich 98 percent), tetramethylammonium chloride (Aldrich 98 percent), and helium or nitrogen gas.

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O. Equipment: Freezer, refrigerator, labels, first-aid kit, plastic-backed, absorbent paper, squirt bottles, wiping tissues, beakers, bottles, maxi-
vials, pipettes, pipette bulbs, tissue paper, laboratory coat, protective eyewear, spatula, stainless-steel pans, glass stir rods, syringes, needles, forceps, and latex gloves.

Proton NMR spectra will be obtained on Battelle's Varian CFT-20 Fourier transform NMR spectrometer located in Room 7237-A of the King Avenue facility.

The HPLC analytical system, to be used consists of the following: HPLC pump, HPLC ultra violet (UV) detector, HPLC injection system (autosampler), analytical column, strip-chart recorder (optional), electronic data system. Any equivalent system may be used once confirmation of performance has been established.

E. Hazards Involved:

1. Solvents: The solvents used in preparing the dilute material may have hazards associated with their use. A copy of the Material Safety Data Sheets (MSDS) is available from the manufacturer or through Battelle's Safety Office at 505 F Avenue. A brief listing of hazards associated with handling the more commonly used solvents has been included:

   a. Acetonitrile: Acetonitrile is a flammable liquid that must be handled as a solvent with a dangerous fire risk. The flash point of acetonitrile is 5.56°C. The 1988-1989 ACGIH TLV for acetonitrile is 40 parts per million (ppm) as an 8-hr TWA and 60 ppm as a 15-min STEL. Skin contact may also represent a significant route of exposure.

   b. Methanol: Methanol is a flammable liquid that must be handled as a solvent with a dangerous fire risk. The flash point (open cup) of methanol is 12.2°C with an autoignition temperature of 464°C. The 1988-1989 ACGIH TLV for methanol is 200 ppm as an 8-hr TWA and 250 ppm as a 15-min STEL. Also, skin contact may represent a significant route of exposure.

   c. Benzene: Benzene is a flammable liquid that must be handled as a solvent with a dangerous fire risk. Benzene is toxic by ingestion, inhalation, and skin absorption. Benzene is regulated as a carcinogen by the Occupational Safety and Health Administration (OSHA) resulting in excess leukemia. Containers must say "DANGER CONTAINS BENZENE CANCER HAZARD." OSHA 3-hr permissible exposure limit (PEL) = 1 ppm, Action Level = 0.5 ppm.
F. Safety Requirements:

1. Hoods: Hood face velocity must average 100 ± 20 l fpm. The average is computed from individual readings taken in approximately each square foot of hood face (usually nine readings). No equipment will be within 20 cm of the face of the hood.

2. Protective Equipment: When working in the laboratory, the following clothing and protective gear are required as a minimum for all personnel. This equipment must be used as directed in the FSSP.
   - lab coat
   - latex gloves (as needed)
   - protective eyewear

   All provisions of the FSSP apply to the checking and testing of gloves, aprons, and other protective equipment.

3. First Aid: The location of the nearest eyewash fountain, shower, and fire extinguisher will be known to all workers before work begins.

G. Procedures:

1. MREF Entry: Before entering the secured facility, note the status of the "Agent-in-Use" light at the turnstile. If the "Agent-in-Use" lights are turned on, note the room location and be sure that upon entry to the laboratory area all safety equipment and procedures described in FSSP SOP MREF-1B are in place. Upon entry of the room, confirm that there are no audible alarms. No operations can be initiated in a room with audible alarms. After entry, personnel will observe the magnetelic gauge on the hood. If inspection reveals that the hood has failed, is marginal in flow, or operates outside the guidelines of FSSP SOP MREF-21, the problem is reported to the MREF Manager and the operation does not begin.

2. Hood Set Up: The operation hood area must be prepared with all materials necessary to perform the operation prior to starting the operation. All materials will be kept behind the 3-inch line in the hood.

3. Sample Preparation: The drug formulation samples provided for analysis will be manipulated so that the interference of solvents and other components associated with the samples is minimized to provide relatively pure drug samples for NMR analysis.

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HPLC analyses may be performed on either the dosing formulations as received, dilutions of the parent materials, or on reference standard solutions of known concentration.

a. Analytical Reference Standard: Solid atropine sulfate standard used as a reference material is dried at 100°C, 0.4 mm Hg for 3 hr prior to use in a vacuum oven. This is performed by placing the solid material contained in its original container which has had its cap removed into a pre-heated oven. The oven is sealed and the vacuum adjusted to 0.4 mm Hg.

b. NMR: For the NMR sample preparation, 1 mL of test sample is made basic with 2.0 mL of 0.1 M sodium hydroxide to reach a pH of approximately 13 (verified by color pHast paper). This solution is stirred rapidly with benzene (5.0 mL) for 15 min and then poured through Whatman 1ps phase separation paper (with 1.0-mL benzene rinse). The filtrate is stirred for 1 min with 2.0-mL deionized water and this mixture is passed again through a fresh phase separation paper (with 1.0-mL benzene rinse). The benzene filtrate is evaporated in a rotary evaporator to yield atropine as its free base. The sulfate is reformed by adding a slight molar excess of dilute D2SO4 in D2O to the free base.

NMR samples are prepared by transfer of the deuterium oxide solution and transferred into an NMR tube (tube capped after transfer) for NMR analysis.

c. HPLC Analysis: Samples are either analyzed directly or can be diluted so that the expected concentration range is between 0.1 and 1.3 mg/mL.


a. NMR: Within a glove bag thoroughly flushed with dry nitrogen or argon, weigh 10 ± 0.1 mg of atropine sulfate onto weighing paper. Transfer the sample into a screw-capped bottle and close tightly. Outside the bag, dissolve the sample in an accurately measured volume of 10.0 mL of deuterium oxide and recap the bottle to minimize the contamination of the sample with undeuterated moisture.

b. HPLC: Weigh 50 ± 0.1 mg of atropine sulfate onto weighing paper. Quantitatively, transfer the sample into a 50-mL volumetric flask containing approximately 40 mL of mobile phase (see Section G.6.b)

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Mix the solution thoroughly on a vortex mixer. Dilute to 50.0 mL with the mobile phase and remix the solution. The resulting concentration of the atropine sulfate will be approximately 1 mg/mL.

Mix and dilute the atropine sulfate stock solution with the mobile phase as follows:

10.0-mL stock + 0.0-mL mobile phase
5.0-mL stock + 5.0-mL mobile phase
2.5-mL stock + 7.5-mL mobile phase
1.0-mL stock + 9.0-mL mobile phase
0.0-mL stock + 10.0-mL mobile phase

The atropine sulfate concentrations obtained are 1.00, 0.50, 0.25, 0.10, and 0.0 mg per mL.

Diluted standard solutions are kept refrigerated until use. Standards may be kept refrigerated for up to 30 days.

5. Analysis Start-Up: NMR is performed to verify the structure of atropine sulfate. HPLC is performed to quantitatively determine the concentration of atropine sulfate and confirm the identity of the atropine in the samples.

a. NMR: Calibrate the NMR instrument and data system according to instructions in the operator's manual. When properly calibrated against the standard reference solutions identified in the manual, proceed with the analysis Section G.7.a.

b. Quantitative HPLC: Prepare HPLC mobile phase for quantitative analysis by dissolving 2.2 g of sodium heptane sulfonate (1-heptane sulfonic acid sodium salt) and 2.7 g of tetramethylammonium chloride in approximately 90 mL of deionized water. Add 1.0 mL of glacial acetic acid and dilute to 1 L and mix. Filter buffer solution before using.

The mobile phase may be established using a gradient system with a 78 percent buffer : 2 percent methanol : 20 percent acetonitrile ratio or mixed prior to analysis. To mix the mobile prior to analysis, add 780 mL of the buffer prepared above to a 1-L glass bottle, add 20 mL of methanol and 100 mL of acetonitrile and mix. Once the buffer has been prepared, it must be filtered and used within 30 days.
Insure that the appropriate analytical column has been installed in the analytical system, and that the injector is equipped with at least a 20μL sample injection loop.

All mobile phase must be filtered and degassed for at least 5 min with nitrogen or helium, prior to use.

The detector and the pump must be turned on for a warm-up period of at least 15 min prior to system evaluation. The pump flow must be set at 1.0 mL/min during the warm-up period. After approximately 15 min, measure the flow for 5 min with a 10-mL graduated cylinder. The flow rate must be set at 1.0 ± 0.1 mL/min. Adjust the flow rate setting on the pump controller if necessary to obtain an actual flow rate within these limits and re-check flow.

After the pump has been on for 30 min, adjust the detector zero with the balance control with the detector attenuation set at the appropriate attenuation. Adjust the recorder to electrical zero at "0" chart units. Adjust the detector zero to slightly above the electrical zero position with the recorder balance control.

c. HPLC Identity Confirmation: Prepare HPLC mobile phase for identity confirmation by adding 6.0 g of sodium lauryl sulfate and 1.0 g of tetrabutylammonium nitrate to a 1-L volumetric flask and dissolve the reagents in approximately 500 mL of deionized water. Add 20 mL of glacial acetic acid to the solution and mix. The volumetric flask is filled to the 1-L mark and the solution re-mixed. Filter the solution with a 5 μm filter and store in a clean glass bottle. Use within 30 days.

The mobile phase may be established using a gradient system with a 60 percent buffer: 40 percent acetonitrile ratio or mixed prior to analysis. To mix the mobile prior to analysis, add 600 mL of the buffer prepared above to a 1-L glass bottle and add 400 mL of acetonitrile and mix. Once the buffer has been prepared it must be used within 30 days.

Insure that a Supelco LC-1 column or equivalent has been connected to the injector and detector and the injector is equipped with a 20 μL sample injection loop.

All mobile phase must be degassed for at least 5 min with helium or nitrogen prior to use.

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The detector and the pump must be turned on for a warm-up period of at least 15 min prior to system evaluation. The pump flow must be set at 1.0 mL/min during the warm-up period. After approximately 15 min, measure the flow for 5 min with a 10-mL graduated cylinder. The flow rate should be 1.0 ± 0.1 mL/min. Adjust the flow rate setting on the pump if necessary to obtain an actual flow rate within these limits and re-check.

After the pump has been on for 30 min, adjust the detector zero with the balance control with the detector set at the appropriate attenuation. Adjust the recorder to electrical zero at "0" chart units. Adjust the detector zero to slightly above the electrical zero position with the recorder balance control.

6. Analysis of Samples: NMR is performed for structural confirmation. HPLC standards and collected samples are analyzed to determine concentration and identity confirmation.

   a. NMR: Multiple acquisitions (> 100 transients) are generally required. Spectra will be printed on standard NMR paper and computer referenced to the chemical shift of sodium 2,2-dimethyl-2-silapentane-5-sulfonate determined on the same day to facilitate interpretation.

   b. Quantitative HPLC: The following is a set of HPLC conditions that have been found to be satisfactory for quantitative analysis of atropine sulfate by HPLC (reference 1):

   Column: C18 u-Bondapak or equivalent, 250-mm long x 4.6-mm inner diameter with 5 micron particle size.

   Mobile Phase: See Section G.6.b

   Detector: UV @ 260 nm

   Flow Rate: 1.8 mL/min

   Injection Volume: 20 µL

For quantitative analysis of atropine sulfate samples, transfer 1-mL duplicate aliquots of each atropine sulfate standard to autosampler vials and place the vials in the autosampler in ascending concentration order. Set up the data system to acquire data for each standard as described in the instruction manual. Transfer 1-mL duplicate aliquots of each sample to autosampler vials and place the vials in the autosampler.

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For every ten samples to be analyzed, one blank sample and one standard must be analyzed as a minimum. All samples must be analyzed under the same conditions as used for the standards.

c. HPLC Identity Confirmation: For confirmation of the identity of atropine sulfate by HPLC, a second set of HPLC conditions is employed. The following is a set of HPLC conditions found to be satisfactory for the confirmation of atropine.

Column: Supelco LC-1, 250-mm long x 4.6-mm inner diameter, with 5 micron particle size.

Mobile Phase: See Section G.6.c

Detector: UV 9 254 nm

Flow Rate: 1 mL/min

Injection Volume: 20 μL

For confirmation purposes, analyze an atropine sulfate standard and a sample from the formulation under these HPLC conditions.

7. Instrument Shut-Down:

a. When the instrument is not to be used for extended periods of time, the system must be shut down following manufacturer's instructions to ensure column life and instrument stability.

b. For overnight shut-down, turn off the UV detector, chart recorder, and pump controller.

c. For weekend shut-down, follow the same procedure as for overnight shut-down but also cap off the analytical column to prevent the solid phase from drying.

8. Data Reduction: The NMR spectra obtained in Section G.7 are compared to reference NMR spectra for atropine to verify structural identity. The HPLC samples analyzed in Section G.7 are compared with results obtained from known reference standards to determine concentration.

a. NMR: Compare the NMR spectrum for the sample with the spectrum obtained for the atropine sulfate reference standard. Verify correspondence of chemical shifts, multiplicities, and intensities for structural verification in conjunction with HPLC findings.
b. Quantitative HPLC: Obtain printouts of the peak areas for each standard and sample as described in the data system instruction manual. Prepare a standard curve from the peak areas versus concentration of the standards.

Determine the atropine sulfate concentration in the samples and control standards using the standard curve. If necessary, correct any dilution made to the samples prior to analysis.

If the response for any of the control standards varies from the predicted response by more than ± 10 percent, then the samples associated with that standard are reanalyzed.

c. HPLC Identity Confirmation: HPLC confirmation of the identity of atropine sulfate is performed by analysis under a second set of HPLC conditions. Compare the retention times and relative responses of the atropine sulfate reference standard and sample peak for structural confirmation in conjunction with the first set of HPLC results and NMR conclusions.

H. Emergency Procedures: All personnel involved in the HML or MREF laboratory operations must be familiar with the respective laboratory's FSSP, and the emergency procedures detailed within this document. All personnel involved in the King Avenue operation must be familiar with HEG H/SP 8-01 and the emergency procedures detailed within this document.

I. First Aid Procedures: First aid and self aid at the MREF are to be conducted as specified in the FSSP.

J. References:


TLH:cah

Revised February 20, 1990
TITLE: Analysis of 3-1-(2-Hydroxviminomethylpyridinium)-2- (4-Carboxyamido-Pyridinium)-Dimethyl Ether Dichloride (MH-6) and 1,1'-Methvlene-Bis [3-(Hydroxviminomethyl)Pyridinium] Dichloride (MHB-3) Using High Performance Liquid Chromatography (HPLC)

LABORATORY: MREF, HML or King Ave. SOP APPROVAL DATE: June 16, 1990

PLACE OF OPERATION OR TEST: Any safety approved laboratory within the approved facilities

This Standard Operating Procedure (SOP) has been prepared as prescribed by Contract DAAD17-89-C-0505 and will be effective for one year from date of approval unless sooner rescinded or superseded.

No deviation from this SOP will be permitted. Whenever the approved method is changed, the SOP will be revised.

Supervisory personnel will assure that all personnel involved with this SOP have been properly trained and instructed in its provisions and attest to this requirement by affixing their signatures on page 3.

A copy of this SOP will be posted at the job site whenever the operation is being performed.

Submitted By: Timothy L. Hayes, June 17, 1990
Principal Research Scientist

Approved By: David L. Stitcher, C.H., Safety/Surety Officer
C.H., Safety/Surety Officer

Approved By: Garrett S. Gill, D.V.M., Manager
D.V.M., Manager
**SIGNATURES**

I have read and understand the contents of MREF SOP-89-64.

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STANDARD OPERATING PROCEDURE 89-64

Extraction and analysis of z 1-(2-Hydroxyiminomethylpyridinium)-2-
(4-Carboxyamido-Pyridinium)-Dimethyl Ether Dichloride (HI-6) and
1,1'-Methylene-Bis [4-(Hydroxyiminomethyl) Pyridinium] Dichloride (MMB-4)
Using High Performance Liquid Chromatography (HPLC)

A. Statement of Work: This SOP describes the method for the determination of
z 1-(2-hydroxyiminomethylpyridinium)-2-(4-carboxyamido-pyridinium)-
dimethyl ether dichloride (HI-6) and 1,1'-methylenedibis [4-(hydroxyiminomethyl) pyridinium] dichloride (MMB-4) in dosing samples.
The prepared sample is analyzed by high performance liquid chromatography
(HPLC). The sample preparation and analysis methods detailed here were
developed in support of pharmacokinetics studies performed at Battelle's
Medical Research and Evaluation Facility (MREF). This method uses HI-6 as
the internal standard when analyzing for MMB-4 and MMB-4 as the internal
standard when analyzing for HI-6. Only samples containing one of the
analytes can be analyzed using this SOP.

B. Responsibility:

All technical staff will be familiar with the safe handling practices of
chemical materials within a laboratory. Personnel performing the
following procedures must read and sign this SOP. They must use personal,
protective equipment required by the Facility Safety and Surety Plan
(FSSP) while working within the MREF and develop safe work habits to
protect themselves and fellow workers from injury and to prevent damage to
material, equipment, and facilities.

The organization involved in this research is the MREF of Battelle
Memorial Institute, 505 King Avenue, Columbus, OH 43201-2693.

C. Materials To Be Used: Potassium phosphate dibasic, 1-heptanesulfonic
acid, triethylamine, acetonitrile, methyl alcohol, trichloroacetic acid,
HI-6, 1,1'-methylenedibis [4-(hydroxyiminomethyl) pyridinium] dichloride
(MMB-4), acetic acid, labels, plastic-backed absorbent paper, brown paper,
and wiping tissues.

D. Equipment: Freezer, refrigerator, first aid kit, plastic-backed, squirt
bottles, beakers, bottles, maxi-vials, pipettes, pipette bulbs, laboratory
coat, protective eyewear, spatula, stainless-steel pans, glass stir rods,
syringes, needles, forceps, and latex gloves.

E. Hazards Involved:

1. Solvents and Chemicals: The solvents and chemicals used in this SOP
may have hazards associated with their use. The Material Safety Data
Sheets (MS3S) are available in the administrative area of the MREF or through Battelle's Safety Services Department at 505 King Avenue.

F. Procedures:

1. Hood Set Up: The operation hood area must be prepared with all materials necessary to perform the operation prior to starting the operation. All materials will be kept behind the 8-inch line in the hood.

   Plastic-backed, absorbent paper must be used to protect the work surface of the hood.

2. Solution Preparation:
   a. Mobile Phase Buffer: Accurately weigh 4.1 ± 0.01 g 1-heptanesulfonic acid, and 17.4 ± 0.01 g potassium phosphate dibasic onto weighing paper. Quantitatively transfer each chemical into a 2-L volumetric flask containing approximately 1500 mL millipore water. Deliver 2.81 mL ± 0.01 of triethylamine (using a 5000-μL gas-tight syringe) to the resulting solution and mix. Adjust the pH of the solution to 4.1 ± 0.1 with glacial acetic acid (approximately 28 mL). Mix well and dilute to volume with millipore water. Filter through a 0.45 μm filter.

   b. 20.0 mg/mL MMB-4 Stock Solution: Weigh 100 ± 1.0 mg of neat MMB-4 in a weighing dish. Quantitatively transfer the weighed material into a 5-mL volumetric flask containing approximately 2-mL millipore water. Vortex mix the solution and dilute to volume with millipore water. Stock solution must be made up fresh daily.

   c. Mobile Phase Diluent with MMB-4 Internal Standard: Accurately measure and dispense 125 μL MMB-4 stock solution at 20.0 mg/mL (using a 250-μL gas-tight syringe) into a 100-mL volumetric flask containing approximately 50 mL mobile phase buffer. Mix well. Dilute to volume with mobile phase buffer. Mix resulting solution again, label and store in refrigerator until use.

   d. 10.0 mg/mL HI-6 Stock Solution: Accurately weigh 100.0 mg ± 1.0 mg of HI-6 on weighing paper. Quantitatively transfer the HI-6 into a 10-mL volumetric flask containing approximately 5 mL millipore water. Mix well until dissolved. Dilute to volume with millipore water and mix again. Stock solution must be made up fresh daily.

   e. Mobile Phase Diluent with HI-6 Internal Standard: Accurately measure and dispense 1,110 μL of the HI-6 stock solution at
10.0 mg/mL (using a 1,250 μL gas-tight syringe) into a 100-mL volumetric flask containing approximately 50 mL mobile phase buffer. Mix well. Dilute to volume with mobile phase buffer. Mix resulting solution again, label and store in refrigerator until use.

f. HI-6 Standards: (Must be made up fresh daily.)

1. 100 μg/mL HI-6 Standard: Into a 10 mL volumetric flask containing approximately 5 mL of the mobile phase diluent spiked with MMB-4 (G.3.c.), deliver 100 μL of the 10 mg/mL HI-6 stock solution (using a 100 μL gas-tight syringe). Mix well and dilute to volume with mobile phase diluent.

2. 50 μg/mL HI-6 Standard: Into a 10-mL volumetric flask containing approximately 5 mL of the mobile phase diluent spiked with MMB-4, deliver 50 μL of the 10 mg/mL HI-6 stock prepared in Section G.3.d. (using a 50 μL gas-tight syringe). Mix well and dilute to volume with mobile phase diluent. Mix on a vortex mixer.

3. 25 μg/mL HI-6 Standard: Into a 10 mL volumetric flask containing approximately 5 mL of the mobile phase diluent spiked with MMB-4, deliver 25 μL of the 10 mg/mL HI-6 stock prepared in Section G.3.d. (using a 25 μL gas-tight syringe). Mix well and dilute to volume with mobile phase diluent. Mix on a vortex mixer.

4. 10 μg/mL HI-6 Standard: Into a 10-mL volumetric flask containing approximately 5 mL of the mobile phase diluent spiked with MMB-4, deliver 10 μL of the 10 mg/mL HI-6 stock prepared in Section G.3.d. (using a 10 μL gas-tight syringe). Mix well and dilute to volume with mobile phase diluent. Mix on a vortex mixer.

5. Blank: The mobile phase diluent prepared in G.3.c. is used as the HI-6 blank.

g. MMB-4 Standards: (Must be made up fresh daily.)

1. 100 μg/mL MMB-4 Standard: Into a 10 mL volumetric flask containing approximately 5 mL of the mobile phase diluent spiked with HI-6 (G.3.e.), deliver 50 μL of the 20 mg/mL MMB-4 stock solution (using a 50 μL gas-tight syringe). Mix well and dilute to volume with mobile phase diluent.

2. 50 μg/mL MMB-4 Standard: Into a 10-mL volumetric flask containing approximately 5 mL of the mobile phase diluent spiked with HI-6, deliver 25 μL of the 20 mg/mL MMB-4 stock

(3) 20 μg/mL MMB-4 Standard: Into a 10-mL volumetric flask containing approximately 5 mL of the mobile phase diluent spiked with H1-6 prepared in Section F.2.e., deliver 10 μL of the 20 mg/mL MMB-4 stock prepared in Section F.2.d. (using a 10 μL gas-tight syringe). Mix well and dilute to volume with mobile phase diluent. Mix on a vortex mixer.

(4) 10 μg/mL MMB-4 Standard: Into a 10-mL volumetric flask containing approximately 5 mL of the mobile phase diluent spiked with H1-6, deliver 5 μL of the 20 mg/mL MMB-4 stock prepared in Section G.3.d. (using a 10 μL gas-tight syringe). Mix well and dilute to volume with mobile phase diluent. Mix on a vortex mixer.

(5) Blank: The mobile phase diluent prepared in F.2.e is used as the MMB-4 blank.

3. Equipment Preparation:

   a. Instrument Preparation: The HPLC is prepared for use with the following recommended initial settings and conditions. These settings and conditions are to be optimized by the operator prior to instrument calibration and sample analysis.

   (1) Column - 15 cm x 4.6 mm inside diameter (I.D.) Zorbax LC-8 with 5 μm partial size or equivalent.

   (2) Guard Column - 2 cm x 4.6 mm I.D. Zorbax LC-8 with 5 μm partial size.

   (3) Mobile Phase: 80 percent buffer (see Section F.2.a) 15 percent Acetone: Isopropanol (80:20) 5 percent spectroscopic grade methyl alcohol.

   (4) Mobile Phase Flow Rate: 1.5 mL/min.

   (5) Injection Volume: 100 μL volume for H1-6. 30 μL volume for MMB-4.

   (6) Detector Integration Wavelength: 304 nm.

   b. Column Conditioning: The column needs approximately 45 min of mobile phase conditioning before it can be used to analyze samples. This conditioning is required so the stationary phase can equilibrate with the ion-pair reagent in the mobile phase.
c. Column Check: The efficiency of the chromatographic system must be checked before samples are analyzed. To determine this a 25 μg/mL HI-6 standard is analyzed and the resolution and peak shape for each analyte peak, HI-6 and MMB-4 is determined. When reduction in column performance is observed, the column and or guard column must either be cleaned or replaced. The guard column should always be replaced and conditioned without the analytical column in position to avoid contamination of the analytical column.

4. Dilution of Samples: Samples are diluted in the mobile phase buffer. Samples having high concentrations of HI-6 or MMB-4 must be sonicated to ensure complete solvation of the material, due to limited solubility of these materials in aqueous solvents, prior to performing dilutions. The expected concentration of each sample is adjusted to within the working calibration range. When sample size permits all samples will be diluted using volumetric glassware. When sample size is limited dilutions must be performed using analytical syringes. Before the samples are diluted, the volume of the stock solution prepared in either F.2.b. or F.2.d. must be calculated and added to the sample dilution scheme. The final concentration of the internal standard in the samples will be either 11 μg/mL for HI-6 or 25 μg/mL for MMB-4.

5. Instrument Calibration: Instrument calibration must be performed each time quantitation of samples is required. The instrument calibration is performed by injecting the analytical standards prepared in Section F.2.f and F.2.g. A complete set of analytical standards is analyzed prior to analysis of any samples. Once the initial calibration and standards have been analyzed and the linearity confirmed, the sample may begin with at least every sixth sample injected being an analytical standard or blank to check the performance of the instrument. A complete set of analytical standards is analyzed following the last sample. All analytical standards analyzed are used to develop a complete calibration curve for quantitation of the samples. No sample amount may be reported that exceeds the range of the analytical standards. Samples that yield responses less than the calibration range will be reported as less than the lower quantitation limit. Any sample response that exceeds the largest analytical standard will be reported as greater than the highest analytical standard, and must be either diluted to within range or the calibration range extended for quantification of the sample.

6. Analysis of Analytical Blank: The appropriate analytical blank prepared in Section F.2.f and F.2.g is analyzed to determine the quality of the reagents used, and to determine if the presence of sample matrix components interfere with the analysis method.
7. **Analysis of Samples:** Samples and calibration standards are analyzed using the procedures described in Section F.5. At least every sixth analysis should be a analytical standard or blank.

8. **Calculations:** The sample amounts are calculated using an internal standard method of calibration. The calibration data is analyzed using a linear regression model to estimate the parameter values for the model.

   a. Using a simple linear regression program, enter the peak area ratio of HI-6 to MB-4 as the ordinate (y-value) and the corresponding standard concentration as the abscissa (x-value). The regression model used to generate the slope, intercept, and correlation coefficient for HI-6 in the calibration data is:

   \[ y = bx + a \]

   b. If a regression program is not available, program the following calculations:

   \[ b = \frac{[\sum(xy)-\sum(x)\sum(y)]}{[\sum(x^2)-\left(\sum(x)\right)^2]} \]

   \[ a = \frac{\sum(x)\sum(xy)-\sum(x)\sum(y)}{[\sum(x^2)-\left(\sum(x)\right)^2]} \]

   \[ r = \frac{[\sum(x^2)(\sum(y^2))-(\sum(x)\sum(y))^2]}{[\sum(x^2)-\left(\sum(x)\right)^2][\sum(y^2)-\left(\sum(y)\right)^2]} \]

   where,

   \[ y = ax + b \]
   \[ a = \text{slope} \]
   \[ b = \text{y-intercept} \]
   \[ r = \text{correlation coefficient} \]
   \[ x = \text{analyte peak area/internal standard peak area} \]
   \[ y = \text{analyte amount in \( \mu g/mL \)} \]
   \[ n = \text{number of replicates} \]

   c. All data points obtained from the analysis of the analytical standards are used to calculate the regression model estimates. In addition the percent relative standard deviation (%RSD) between replicate standards should be monitored to insure that the instrument response does not change excessively during analysis.
The %RSD for any set of analytical standards should not be greater than 10 percent. If the %RSD for any set is greater than 10 percent, the analysis is stopped and the problem corrected prior to resuming analyses. Do not include the results of the analytical blank analyses in the calibration calculations as this will weigh the regression toward zero.

d. Identify the analyte and internal standard peaks in the unknown sample chromatograms and record each peak area. The ratio of the analyte vs the internal standard is calculated and the value recorded. Using the regression values calculated from the calibration data, calculate the found concentration for each sample using the formula above.

9. Column Clean-Up: After each set of samples are analyzed, the column must be cleaned to remove proteins and peptides adhering to the stationary phase. This is accomplished by flushing the column with a series of solvents over a period of time. The sequence recommended for this clean-up is:

Millipore water at 1.5 mL/min for 60 min.

ACN at 1.5 mL/min for 10 min.

IPA at 1.0 mL/min 10 min.

50:50 IPA/chloroform ramped program
   1.5 mL/min - 2.0 mL/min in 10 min
   - 2.0 mL/min for 50 min

IPA at 1.5 mL/min for 5 min.

ACN at 1.5 mL/min for 10 min.

10. Instrument Shut-Down: When the instrument is not to be used for extended periods of time, the system must be shut down following manufacturer's instructions to ensure column life and instrument stability. The column clean-up procedure (Section G.10) is followed and the column is stored with 100 percent ACN wetting the stationary phase. The column must be tightly capped during storage to prevent solvent evaporation. The column must never be stored with buffer remaining on the support.

G. Emergency Procedures: All personnel involved in the HML or MREF laboratory operations, must be familiar with the respective laboratory's FSSP, and the emergency procedures detailed within this document. All personnel involved in the King Avenue operation must be familiar with HEG H/SP 8-01 and the emergency procedures detailed within this document.
H. First Aid Procedures: First aid and self aid at the MREF and HML are to be conducted as specified in the FSSP.

TLH: cah
APPENDIX C

INDIVIDUAL ANIMAL PATHOLOGY DATA
Animal No. 89-1546
Necropsy Date: 4/24/90
Group: HI-6 Treatment Efficacy
Dose: 13.0 μg/kg GD
Necropsy Results: Heart, epicardial surface: hemorrhage, minimal.
Histopathology Results: Not applicable.

Animal No. 89-1558
Necropsy Date: 6/12/90
Group: HI-6 Treatment Efficacy
Dose: 32.0 μg/kg GD
Necropsy Results: Lung, multiple lobes: hemorrhage/congestion, mild
Comment: Minimal yellow-green foam in bronchi; this monkey may have aspirated some stomach contents as a terminal event. Stomach very distended with yellow/green fluid.
Histopathology Results: Not applicable.

Animal No. 89-1572
Necropsy Date: 6/13/90
Group: HI-6 Treatment Efficacy
Dose: 42.1 μg/kg GD
Necropsy Results: No gross lesions found.
Histopathology Results: Not applicable.
<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Necropsy Date</th>
<th>Group</th>
<th>Dose</th>
<th>Necropsy Results</th>
<th>Histopathology Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>89-1335</td>
<td>4/13/90</td>
<td>HI-6</td>
<td>34.7 µg/kg GD</td>
<td>Liver: adhesions, fibrous, minimal.</td>
<td>Not applicable</td>
</tr>
<tr>
<td>89-1588</td>
<td>6/13/90</td>
<td>HI-6</td>
<td>28.4 µg/kg GD</td>
<td>No gross lesions found.</td>
<td>Not applicable</td>
</tr>
<tr>
<td>89-1591</td>
<td>4/24/90</td>
<td>HI-6</td>
<td>18.3 µg/kg GD</td>
<td>No gross lesions found.</td>
<td>Not applicable</td>
</tr>
</tbody>
</table>
Animal No. 89-1602
Necropsy Date: 4/24/90
Group: HI-6 Treatment Efficacy
Dose: 25.9 µg/kg GD
Necropsy Results: Brain, subdural: hemorrhage, moderate
Comment: Subdural hemorrhage presumably associated with trauma/convulsions.
Heart, epicardial surface: hemorrhage, minimal
Histopathology Results: Not applicable.

Animal No. 89-1604
Necropsy Date: 4/19/90
Group: HI-6 Treatment Efficacy
Dose: 30.1 µg/kg GD
Necropsy Results: Heart, epicardial surface: hemorrhage, minimal
Histopathology Results: Not applicable.

Animal No. 89-1618
Necropsy Date: 4/19/90
Group: HI-6 Treatment Efficacy
Dose: 14.7 µg/kg GD
Necropsy Results: No gross lesions found.
Histopathology Results: Not applicable.
Animal No. 89-1632
Necropsy Date: 4/18/90
Group: HI-6 Treatment Efficacy
Dose: 25.1 µg/kg GD
Necropsy Results: No gross lesions found.
Histopathology Results: Not applicable.

Animal No. 89-1900
Necropsy Date: 6/13/90
Group: HI-6 Treatment Efficacy
Dose: 21.5 µg/kg GD
Necropsy Results: Testis, right: aplasia
Comment: Incidental to GD exposure.
Histopathology Results: Not applicable.

Animal No. 39-1536
Necropsy Date: 4/17/90
Group: MMB-4 Treatment Efficacy
Dose: 11.0 µg/kg GD
Necropsy Results: No gross lesions found.
Histopathology Results: Not applicable.
<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Necropsy Date</th>
<th>Group</th>
<th>Treatment Efficacy</th>
<th>Dose</th>
<th>Necropsy Results</th>
<th>Histopathology Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>89-1552</td>
<td>4/17/90</td>
<td>MM8-4</td>
<td></td>
<td>15.7 μg/kg GD</td>
<td>Skeletal muscle, temporalis: hemorrhage</td>
<td>Not applicable.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Comment: Compatible with convulsion-associated trauma.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Skin, lips: ulcers, multiple, subacute</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Comment: Possible ruptured vesicles; believed to be viral in etiology and incidental to GD exposure.</td>
<td></td>
</tr>
<tr>
<td>Animal No.</td>
<td>4/26/90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>89-1566</td>
<td>5/23/90</td>
<td>MM8-4</td>
<td></td>
<td>9.3 μg/kg GD</td>
<td>No gross lesions found.</td>
<td>Not applicable.</td>
</tr>
<tr>
<td>Animal No.</td>
<td>4/26/90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>89-1584</td>
<td>4/26/90</td>
<td>MM8-4</td>
<td></td>
<td>8.4 μg/kg GD</td>
<td>Heart, L &amp; R ventricles, epicardial surface: hemorrhage, ecchymotic, multifocal, mild</td>
<td>Not applicable.</td>
</tr>
</tbody>
</table>
Animal No. 89-1520
Necropsy Date: 4/17/90
Group: MM9-4 Treatment Efficacy
Dose: 29.8 µg/kg GD
Necropsy Results: Skin, lips: crusts, multiple
Comment: Appear to be healing vesicles, interpreted to be viral-induced.
Histopathology Results: Not applicable.

Animal No. 89-1524
Necropsy Date: 4/24/90
Group: MM9-4 Treatment Efficacy
Dose: 12.8 µg/kg GD
Necropsy Results: Skeletal muscle, L temporalis: hemorrhage
Comment: Hemorrhage presumably related to trauma associated with convulsions.
Histopathology Results: Not applicable.

Animal No. 89-1634
Necropsy Date: 4/24/90
Group: MM9-4 Treatment Efficacy
Dose: 9.7 µg/kg GD
Necropsy Results: No gross lesions found.
Histopathology Results: Not applicable.
Animal No. 89-1600
Necropsy Date: 6/14/90
Group: LC₂₉
Dose: 4.5 µg/kg GD
Necropsy Results: No gross lesions found.
Histopathology Results:
Eye - no significant lesion (nsl)
Brain - neuronal necrosis, mild to severe
(frontal, entorhinal and parietal cortex, amygdaloid, hippocampus, thalamus, olfactory bulb)
Pituitary - nsl
Spinal Cord - nsl
Sciatic Nerve - nsl
Brachial Plexus - nsl
Ulnar Nerve - nsl
Radial Nerve - nsl
Phrenic Nerve - nsl
Adrenal Glands - mineralization, minimal
Liver - nsl
Kidneys - nsl
Lung - nsl (lung mite pigment noted)
Ileum - nsl
Stomach - nsl
Diaphragm - nsl
Biceps Muscle - myofibril degeneration and necrosis, multifocal, minimal
Common Digital Extensor Muscle - nsl
Heart - myocyte degeneration, minimal

Animal No. 89-1540
Necropsy Date: 3/22/90
Group: LD₃₀
Dose: 7.0 µg/kg GD
Necropsy Result: No gross lesions found.
Histopathology Results: Not applicable.
Animal No. 89-1578
Necropsy Date: 6/13/90
Group: LD_{50}
Dose: 6.8 \mu g/kg GD
Necropsy Results: Skeletal muscle, R t.mporalis: hemorrhage, acute, mild
Comment: Compatible with traumatic injury during convulsions.
Histopathology Results: Not applicable.

Animal No. 89-1532
Necropsy Date: 6/15/90
Group: LD_{50}
Dose: 4.9 \mu g/kg GD
Necropsy Results: No gross lesions found.
Histopathology Results:
  Eye - no significant lesion (nsl)
  Brain - neuronal necrosis, minimal to moderate
    (frontal, entorhinal and parietal cortex, amygdaloid, caudate, hippocampus, thalamus, midbrain)
  Pituitary - nsl
  Spinal Cord - nsl
  Sciatic Nerve - nsl
  Brachial Plexus - nsl
  Ulnar Nerve - nsl
  Radial Nerve - nsl
  Phrenic Nerve - nsl
  Adrenal Glands - nsl
  Liver - fatty change, mild, diffuse
  Kidneys - nsl
  Lung - nsl
  Ileum - nsl
  Stomach - nsl
  Diaphragm - no section available for micro exam
  Biceps Muscle - nsl
  Common Digital Extensor Muscle - myofibril degeneration and necrosis, focal, minimal
  Heart - nsl
Animal No. 89-1625
Necropsy Date: 3/28/90
Group: LD₉₅
Dose: 6.0 μg/kg GD
Necropsy Results:
- Brain, meninges: congestion, diffuse
- Heart, epicardial surface: congestion, diffuse
- Lung: adhesions, multiple
- Thymus: hemorrhage, petechial, multiple

Histopathology Results: Not applicable.

Animal No. 89-1638
Necropsy Date: 6/15/90
Group: LD₉₅
Dose: 5.0 μg/kg GD
Necropsy Results: No gross lesions found.

Histopathology Results:
- Eye - no significant lesion (nsl)
- Brain - neuronal necrosis, minimal to moderate
  (frontal, entorhinal and parietal cortex, amygdaloid, hippocampus,
  thalamus, pons, olfactory bulb)
- Pituitary - nsl
- Spinal Cord - nsl
- Sciatic Nerve - nsl
- Brachial Plexus - nsl
- Ulnar Nerve - nsl
- Radial Nerve - nsl
- Phrenic Nerve - nsl
- Adrenal Glands - nsl
- Liver - fatty change, minimal, diffuse
- Kidneys - tubular necrosis, multifocal, mild
- Lung - nsl
- Ileum - nsl
- Stomach - nsl
- Diaphragm - no section available for micro exam

Biceps Muscle - myofibril degeneration and necrosis, multifocal, minimal
Common Digital Extensor Muscle - myofibril degeneration and necrosis,
  disseminated, moderate
- Heart - nsl
Animal No. 89-1640
Necropsy Date: 3/20/90
Group: LD₅₀
Dose: 8.8 µg/kg GD
Necropsy Results: No significant lesions found.
Note: Bruise over L eyebrow and R temporalis muscle compatible with incidental trauma of convulsions.
Histopathology Results: Not applicable.