DEPARTMENT OF THE ARMY
U S ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
FORT DETRICK, FREDERICK, MD 21702-5012

AD-B151643

MCMR-RMI-S (70-1y)

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OMI, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statements

1. The U.S. Army Medical Research and Materiel Command, has reexamined the need for the limited distribution statement on technical reports for Contract No. DAMD17-89-C-9050. Request the limited distribution statement for AD Nos. **ADB162425, ADB155185, ADB151643, ADB173995, ADB154041, ADB154015 and ADB165708**, be changed to "Approved for public release; distribution unlimited," and that copies of these reports be released to the National Technical Information Service.

2. The point of contact for this request is Ms. Judy Pawlus, DSN 343-7322.

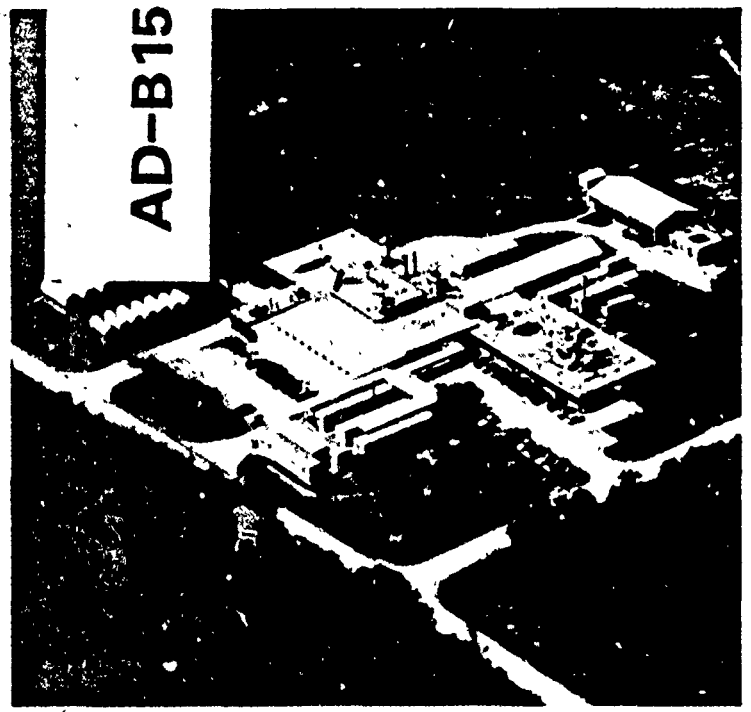
FOR THE COMMANDER

for Cornelius R. Fay III
CORNELIUS R. FAY III
Lieutenant Colonel, MS
Deputy Chief of Staff for
Information Management

REPORT

DAMD17-89-C-9050

FINAL REPORT



Task 89-08: An Efficacy and Pharmacokinetic Evaluation of a Dose of Diazepam That Will Reduce The Incidence of Convulsions in Indian Rhesus Monkeys Pretreated With Pyridostigmine Bromide, Challenged With Soman, and Treated With Atropine and Pralidoxime Chloride With The Diazepam

To

U.S. Army Medical Research and Development Command

Battelle
Putting Technology To Work

DECEMBER, 1990

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Distribution authorized to U.S. Government Agencies and their contractors: critical technology. 14 Dec 90. ATTN: SGRD-RMI-S
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Battelle Memorial Institute; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION: U.S. Army Medical Research Insitutue of Chemical Defense
6c. ADDRESS (City, State, and ZIP Code): 505 King Avenue, Columbus, Ohio 43201-2693	7b. ADDRESS (City, State, and ZIP Code): Aberdeen Proving Ground, MD 21010-5425
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: Contract No. DAMD17-89-C-9050
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 63751A; PROJECT NO. 3M4-63751A; TASK NO. BB; WORK UNIT ACCESSION NO. 023
11. TITLE (Include Security Classification): Olson, C.T., Dill, G.S., Menton, R.G., Kiser, R.C., Hayes, T.L., Snider, T.H., Singer, A.W.	
12. PERSONAL AUTHOR(S)	
13a. TYPE OF REPORT: Final Report; 13b. TIME COVERED: FROM 890501 TO 901101; 14. DATE OF REPORT (Year, Month, Day): 12/90; 15. PAGE COUNT: 233	
16. SUPPLEMENTARY NOTATION: Task 89-08: An Efficacy and Pharmacokinetic Evaluation of a Dose of Diazepam That Will REduce the Incidence of Convulsions in Indian Rhesus Monkeys Pretreated with Pyridostigmine Bromide, Challenged with Soman, and Treated with Atropine and Pralidoxime Chloride with the Diazepam	

17. FIELD	GROUP	SUB-GROUP
06	15	
06	20	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number): Diazepam, rheus monkeys, Soman (GD) pyridostigmine, atropine, pralidoxime chloride (2-PAM), convulsions, pharmacokinetics, efficacy.

19. ABSTRACT (Continue on reverse if necessary and identify by block number): A study was initiated to determine the smallest dose of diazepam that would result in no more than a 20 percent incidence of convulsions in male rhesus monkeys pretreated with pyridostigmine, challenged with soman, and post treated with atropine and 2-PAM in conjunction with diazepam. Additionally, the pharmacokinetic parameters for diazepam in monkeys were determined using the effective dose of diazepam determined in the efficacy portion. The estimated 90 min diazepam ED 80 was 112 micrograms/kg and the 4 hr ED 80 was 230 micrograms/kg both with wide confidence limits. The pharmacokinetics were run at 70, 110, and 220 micrograms/kg of diazepam.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT: ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ OTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION: Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Mary Frances Bostian	22b. TELEPHONE (Include Area Code): 301-663-7327; 22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

FINAL REPORT

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

on

TASK 89-08: AN EFFICACY AND PHARMACOKINETIC EVALUATION OF A DOSE OF DIAZEPAM THAT WILL REDUCE THE INCIDENCE OF CONVULSIONS IN INDIAN RHESUS MONKEYS PRETREATED WITH PYRIDOSTIGMINE BROMIDE, CHALLENGED WITH SOMAN, AND TREATED WITH ATROPINE AND PRALIDOXIME CHLORIDE WITH THE DIAZEPAM

to

U.S. ARMY MEDICAL RESEARCH
AND DEVELOPMENT COMMAND

December, 1990

Dr. Carl T. Olson
Dr. Garrett S. Dill
Dr. Ronald G. Menton
Ms. Robyn C. Kiser
Mr. Timothy L. Hayes
Mr. Thomas H. Snider
Dr. Allen W. Singer

BATTELLE COLUMBUS OPERATIONS
505 King Avenue
Columbus, OH 43201-2693

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health (NIH), Publication No. 86-23, Revised 1985).

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FINAL REPORT

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TASK 89-08: AN EFFICACY AND PHARMACOKINETIC EVALUATION OF A DOSE OF DIAZEPAM THAT WILL REDUCE THE INCIDENCE OF CONVULSIONS IN INDIAN RHESUS MONKEYS PRETREATED WITH PYRIDOSTIGMINE BROMIDE, CHALLENGED WITH SOMAN, AND TREATED WITH ATROPINE AND PRALIDOXIME CHLORIDE WITH THE DIAZEPAM

to

U.S. ARMY MEDICAL RESEARCH
AND DEVELOPMENT COMMAND

December, 1990

Garrett S. Dill, D.V.M. — Date 12/4/90
Principal Investigator

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QUALITY ASSURANCE STATEMENT

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

Phase	Date
MREF Protocol 52 review	05/24/89
Syringe preparation, weighing of syringes, dosing, clinical observations, acetylcholinesterase assays	10/26/89, 11/6/89
Body weights, blood collection, restraint board acclimation	10/26/89, 11/6/89
Data audits	7/5/89, 11/3/89, 11/29/89, 1/3/90, 2/14/90, 4/4/90, 5/2/90, 7/16/90
Draft Final Report audit	9/25/90
Final Report audit	10/9/90

Report to study director and management: 7/5/89, 11/3/89, 11/6/89, 11/29/89, 1/3/90, 2/14/90, 4/4/90, 5/2/90, 7/16/90, 9/25/90

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

Richard H. Stewart 10-11-90

Quality Assurance Unit Date
Health and Environment Group

GOOD LABORATORY PRACTICES COMPLIANCE STATEMENT

To the best of my knowledge, all aspects of the efficacy portion of this study were conducted in compliance with the U.S. Food and Drug Administration's Good Laboratory Practices regulations (21 CFR Par. 58). 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 10-4-90

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

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1.0 INTRODUCTION

Current standard therapy in research with non-human primates exposed to Soman (pinacolyl methylphosphonofluoridate; GD) is pretreatment with pyridostigmine bromide and treatment with atropine and pralidoxime chloride (2-PAM). GD-induced convulsions often occur during these studies. Because GD-induced convulsions have been shown to increase the incidence of brain lesions in non-human primates,[1,2] it is likely that similar lesions could occur in man. It is therefore desirable to add an anticonvulsant to the treatment regimen for nerve agent intoxication to prevent convulsions and to increase the chances of survival and return to normalcy. This task was initiated at Battelle's Medical Research and Evaluation Facility (MREF) to determine the smallest dose of diazepam (ED_{50}) which results in no more than a 20 percent incidence of convulsions in monkeys pretreated with pyridostigmine bromide, exposed to 5 X LD_{50} dose of GD, and given a standard treatment regimen of atropine and 2-PAM in conjunction with diazepam. A second objective of this task was to estimate pharmacokinetic parameters for diazepam in monkeys from this same population using an effective anticonvulsant dose of diazepam, as determined in the efficacy portion of this task, and one dose above and one dose below this level. Pharmacokinetic investigations were conducted using a cross-over design -- all three diazepam doses being given to each of nine animals with approximately one month between doses.

2.0 EXPERIMENTAL DESIGN

2.1 Test Animals

Male rhesus monkeys, Macaca mulatta, were specified for this study because there is considerable scientific evidence that the monkey is predictive of responses in man. Male rhesus monkeys exhibit pyridostigmine, atropine, and 2-PAM pharmacokinetics similar to that of human beings.[3]

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.[4] Monkeys for use in this study were provided by the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD).

Monkeys were housed individually in stainless-steel cages approximately 24 inches wide, 34 inches high, and 26 inches deep. Room temperatures were maintained at 77 ± 5 F and relative humidity at 50 ± 10 percent. (A small number of excursions outside these ranges occurred and are on file at the MREF.) Fluorescent lighting on a light/dark cycle of 12 hr each per day was used. Purina Certified Primate 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 ad libitum 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 food or water.

All animals arrived with tattoos so that positive identification could be maintained. Monkeys were maintained in quarantine for one month, during which time they were examined by the study veterinarian and blood samples taken for hematology, serum chemistries, and erythrocyte acetylcholinesterase (AChE) activity measurements. One monkey was found to have an abnormal, palpable mass in the abdomen. Upon further diagnostic evaluation, including radiography and examination of a biopsy specimen, this animal was found to have bilateral dilatation of renal pelves and ureters, a low urine specific gravity, fluid surrounding the left kidney, and a subacute tubulointerstitial nephropathy. This animal, 6TN, was eliminated from the study group. Fecal samples were taken for intestinal parasite evaluation, and three tuberculin tests were performed at two week intervals. Because of a problem with chronic diarrhea in some animals and the passage of large numbers of tapeworms, all monkeys were treated once with praziquantel (Droncit®) using a 56.8 mg/mL solution at 0.1 mL/kg given intramuscularly (IM). This was

accomplished after quarantine but more than a month prior to the start of studies. Because monkeys were relatively immature and light in weight compared to other monkeys used in studies at the MREF, the start of the experiment was delayed approximately 4 months.

Based upon results of physical examinations and clinical laboratory findings, all monkeys other than 6TN were found to be acceptable for study. Monkeys were randomized, based on body weight, into a group of 10 animals for a GD 48-hr LO_{50} determination, 50 animals for efficacy testing, and nine for pharmacokinetic studies to obtain group homogeneity of weight, as possible, across phases and stages of the experiment. Prior to the start of studies, monkeys were acclimated to placement on a slotted, V-shaped platform where limbs were restrained by lanyards. This restraint was used for obtaining body weights and blood samples (femoral venipuncture), for placing catheters within the saphenous vein for pharmacokinetic studies, for pretreating with pyridostigmine (nasogastric tube), and for injection of GD and treatment compounds.

2.2 Materials and Methods

For diazepam efficacy evaluations, atropine and 2-PAM were supplied by USAMRICD. Pyridostigmine bromide in a syrup base (Mestinon®) was purchased locally and diazepam (Valium®) was obtained from Roche Laboratories (Nutley, NJ). Pyridostigmine bromide identity was confirmed by nuclear magnetic resonance (NMR) and concentration determined by high performance liquid chromatography (HPLC; MREF SOP-89-58). Verification and analysis of atropine (MREF SOP 89-55), 2-PAM (MREF SOP 88-39), and diazepam (MREF SOP 89-63) solutions were accomplished by HPLC.

GD was supplied by USAMRICD. Purity of GD stored at Battelle is periodically confirmed by Battelle chemists. For animal dosing, solutions of a nominal 1.50 mg/mL GD in physiologic saline were prepared for this study. Six-mL aliquots were stored in amber 10-mL serum vials in an approximately -70 C freezer. After preparation, samples of these stock solutions, and after each dosing day, samples of dosing aliquots were analyzed by gas chromatography (MREF SOP 88-31).

injections. Individually labeled syringes were loaded with the calculated volume of GD (based on animal weights taken at the time of the last pyridostigmine dosing) for each monkey in that group prior to the start of GD dosing. Syringes were weighed, placed on ice until used, and after each day's dosing was completed, syringes were reweighed to determine weight loss and calculate the volume delivered. Pre- and post-treatment weighing was also accomplished with those syringes used to inject atropine, 2-PAM, and diazepam. Monkeys were returned, immediately after treatment, to an animal holding room where video recordings were made of each animal. A 40-min time period following dosing of each animal was allowed for taping. All monkeys were closely and continuously observed for a minimum of 2 hr following dosing and at intervals thereafter with observations annotated at 4, 6, 8, 12, 18, 24, 36, and 48 hr. Signs specifically monitored were muscle fasciculations, tremors, convulsions, prostration, salivation/bronchial discharge, miosis/mydriasis, and death.

It was recognized prior to the start of the study that a consistent interpretation of the incidence of convulsions was required. The study director and two technician supervisors from Battelle along with four principal investigators from USAMRICD reviewed videotapes of monkeys given GD at USAMRICD. A videotape was also sent to Battelle for training additional technicians. Signs of intoxication for the first two hours were consistently recorded by the same four experienced, trained personnel, and in case of doubt about occurrence of a particular sign, a consensus was obtained with concurrence of the study director. Additional experience was obtained by observing animals in the LD_{50} phase of the study conducted prior to the diazepam efficacy portion. As further experience was gained, definitions for fasciculations, tremors, and convulsions were refined. Fasciculations were defined as local areas of involuntary muscular contraction and relaxation as exemplified by a tic or twitch. Tremors were defined as involuntary trembling or quivering of a limb or portion of the body due to repeated contraction and relaxation of groups of muscles as exemplified by shivering. Convulsions were further defined as rapid, repetitive, violent, involuntary, often rhythmic, muscular contractions usually involving the whole body and resulting in positional changes and associated with an altered state of consciousness.

Occasionally, an animal would experience what was called a spasm, a short duration stiffening of the whole body not immediately followed by a similar event.

Monkeys that died prior to the end of the 48-hr observation period in both the LD_{50} and efficacy phases of the study were necropsied by an experienced veterinary pathologist and gross lesions recorded. Two monkeys which died following the 48-hr observation period were necropsied and tissue samples collected for histopathologic evaluation. One monkey was anesthetized at the end of the 48-hr observation period, tissues perfusion-fixed and samples taken for histopathologic evaluation. Following necropsy, all animal remains were incinerated.

Nine monkeys were used in a diazepam pharmacokinetic study using three different doses of diazepam (selected by USAMRICD personnel following completion of the efficacy phase of the study) in each monkey with an approximately 1-month recovery period between administration of doses. Prior to the start of this study, monkeys were acclimated to placement in a restraint chair. A 21-gauge, 4.5-inch long intravenous catheter (Intrafusor®, Sorenson Research Co., Salt Lake City, UT) for blood collection was introduced through a 19-gauge, 1.5-inch needle into a saphenous vein, and the monkey was placed in a restraint chair so that he could not remove the catheter. The selected dose of diazepam was injected in the area of the vastus lateralis muscle of the opposite leg from that catheterized. Each syringe was weighed after being loaded with the calculated desired volume of diazepam solution and reweighed after injection of the diazepam. The measured density of the diazepam solution (1.0183 g/mL) was used to determine the actual volume of diazepam delivered. On each day of experimentation, an equal number of monkeys was given each dose of diazepam. One and one-half mL blood samples were obtained prior to dosing and at 2.5, 5, 10, 15, 25, 40, 60, 90, 120, 180, and 240 min after dosing using a heparinized disposable 3-mL syringe (Becton Dickinson, Rutherford, NJ). A 0.5-mL volume of heparinized saline (30 units of heparin/mL solution) was placed in the catheter between blood sampling times to prevent the formation of a clot in the catheter. A 0.7-mL volume of heparinized saline and blood was removed from the catheter prior to drawing the 1.5-mL blood sample for diazepam analysis. Following the drawing of the

240-min blood samples, catheters were removed and after any bleeding had been controlled, monkeys were returned to their cages. Blood samples at 480 and 1,440 min were obtained by femoral venipuncture in the same leg used for prior blood sampling using a 2-mL heparinized vacutainer® (Becton Dickinson, Rutherford, NJ) and a 22-gauge, 1-inch needle.

Blood samples were centrifuged for six min at approximately 1,500 x G and the plasma removed by transfer pipette, placed in a clean, labeled polypropylene tube and stored at approximately -70 C until analyzed. On each day of analysis, plasma samples for all time points from three monkeys which received different diazepam dose levels were prepared. Extraction and analysis procedures are described in MREF SOP-89-60, Analysis of Serum or Plasma Samples for Diazepam and Metabolite, Desmethyldiazepam, by Gas Chromatography (GC) (See Appendix B). Briefly, known volumes of plasma (samples and diazepam calibration standards alike) were brought to room temperature, spiked with a medazepam® (a benzodiazepine with a distinct and separate GC peak; Sigma No. M-0521, lot 35F-0255) solution surrogate extraction control, mixed with benzene for 30 min on a rotary extraction apparatus, and centrifuged for 30 min at approximately 1,500 x G. Five hundred microliters of the benzene (top) layer were transferred to a GC autosampler vial. Each 500 µL extract was spiked with midazolam® (another benzodiazepine; Hoffmann-LaRoche No. RO 21-3981/000, lot 022115), which acted as an analytical internal standard, and vortex mixed for 10 sec. Samples were analyzed by GC using a 25 m x 0.32 mm inside diameter RSL-300 (bonded OV-17, Alltech Associates, Deerfield, IL) column and a nitrogen phosphorous detector. Calibration standards with concentrations in the area of interest were interspersed with samples and analyzed, and for each calibration standard and sample injection, a corrected peak area ratio (CPAR) was calculated. The diazepam peak area was divided by the internal standard midazolam peak area to correct for detection efficiency. This was then divided by the quotient of the medazepam peak area divided by the midazolam peak area to correct for extraction efficiency. The result was then divided by the average of the medazepam peak area/midazolam peak area for all calibration standards to normalize the data. Using a linear regression program, the slope, intercept, and correlation coefficient of the diazepam CPAR versus diazepam concentration

of calibration standards were generated. The resulting standard calibration curve was used to determine concentrations of diazepam in the samples. Similar procedures were used to determine the concentration of desmethyldiazepam, an active metabolite of diazepam, in each sample. (See Appendix D.) When diazepam analyses were completed, plasma concentrations as a function of time, maximum concentrations, time to maximum concentrations, areas under the plasma concentration-time curves, absorption and elimination rate constants, and apparent volumes of distribution were estimated.

The analytical methodology for diazepam developed for this task has virtually 100 percent diazepam extraction efficiency from plasma or serum, and is quantitative between concentrations of 5 and 500 ng/mL with less than a 10 percent relative error in analysis. A letter report[5] describing the technique was submitted to USAMRICD prior to the pharmacokinetic study, and a validation of the technique was performed by analyzing diazepam-spiked plasma samples supplied by USAMRICD.[6]

Prior to the start of the pharmacokinetic study, a blood sample from each monkey to be used in the study was obtained from a femoral vein using a heparinized vacutainer. The blood was centrifuged and the plasma removed and stored in a freezer at approximately -70 C until it was shipped on dry ice to the laboratory of Dr. David J. Greenblatt, Division of Clinical Pharmacology, Tufts-New England Medical Center. Dr. Greenblatt determined the percent of diazepam-plasma protein binding in these monkey plasma samples using radiolabeled diazepam and dialysis techniques.[7]

2.3 Statistical Analyses

Monkeys were dosed one or two at a time using a modified up-down approach 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 by a statistician in order to most efficiently estimate the 48-hr GD LD_{50} in the present population of monkeys. The experiment was designed to use a maximum of ten monkeys to determine the

48-hr GD LD_{50}, but if, after a minimum of three monkeys had been challenged, the estimated LD_{50} fell within the 95 percent confidence limits of Battelle's historic Indian rhesus monkey 48-hr LD_{50}, that historic LD_{50} value would be accepted as the approximate LD_{50} for the present population of monkeys and further experimentation for determination of the LD_{50} would not be conducted.

A GD dose of five times the 48 hr GD LD_{50} of untreated monkeys would be used to assess the efficacy of diazepam in reducing the incidence of convulsions in monkeys pretreated with pyridostigmine bromide and treated with atropine and 2-PAM in conjunction with the diazepam. Assuming that at 5 X GD LD_{50} a diazepam dose-convulsion incidence response exists, a stagewise design experiment, using different doses of diazepam, was to be used to determine the minimum dose of diazepam that resulted in no more than a 20 percent incidence of convulsions in monkeys given 5 X the GD LD_{50}. Diazepam doses were to be selected by a statistician based upon predictions of the dose-response curve slope and estimated percentiles of response based on probit analyses. As data were obtained, all information available would be used to select the diazepam doses for the succeeding animals in the next stage of experimentation. This approach allows estimation of the diazepam dose-convulsion response relationship with a minimal number of animals. The number of monkeys required to determine a diazepam dose which would limit the incidence of convulsions to 20 percent is dependent upon the slope of the diazepam dose-convulsion response curve and the degree of accuracy required in the estimate. The study was designed to end when a 10 percent or less standard error in the estimate of the required diazepam dose was reached or when a maximum of 50 monkeys had been tested.

3.0 RESULTS

3.1 Chemistry

Pyridostigmine bromide syrup (Mestinon® manufactured by Roche Laboratories, Nutley, NJ) with a reported concentration of 12 mg/mL was purchased locally. Confirmation of identity of pyridostigmine bromide was confirmed by NMR, and HPLC confirmed a concentration of approximately 12 mg/mL

which was used to determine volumes of doses to be delivered intragastrically. Atropine identity was confirmed and concentration determined by HPLC. Atropine sulfate equivalent concentration was determined to be 2.74 mg/mL (2.28 mg atropine free base per mL of solution), and atropine treatments were given IM using a volume to deliver desired amounts of atropine free base per kilogram body weight. 2-PAM identity was confirmed and concentration determined by HPLC. Concentration of 2-PAM was determined to be 301 mg/mL and animals were dosed at 25.71 mg/kg body weight using this concentration to determine volumes of 2-PAM to inject. Diazepam (Valium®) was received from Roche Laboratories (Batch 0085, Lot # NDC 0140-1931-06) at a reported concentration of 5 mg/mL. HPLC analysis confirmed a 5 mg/mL concentration and this figure was used to determine volume of treatments.

Two separate GD dosing solutions were prepared and used in this task. The first, in which ten 6-mL aliquots of GD in physiologic saline were prepared and frozen at approximately -70 C, was used in LD_{50} studies and in the first four stages of the diazepam efficacy evaluation. GC analyses of this dosing solution gave a 1,470 µg/mL concentration. A second dosing solution, prepared 3 months later and used in the fifth through tenth stages of diazepam efficacy testing, was analyzed at 1,450 µg/mL. Chemical analyses of daily dosing solutions yielded an average within 6 percent of these initial analyzed concentrations.

3.2 GD LD_{50} Study

A modified up-down type approach was used to estimate the 48-hr GD LD_{50} in untreated monkeys of this population. The only historic Battelle 48-hr GD LD_{50} information in rhesus monkeys of Indian origin is from Task 85-18, and the LD_{50} in that task was reported as 15.2 µg/kg body weight. After a few animals were exposed to GD under Task 89-08, it became apparent that the 48-hr GD LD_{50} in the present population of monkeys was considerably less than 15.2 µg/kg. A total of ten untreated monkeys were challenged with GD in Task 89-08 and the doses and results are listed in Table 1.

TABLE 1. RESULTS OF TASK 89-08 48 HR GD LD_{50} STUDY

Animal ID	Weight (kg)	GD Dose (μg/kg)	Onset of Convulsions	Outcome
79B	3.9	12.8	None	Died at 14 min
6EA	2.9	9.3	7 min	Died at 23 min
6PS	3.3	7.7	8 min	Lived
6PF	3.3	7.4	8 min	Died at 37 min
6LF	3.0	7.0	5 min	Died about 74 hr
6EM	3.4	6.4	10 min	Died at 29 hr
6EU	3.3	6.3	17 min	Lived
78N	2.9	5.9	23 min	Lived
69E	3.3	5.7	8 min	Died at 51 hr
6FS	3.3	5.1	22 min	Lived

Historical data from 36 monkeys of Indian origin from Task 85-18, 19 Chinese monkeys from Task 87-34, and 9 Chinese monkeys from Task 89-12 were combined with the data from Task 89-08 to estimate the slope of the GD dose-lethal response curve. The historical data were included to increase the precision of the slope estimate. A probit dose-response model was fitted to the 48-hr lethality data using the logarithm of the GD dose as the independent variable. This model assumed that the dose-response relationships for the four groups of monkeys had a common slope, but different intercepts. The estimate of the common slope was 12.1, with a standard deviation of 2.86 and 95 percent confidence limits of 6.3 to 17.8. Table 2 displays the 48-hr GD LD_{50}s with 95 percent confidence intervals for monkeys from each task, based on the results of the common slope model. The estimate of the 48-hr GD LD_{50} for the untreated monkeys studied in Task 89-08 was 7.4 μg/kg, with a 95 percent confidence interval of 6.1 to 9.3 μg/kg. The estimated 48-hr GD LD_{50} in untreated Indian monkeys of Task 89-08 is identical to that estimated, based on weight differentials of syringes, in 19 rhesus monkeys of Chinese origin injected by USAMRICD personnel (Task 87-34),[8] but significantly different than that estimated in rhesus monkeys of Indian origin used in Task 85-18.[3]

TABLE 2. ESTIMATED 48 HR GD LD_{50}s WITH 95 PERCENT CONFIDENCE LIMITS FOR UNTREATED MONKEYS BASED ON COMMON SLOPE MODEL

Task	Estimated LD_{50} (95 Percent Confidence Bounds) µg/kg
85-18	15.2 (13.4, 16.9)
87-34	7.4 (6.4, 8.7)
89-08	7.4 (6.1, 9.3)
89-12	6.0 (4.9, 7.5)

In addition to fitting a common slope model to the combined data from the four groups of monkeys, separate probit models were fitted to each group of monkeys. 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 the log-likelihood ratio test, the null hypothesis of a common slope 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.

3.3 Diazepam Efficacy Study

Five times the GD LD_{50}, or 37 µg/kg, was initially used to dose monkeys to evaluate the efficacy of various doses of diazepam in preventing convulsions. Six monkeys were given four doses of 1.2 mg/kg pyridostigmine bromide at 8 hr intervals (q8h), dosed with GD 4 hr following the last pyridostigmine dose and then given 0.4 mg/kg atropine free base, 25.71 mg/kg 2-PAM, and various levels of diazepam in succession at 1 min after GD injection. Two of the six monkeys were given no diazepam, and the other four received diazepam doses of 10, 24, 140, or 208 µg/kg. Only mild convulsions of relatively short duration were seen in two animals, one that received

24 μg/kg diazepam and one that received no diazepam. The monkey given no diazepam that convulsed died 18 hr following GD injection. Six additional monkeys were pretreated with pyridostigmine q8h but, based on results following GD injection in the first two monkeys, only two were given a GD, atropine, and 2-PAM regimen with no diazepam. One of the two monkeys convulsed. The low incidence of convulsions in monkeys given this challenge and therapy with no diazepam prevented an assessment of the efficacy of diazepam in lowering the incidence of convulsions. At the direction of USAMRICD, six additional monkeys were given pyridostigmine pretreatment and four were given a 37 μg/kg GD challenge and atropine/2-PAM treatment but with the dose of atropine free base limited to 0.2 mg/kg. Two of the four monkeys received no diazepam, and the other two received a diazepam dose of 20 or 44 μg/kg. One of the two monkeys which received no diazepam convulsed at 48 min and the monkey receiving 44 μg/kg diazepam convulsed at 8 hr following GD injection.

In the next stage, the GD dose was increased to 10 X GD 48-hr LD_{50} (74 μg/kg) and the atropine free base dose was kept at 0.2 mg/kg in order to increase the incidence of convulsions to a level such that the effect of diazepam on the incidence and severity of convulsions following the treatment could be evaluated. Six monkeys were pretreated with pyridostigmine, dosed with GD and treated 1 min later. Four monkeys received no diazepam, one received a dose of 47 μg/kg and one received 105 μg/kg. All animals not receiving diazepam convulsed within 90 min and neither monkey receiving diazepam was observed to convulse. This regimen was maintained in the dosing of 32 additional monkeys, using various doses of diazepam. The six monkeys pretreated with pyridostigmine but not dosed with GD, atropine, 2-PAM, or diazepam (animals 6V5, 6RX, 6N5, 69M, 6EY, and 6PC) were placed at the end of the study, at least 60 days following the first pretreatment. For experimental planning and analysis of data on convulsion incidence, the window for onset of clinically relevant convulsions was set by the USAMRICD Task Area Manager as 1 to 90 min following GD injection. One monkey (78Z) started convulsing prior to diazepam injection but ceased within the first minute of diazepam injection, and convulsions did not recur within the observation period. That diazepam dose was deemed an effective anticonvulsant and the

occurrence of convulsions in that animal was not used in statistical analyses. Even though convulsions prior to injection of diazepam or after 90 min (the biological half-life of serum atropine) were judged to be of limited significance, the incidence of convulsions during the first 4 hr following GD administration was also analyzed. The doses of diazepam used (determined by weight differential of loaded and spent syringes) and responses observed are presented in Table 3. A total of 38 monkeys were challenged with 74 μg GD/kg body weight and treated with 0.2 mg/kg atropine free base, 25.71 mg/kg 2-PAM, and various doses of diazepam. Animals 6RX (86 μg/kg diazepam) and 5VA (63 μg/kg diazepam) died prior to 90 min and were not included in the analysis of the incidence of convulsions. The convulsion incidence in ten monkeys dosed with 76.5 μg GD/kg in a preliminary diazepam efficacy study conducted at USAMRICD[9] was used to increase the precision of the slope estimate of the convulsion incidence-diazepam dose response curve, and thereby increase the precision of the diazepam 90 min ED_{50} calculated in the present experiment.

3.3.1 Analysis of Convulsion Incidence Within 90 Min

Each animal was scored as "one" if it did not convulse within the first 90 min after GD challenge, and zero if it did convulse during the first 90 min. Since convulsion incidence was anticipated to decrease with diazepam dose, absence of convulsions rather than incidence of convulsions was selected as the dependent variable. A probit dose-response model in log dose of diazepam was fitted to the 90 min convulsion results for the 36 Task 89-08 monkeys and the ten monkeys exposed at USAMRICD. Because the logarithm of zero is not defined, a small positive number was added to each diazepam dose before taking logarithms. The model assumed that the dose-response relations for the two groups of monkeys had a common slope but different intercepts. The common slope assumption allowed the use of the data from both groups of monkeys to estimate the common slope. It results in a more precise estimate of the common slope and the dose-response distribution percentiles when the assumption of a common slope is correct. The parameter estimates and results from fitting the common slope model are summarized in Table 4. The estimate of the common slope is 1.34 with a standard error of 0.41.

TABLE 3. TREATMENT REGIMEN[a] AND CONVULSION INCIDENCE FROM DIAZEPAM EFFICACY EXPERIMENT

Animal ID	Weight (kg)	GD Dose (μg/kg)	Atropine Dose (mg/kg)	Diazepam Dose (μg/kg)[b]	Onset of Convulsions	AChE Inhibition (percent)
73B	2.7	37	0.4	24	1 min	8
5U7	3.5	37	0.4	208	-	27
5W5	3.3	37	0.4	10	-	19
6PR	3.8	37	0.4	140	-	24
5SS	3.0	37	0.4	-	-	15
6F8[c]	3.5	37	0.4	-	7 min	18
6C6	3.4	37	0.4	-	-	17
6NB	3.1	37	0.4	-	9 min	15
62H	3.1	37	0.2	20	-	18
6RJ	3.0	37	0.2	44	8 hr	25
5X9	3.4	37	0.2	-	-	18
6UM	2.8	37	0.2	-	48 min	18
68F	3.1	74	0.2	-	66 min	16
6MG[d]	3.1	74	0.2	-	9 min	23
6ET	4.1	74	0.2	-	10 min	23
6DW	3.7	74	0.2	-	46 min	27
79G	3.1	74	0.2	47	-	18
5W8	3.3	74	0.2	105	-	28
6RY	3.8	74	0.2	2	1 min	28
6N6	4.0	74	0.2	9	-	39
6F2	3.5	74	0.2	28	163 min	25
78Z	3.2	74	0.2	33	-	20
77E	2.9	74	0.2	44	-	25
6R6[e]	3.0	74	0.2	92	41 min	16
5XB	3.5	74	0.2	13	-	19
5UT	3.3	74	0.2	12	4 min	17
5VA[f]	3.9	74	0.2	63	-	14
6P9	3.4	74	0.2	109	-	29
6PH	3.4	74	0.2	149	66 min	18
7DE	3.1	74	0.2	207	19 hr	15
6BK	3.5	74	0.2	130	-	17
79K	3.6	74	0.2	182	-	26
6F6	3.7	74	0.2	203	-	31
6Z9	3.4	74	0.2	242	-	26
6MJ	4.1	74	0.2	304	9.5 hr	28
61J	3.4	74	0.2	305	-	20

TABLE 3.
(Continued)

Animal ID	Weight (kg)	GD Dose (μg/kg)	Atropine Dose (mg/kg)	Diazepam Dose (μg/kg)[b]	Onset of Convulsions	AChE Inhibition (percent)
6F4	3.1	74	0.2	21	-	11
558	3.7	74	0.2	71	3.7 hr	22
78G	4.2	74	0.2	80	-	23
6VL	3.1	74	0.2	143	2 min	24
6G2	3.9	74	0.2	175	90 min	28
6XE	3.8	74	0.2	224	-	21
6EY[g]	3.8	74	0.2	67	3 min	33
6RX[h]	3.4	74	0.2	86	-	19
6V5[i]	3.4	74	0.2	128	-	14
6W7	4.1	74	0.2	164	-	26
6PC	4.6	74	0.2	62	-	0
6N5	3.4	74	0.2	92	54 min	16
5YI	3.7	74	0.2	109	107 min	19
69M	4.1	74	0.2	117	183 min	8

[a]All animals were given 4 pretreatments of 1.2 mg/kg pyridostigmine at 8 hr intervals prior to GD challenge and treated with atropine and 25.71 mg/kg 2-PAM at 1 min after GD injection.
[b]Dose of diazepam as determined by weight differentials of loaded and spent syringes.
[c]Animal died at 18 hr.
[d]Animal died at 74 hr.
[e]Animal died at 22 hr.
[f]Animal died at 52 min.
[g]Animal died at 24 hr.
[h]Animal died at 10 min.
[i]Animal died at 67 hr.

TABLE 4. PARAMETER ESTIMATES AND MODEL RESULTS FROM FITTING COMMON SLOPE MODEL TO THE 90-MIN CONVULSION RESPONSES IN MREF AND ICD MONKEYS

Non-Linear Least Squares Summary Statistics

Dependent Variable (Absence of Convulsions in First 90 min)

Source	DF	Weighted SS	Weighted MS
Regression	3	156.23	52.08
Residual	38	38.92	1.02
Uncorrected Total	41	195.15	
(Corrected Total)	40	64.28	
Sum of Loss		47.03	

Parameter[a]	Estimate	Asymptotic Standard Error	Asymptotic 95% Confidence Interval Lower	Upper
B1	1.34	0.41	0.51	2.16
B01	2.90	0.86	1.17	4.63
B02	3.10	0.75	1.55	4.59

[a]B1 is the estimate of the common slope, B01 is the estimate of the intercept for the ICD monkeys and B02 is the estimate of the intercept for the MREF monkeys.

In addition to fitting a common slope model to the combined Task 89-08 and USAMRICD monkey data, separate probit models were fitted to each group of monkeys. 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 two 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 common slope model was used to predict the probability of not convulsing within 90 min of diazepam dosing for each monkey; the calculated probabilities are shown in the last column of Table 5. The common slope model was used to estimate the percentiles of the dose-response distribution for absence of convulsions in the Task 89-08 monkeys. Table 6 displays the estimated 20th, 50th, and 80th percentiles of the diazepam dose-absence of convulsion response and their 95 percent confidence limits. The estimate of the diazepam 90 min ED_{50} from the common slope model is 112 μg/kg with a 95 percent confidence interval of 47 to 588 μg/kg. The rather wide confidence interval estimated for the diazepam 90 min ED_{50} is a result of the relatively flat slope estimate (1.34) for the dose-response relationship.

3.3.2 Analysis of Convulsion Incidence Within 4 Hr

Probit models were also fitted for data on the incidence of convulsions within 4 hr of GD injection. Again, probit models were fitted to the 4 hr convulsion data for the 36 Task 89-08 and the 10 USAMRICD monkeys, and the model assumed that the dose-response relations for the two groups of monkeys had a common slope but different intercepts. The parameter estimates and results from fitting the common slope model are summarized in Table 7. The estimate of the common slope is 1.23 with a standard error of 0.41.

In addition to fitting a common slope model to the combined monkey data, separate probit models were fitted to each group of monkeys. 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, and based on the results of a log-likelihood ratio test, the null hypothesis of a common slope for the two groups of monkeys can not be rejected at the 5 percent significance level.

The common slope model was used to predict the probability of not convulsing within 4 hr of diazepam dosing for each monkey; the calculated probabilities are shown in the last column of Table 8. The common slope model was used to estimate the percentiles of the dose-response distribution for absence of convulsions in the Task 89-08 monkeys. Table 9 presents the estimated 20th, 50th, and 80th percentile doses and their 95 percent

TABLE 5. EXPERIMENTAL AND PREDICTED RESULTS FOR ABSENCE OF CONVULSIONS DURING THE FIRST 90 MIN AFTER GD DOSING BASED ON COMMON SLOPE MODEL FITTED TO TASK 89-08 AND USAMRICD MONKEY DATA

Diazepam Dose (μg/kg)	USAMRICD Results: Observed Proportion without Convulsions	USAMRICD Results: Predicted Proportion without Convulsions within 90 Min
0	0/2	0.12
50	1/2	0.59
100	3/4	0.73
150	1/1	0.80
250	1/1	0.87

Diazepam Dose (μg/kg)	Stage	Task 89-08 Results: Absence of Convulsions within 90 Min	Task 89-08 Results: Predicted Proportion without Convulsions within 90 Min
0	1	No	0.16
0	1	No	0.16
0	1	No	0.16
0	1	No	0.16
2	2	No	0.21
9	2	Yes	0.35
12	3	No	0.39
13	3	Yes	0.40
21	5	Yes	0.49
28	2	Yes	0.54
33	2	Yes	0.57
44	2	Yes	0.63
47	1	Yes	0.64
62	7	Yes	0.70
63	3	(Died)	0.70
67	6	No	0.71
71	5	Yes	0.72
80	5	Yes	0.74
86	6	(Died)	0.76
92	2	No	0.77
92	7	No	0.77
105	1	Yes	0.79
109	3	Yes	0.80
109	7	Yes	0.80
117	7	Yes	0.81
128	6	Yes	0.82
130	4	Yes	0.82
143	5	No	0.84
149	3	No	0.84
164	6	Yes	0.85
175	5	No	0.86
182	4	Yes	0.87
203	4	Yes	0.88
207	3	Yes	0.88
224	5	Yes	0.89
242	4	Yes	0.90
304	4	Yes	0.92
305	4	Yes	0.92

TABLE 6. ESTIMATED DIAZEPAM DOSE-RESPONSE PERCENTILES FOR ABSENCE OF CONVULSIONS DURING THE FIRST 90 MIN AFTER DOSING TASK 89-08 MONKEYS BASED ON COMMON SLOPE MODEL

Percentile	Diazepam Dose Estimate (μg/kg)	Lower 95 Percent Confidence Limit (μg/kg)	Upper 95 Percent Confidence Limit (μg/kg)
20	1.5	0.0	13.4
50	22.6	0.6	56.8
80	112	47.5	588

TABLE 7. PARAMETER ESTIMATES AND MODEL RESULTS FROM FITTING COMMON SLOPE MODEL TO THE 4 HOUR CONVULSION RESPONSES IN TASK 89-08 AND USAMRICD MONKEYS

Non-Linear Least Squares Summary Statistics — Dependent Variable (Absence of Convulsions within 4 hr)

Source	DF	Weighted SS	Weighted MS
Regression	3	93.27	31.09
Residual	38	37.42	0.98
Uncorrected Total	41	130.70	
(Corrected Total)	40	63.06	
Sum of Loss		52.39	

Parameter[a]	Estimate	Asymptotic Standard Error	Asymptotic 95 Percent Confidence Interval Lower	Upper
B1	1.23	0.41	0.42	2.06
B01	3.08	0.85	1.37	4.79
B02	2.90	0.78	1.33	4.47

[a]B1 is the estimate of the common slope, B01 is the estimate of the intercept for the USAMRICD monkeys and B02 is the estimate of the intercept for the Task 89-08 monkeys.

TABLE 8. EXPERIMENTAL AND PREDICTED RESULTS FOR ABSENCE OF CONVULSIONS DURING THE FIRST 4 HOURS AFTER DOSING BASED ON COMMON SLOPE MODEL FITTED TO TASK 89-08 AND USAMRICD MONKEY DATA

Diazepam Dose (μg/kg)	USAMRICD Results: Observed Proportion without Convulsions	Predicted Proportion without Convulsions within 4 Hr
0	0/2	0.15
50	1/2	0.59
100	3/4	0.72
150	1/1	0.79
250	1/1	0.86

Diazepam Dose (μg/kg)	Stage	Task 89-08 Results: Absence of Convulsions within 4 Hr	Predicted Proportion without Convulsions within 4 Hr
0	1	No	0.11
0	1	No	0.11
0	1	No	0.11
0	1	No	0.11
2	2	No	0.15
9	2	Yes	0.25
12	3	No	0.28
13	3	Yes	0.29
21	5	Yes	0.37
28	2	No	0.41
33	2	Yes	0.44
44	2	Yes	0.50
47	1	Yes	0.51
62	7	Yes	0.57
63	3	(Died)	0.57
67	6	No	0.58
71	5	No	0.59
80	5	Yes	0.62
86	6	(Died)	0.63
92	2	No	0.64
92	7	No	0.64
105	1	Yes	0.67
109	3	Yes	0.67
109	7	No	0.67
117	7	No	0.69
128	6	Yes	0.70
130	4	Yes	0.71
143	5	No	0.72
149	3	No	0.73
164	6	Yes	0.75
175	5	No	0.76
182	4	Yes	0.76
203	4	Yes	0.78
207	3	Yes	0.78
224	5	Yes	0.80
242	4	Yes	0.81
304	4	Yes	0.84
305	4	Yes	0.84

confidence limits. The estimate of the diazepam 4 hr ED_{80} from the common slope model is 230 μg/kg with a 95 percent confidence interval of 94 to 3,160 μg/kg. The rather wide confidence interval estimated for the diazepam 4 hr ED_{80} results from the relatively flat slope estimate (1.23) for the dose-response relationship.

TABLE 9. ESTIMATED DIAZEPAM DOSE-RESPONSE PERCENTILES FOR ABSENCE OF CONVULSIONS DURING THE FIRST 4 HR AFTER DOSING TASK 89-08 MONKEYS BASED ON COMMON SLOPE MODEL

Percentile	Diazepam Dose Estimate (μg/kg)	Lower 95 Percent Confidence Limit (μg/kg)	Upper 95 Percent Confidence Limit (μg/kg)
20	5.3	0.0	23.0
50	44.3	7.7	117.0
80	230	94.5	3,160

3.3.3 Analysis of Clinical Signs

In addition to determining the incidence of convulsions within 90 min and 4 hr, each animal was monitored for clinical signs of GD intoxication following administration of the GD dose. Monkeys were observed continuously during the first 2 hr following GD challenge, and then at decreasing frequency for 48 hr. Clinical signs monitored included muscle fasciculations, tremors, convulsions, prostration, salivation/bronchial discharge, miosis/mydriasis, and death.

Onset and duration of tremors and/or convulsions within the first 90 min following GD challenge, and onset and duration of prostration were compiled for statistical analysis. Duration of tremors and convulsions were restricted to the first 90 min because of the decision to concentrate on the incidence of convulsions during the first 90 min after GD administration. Annotated clock times (when available) from the clinical signs observation sheets and letter codes designating the period in which events were observed are given in Table 10 for time to death, onset and duration of tremors or

convulsions and onset and cessation of prostration. Length of time from agent injection to onset of tremors, convulsions, and prostration, and duration of tremors and prostration were calculated from the data in Table 10 using annotated times. Durations of convulsions within the first 90 min following GD challenge were tallied from the clinical observation sheets by summing up the total amount of time the animal was convulsing during the first 90 min following GD challenge. When no clock times were given for cessation of tremors or prostration, the midpoint of the time block in which the sign was last observed was used.

Six of the 38 monkeys challenged at 74 μg GD/kg died, four of these within 48 hr. Table 11 displays the times to death and diazepam doses for these six animals. Since diazepam was being evaluated for its effect in reducing the incidence of convulsions, not the lethality rate, it is not surprising that the lethalities and times to death do not appear to be associated with the dose of diazepam. The 48-hr lethality rate for animals pretreated with pyridostigmine, injected with 74 μg/kg GD, and treated with 0.2 mg/kg atropine free base and 25.71 mg/kg 2-PAM was calculated to be 4/38 = 0.11, with a 95 percent confidence interval of 0.03 to 0.25 for this group of 38 monkeys. Confidence limits were calculated using techniques appropriate for the proportion of a binomial distribution.[18].

The onset of tremors and prostration occurred within the first 15 min for all animals. Therefore, these two endpoints were not analyzed. Of the 36 animals that survived longer than 90 min; 28 had tremors throughout the first 90 min. Table 12 displays the diazepam doses for the eight animals that ceased tremoring prior to 90 min. Duration of tremors within the first 90 min following GD challenge was not analyzed further.

Onset and duration of convulsions within the first 90 min following GD challenge, and duration of prostration were the only clinical signs statistically modeled. Table 13 displays the time of onset of convulsions, duration of convulsions occurring within 90 min, and duration of prostration. Onset of convulsions is the length of time between agent injection and convulsions using the annotated clock times given in Table 10. Eighteen animals were not observed to convulse during the 48-hr period. Thus, 48 hr was used as an upper bound on the time to onset of convulsions, and was

TABLE 18. OBSERVATIONS FOR ONSET AND DURATION OF TREMORS AND CONVULSIONS, AND ONSET AND CESSATION OF PROSTRATION FOR TASK 89-88 MONKEYS CHALLENGED AT 74 µg/kg GD AND TREATED WITH ATROPINE AT 8.2 mg/kg

Monkey ID Number	Date	Animal Weight (kg)	Agent Inj. Time	Diazepam Dose (at) (µg/kg)	Time of Death		Duration of Tremors (+1 min. to +90 min.)				Duration of Convulsions (+1 min. to +90 min)			Duration of Prostration (up to +48 hr)			
							Onset		Cease		Onset			Onset		Cease	
					Block Code	Noted Time	Block Code	Noted Time	Block Code	Noted Time	Block Code	Noted Time	Total Time (hh:mm)	Block Code	Noted Time	Block Code	Noted Time
68F	12-13-89	3.1	12:00	0	Z		A	.	F	.	E	13:06	00:03	A	12:02	K	20:00
6MQ	12-13-89	3.1	12:10	0	Z		A	.	F	.	A	12:19	00:22	A	12:13	K	20:10
6ET	12-13-89	4.1	12:40	0	Z		A	.	F	.	A	12:58	00:17	A	12:42	G	14:11
6DW	12-13-89	3.7	12:50	0	Z		A	.	F	.	D	13:36	00:05	A	12:52	I	15:19
790	12-13-89	3.1	13:20	047	Z		A	.	B	.	AA			A	13:22	J	19:20
6W8	12-13-89	3.3	13:30	105	Z		A	.	F	.	AA			A	13:32	I	16:34
78Z	01-09-90	3.2	12:00	033	Z		A	.	E	.	AA	*		A	12:01	L	24:00
77E	01-09-90	2.9	12:10	044	Z		A	.	F	.	AA			A	12:12	G	.
6F2	01-09-90	3.6	12:40	028	Z		A	.	F	.	I	15:23	00	A	12:42	L	.
6R6	01-09-90	3.0	12:50	092	M	18:20	A	.	F	.	C	13:31	00:25	CC			
6N8	01-09-90	4.0	13:20	009	Z		A	.	F	.	AA			A	13:22	N	16:20
6RY	01-09-90	3.6	13:30	002	Z		A	.	E	.	A	13:31	00:33	A	13:31	K	.
5X8	01-16-90	3.6	12:00	013	Z		A	.	F	.	AA			A	12:02	L	21:21
7DE	01-16-90	3.1	12:10	207	Z		A	.	F	.	N	06:49	00	A	12:12	L	21:10
6P9	01-16-90	3.4	12:40	109	Z		A	.	F	.	AA			A	12:44	Q	.
6PH	01-16-90	3.4	12:50	149	Z		A	.	F	.	E	13:56	00:01**	A	12:51	K	20:50
5UT	01-16-90	3.3	13:20	012	Z		A	.	F	.	A	13:24	00:24	A	13:23	L	24:00
5VA	01-16-90	3.0	13:30	063	D	14:22	A	.	DD	.	AA			CC			
6BK	01-23-90	3.6	12:00	130	Z		A	.	B	.	AA			A	12:01	I	15:02
6WJ	01-23-90	4.1	12:10	304	Z		A	.	F	.	L	21:46	00	A	12:11	I	15:05
79K	01-23-90	3.6	12:40	182	Z		A	.	F	.	AA			A	12:42	I	14:43
81J	01-23-90	3.4	12:50	305	Z		A	.	F	.	AA			A	12:52	H	14:38
6F8	01-23-90	3.7	13:20	203	Z		A	.	F	.	AA			A	13:26	I	15:48
6Z9	01-23-90	3.4	13:30	242	Z		A	.	B	.	AA			A	13:32	E	.
6XE	01-30-90	3.8	12:00	224	Z		A	.	F	.	AA			A	12:01	K	20:00
558	01-30-90	3.7	12:10	071	Z		A	.	A	.	I	14:31	00	A	12:12	K	20:10
78Q	01-30-90	4.2	12:40	080	Z		A	.	F	.	AA			A	12:41	H	14:33
6VL	01-30-90	3.1	12:50	143	Z		A	.	F	.	A	12:52	00:04	A	12:51	I	15:34
6G2	01-30-90	3.9	13:20	176	Z		A	.	F	.	F	14:50	00	A	13:22	E	14:35
6F4	01-30-90	3.1	13:30	021	Z		A	.	F	.	AA			A	13:32	F	14:49

TABLE 18.
(Continued)

Monkey ID Number	Date	Animal Weight (kg)	Agent Inj. Time	Diazepam Dose (wt) (μg/kg)	Time of Death		Duration of Tremors (+1 min. to +90 min.)				Duration of Convulsions (+1 min. to +90 min)			Duration of Prostration (up to +48 hr)			
							Onset		Cease		Onset			Onset		Cease	
					Block Code	Noted Time	Block Code	Noted Time	Block Code	Noted Time	Block Code	Noted Time	Total Time (hh:mm)	Block Code	Noted Time	Block Code	Noted Time
6EY	02-06-90	3.8	12:00	067	N	12:05	A	.	F	.	A	12:03	00:12	CC			
6V6	02-08-90	3.4	12:10	128	Z		A	.	A	.	AA			A	12:12	L	23:40
6W7	02-08-90	4.1	12:40	184	Z		A	.	F	.	AA			A	12:42	E	13:55
6RX	02-06-90	3.4	12:50	088	A	13:00	A	.	DD		AA			CC			
6N5	02-20-90	3.4	12:00	092	Z		A	.	F	.	D	12:54	00:14	A	12:02	K	20:00
69W	02-20-90	4.1	12:10	117	Z		A	.	B	.	I	14:53	BB	A	12:12	M	11:58
5YI	02-20-90	3.7	12:40	109	Z		A	.	F	.	H	14:27	BB	A	12:42	K	20:36
6PC	02-20-90	4.8	12:50	062	Z		A	.	F	.	AA			A	12:52	E	14:01

*Animal convulsed only until diazepam was delivered. Onset and duration were not recorded.
**Animal's first convulsion recorded at 12:51. Convulsion ended immediately after diazepam was delivered. Duration for this convulsion was not recorded. The next record of convulsion was noted at 13:56. Duration of this convulsion is recorded above.

Glossary of Letter Code Designations:

A. 0-15 min.	E. 60- 75 min.	I. 2- 4 hr.	M. 12-24 hr.
B. 15-30 min.	F. 75- 90 min.	J. 4- 6 hr.	N. 24-36 hr.
C. 30-45 min.	G. 90-105 min.	K. 6- 8 hr.	O. 36-48 hr.
D. 45-60 min.	H. 105-120 min.	L. 8-12 hr.	P. 48+ hr.

Z. Animal did not die within 48 hr time period.
AA. Event did not occur within 48 hr time period.
BB. Animal's first convulsion occurred +90 min from dosing. Onset is recorded, duration is not recorded.
CC. Animal died within +48 hr. Duration of prostration is not recorded.
DD. Animal did not live to +90 min. Onset of tremors and convulsions are recorded duration is not recorded.

TABLE 11. TIME TO DEATH FOR LETHALITIES AT 74 μg/kg GD

Animal ID	Animal Weight (kg)	Diazepam Dose (μg/kg)	Time to Death
6MG	3.1	0	74 hr
5VA	3.9	63	52 min
6EY	3.8	67	24 hr
6RX	3.4	86	10 min
6R6	3.0	92	22 hr
6V5	3.4	128	67 hr

TABLE 12. DURATION OF TREMORS FOR ANIMALS WITH DURATIONS LESS THAN 90 MINUTES

Animal ID	Diazepam Dose (μg/kg)	Animal Weight (kg)	Duration of Tremors (min)
6RY	2	3.8	68
78Z	33	3.2	68
79G	47	3.1	23
558	71	3.7	8
69M	117	4.1	23
6V5	128	3.4	8
6BK	130	3.5	23
6Z9	242	3.4	23

TABLE 13. CALCULATED TIMES FOR ONSET AND DURATION OF CONVULSIONS, AND DURATION OF PROSTRATION FOR MONKEYS CHALLENGED WITH GD AT 74 μg/kg AND TREATED WITH 0.2 mg/kg ATROPINE FREE BASE, 25.71 mg/kg 2-PAM AND VARIOUS DOSES OF DIAZEPAM

Animal ID	Diazepam Dose (μg/kg)	Convulsions		Duration of Prostration (min)
		Onset (min)	Duration[a] (min)	
68F	0	66	3	478.0
6MG	0	9	22	477.0
6ET	0	10	17	89.0
6DW	0	46	5	147.0
LRY	2	1	33	420.0
6N6	9	>2,880	0	118.0
5UT	12	4	24	637.0
5XB	13	>2,880	0	559.0
6F4	21	>2,880	0	77.0
6F2	28	163	0	600.0
78Z	33	>2,880	0	719.0
77E	44	>2,880	0	97.5
79G	47	>2,880	0	358.0
6PC	62	>2,880	0	69.0
5VA	63	[b]	[b]	[b]
6EY	67	3	12	[c]
558	71	141	0	478.0
78G	80	>2,880	0	112.0
6RX	86	[b]	[b]	[b]
6R6	92	41	25	[c]
6N5	92	54	14	478.0
5W8	105	>2,880	0	122.0
6P9	109	>2,880	0	97.5
5YI	109	107	0	476.0
69M	117	163	0	1,426.0
6V5	128	>2,880	0	688.0
6BK	130	>2,880	0	181.0
6VL	143	2	4	163.0
6PH	149	66	1	479.0
6W7	164	>2,880	0	73.0
6G2	175	90	0	73.0
79K	182	>2,830	0	121.0
6F6	203	>2,880	0	143.0
7DE	207	1,119	0	538.0
6XE	224	>2,880	0	479.0
6Z9	242	>2,880	0	67.5
6MJ	304	576	0	174.0
61J	305	>2,880	0	106.0

[a]Duration of convulsions within first 90 min following GD challenge.
[b]Animal died prior to 90 min and was omitted from analysis.
[c]Animal died prior to 48 hr and was omitted from the analysis of the duration of prostration.

treated as a right-censored observation. Right-censored observations are preceded by a ">" sign in Table 13, indicating that the event was not observed.

Duration of prostration was calculated as the length of time between onset of prostration and cessation of prostration given in Table 10. Annotated times were used when available. If no time was given for cessation of prostration, then the midpoint of the time interval in which prostration was last observed was used.

A common approach was used for statistically analyzing onset and duration of convulsions, and duration of prostration. To simplify the explanation, details of the statistical approach are described below in terms of onset of convulsions.

The ln-ln linear regression model

$$T = \alpha + \beta * X + \epsilon$$

was fitted to the onset of convulsions data where

- T = natural logarithm (ln) of onset of convulsions,
- X = natural logarithm of the diazepam dose
- α = intercept of the linear regression model,
- β = slope of the linear regression model relating ln dose of diazepam to T, and
- ϵ = is assumed to have a normal distribution with mean zero and standard deviation σ.

Estimates of α, β, and σ were calculated using maximum likelihood estimation. Let $\hat{\alpha}$, $\hat{\beta}$, and $\hat{\sigma}$ denote the maximum likelihood estimates of α, β, and σ. If a relationship does not exist between the dose of diazepam and onset of convulsions, then the slope of the linear regression model would be zero. A hypothesis test was conducted to determine if $\hat{\beta}$ was statistically significantly different from zero. If the data are compatible with the null hypothesis of $\beta = 0$, then the distribution of the maximum likelihood estimate of the slope divided by its standard deviation $(\hat{\beta}/SD(\hat{\beta}))$ can be approximated by the standard normal distribution. Thus, a ratio of $\hat{\beta}/SD(\hat{\beta})$ larger than 1.96 in absolute value leads to the rejection of the null hypothesis $\beta = 0$ at the 5 percent significance level.

For each ln dose of diazepam, the estimated parameters were used to predict the natural logarithm of the time at which 50 percent of the animals would exhibit convulsions, LT_{50}, by

$$LT_{50} = \hat{\alpha} + \hat{\beta} X.$$

The standard deviation of LT_{50} was estimated as

$$SD(LT_{50}) = \{ Var(\hat{\alpha}) + 2XCov(\hat{\alpha}, \hat{\beta}) + Var(\hat{\beta})*X^2 \}^{1/2}$$

where $Var(\hat{\alpha})$, $Var(\hat{\beta})$, and $Cov(\hat{\alpha}, \hat{\beta})$ are estimates of the variance of $\hat{\alpha}$, variance of $\hat{\beta}$, and the covariance of $\hat{\alpha}$ and $\hat{\beta}$, respectively. Upper and lower 95 percent confidence limits were computed for LT_{50} by adding and subtracting 1.96 standard deviations to the estimated value of LT_{50}. For each dose of diazepam, the time at which 50 percent of the animals would be predicted to exhibit convulsions, T_{50}, was calculated from the predicted value for LT_{50} by exponentiation. Upper and lower 95 percent confidence bounds for T_{50} were calculated from the 95 percent confidence limits for LT_{50} by exponentiation.

In addition to the above analyses carried out for all three endpoints, the fitted model for onset of convulsions was used to estimate the dose of diazepam at which 80 percent of the monkeys would be free from convulsions during the first 90 min following GD challenge. If p percent of the animals exposed to ln dose X of diazepam exhibit convulsions at ln time T_g, then (1-p) percent of the animals at ln dose X of diazepam will be free from convulsions until ln time T_g. At any given dose,

$$\hat{\alpha} + \hat{\beta} X$$

estimates the natural logarithm of the time during which 50 percent of the monkeys are predicted to be free from convulsions. Because the natural logarithms of the time to onset of convulsions at ln dose X were assumed to have a normal distribution with mean $\alpha + \beta X$ and standard deviation σ, 80 percent of the monkeys at ln dose X are predicted to be free from convulsions until ln time

$$\hat{\alpha} + \hat{\beta}X - 0.842\hat{\sigma}.$$

The ln dose of diazepam at which 80 percent of the monkeys would be free from convulsions during the first 90 minutes following GD challenge was estimated as

$$\ln ED_{80} = \frac{\ln(90) + 0.842\hat{\sigma} - \hat{\alpha}}{\hat{\beta}} .$$

The standard deviation of the estimated diazepam 90 min ln ED_{80} was calculated using the delta method.[18] The diazepam 90 min ED_{80} and its 95 percent confidence limits were calculated from the values calculated for the ln ED_{80} using exponentiation.

It is interesting to compare the traditional probit approach to estimating the diazepam 90 min ED_{80} previously discussed with the approach based on modeling the time to onset of convulsions described above. Both approaches make essentially the same distributional assumptions about the relationship between diazepam dose and convulsions, and differ mostly in how they utilize the data to estimate the dose-response relationship between diazepam dose and convulsions. While the modeling of the time to onset of convulsions used all the quantitative information collected from each animal, the probit approach employed only the qualitative information on the incidence of convulsions observed for each animal.

Table 14 summarizes the statistical modeling of time to onset of convulsions, duration of convulsions during the first 90 min following GD challenge, and duration of prostration. For each endpoint, the maximum likelihood estimates of the intercept ($\hat{\alpha}$), slope ($\hat{\beta}$), and standard deviation ($\hat{\sigma}$) are contained in second, third, and fourth columns of the table, respectively. Also presented in the table are the estimates of the variance of $\hat{\alpha}$ ($Var(\hat{\alpha})$), covariance of $\hat{\alpha}$ and $\hat{\beta}$ ($Cov(\hat{\alpha}, \hat{\beta})$), and variance of $\hat{\beta}$ ($Var(\hat{\beta})$). The last two columns summarize the results of the hypothesis tests of $\beta = 0$. The null hypothesis of $\beta = 0$ was rejected at the 5 percent significance level for each endpoint with an observed significance level less than 0.05. Consequently, the associations between ln dose of diazepam and ln time to onset of convulsions and duration of convulsions were determined to be statistically significant, and the association between ln dose of diazepam and ln duration of prostration was determined not to be statistically significant.

TABLE 14. SUMMARY OF REGRESSION MODELING OF TIME TO ONSET OF CONVULSIONS, DURATION OF CONVULSIONS DURING FIRST 90 MIN FOLLOWING GD CHALLENGE, AND DURATION OF PROSTRATION

Endpoint	$\hat{\alpha}$	$\hat{\beta}$	$\hat{\sigma}$	$Var(\hat{\alpha})$	$Cov(\hat{\alpha},\hat{\beta})$	$Var(\hat{\beta})$	Hyp. Test of $\beta = 0$[a] Test Statistic	Hyp. Test of $\beta = 0$[a] Observed Significance Levels
Onset of Convulsions	3.38	1.03	3.98	2.85	-0.613	0.172	2.49	0.013
Duration of Convulsions	14.5	-2.59	7.54	9.76	-2.11	0.545	-4.63	<0.001
Duration of Prostration	5.71	-0.837	0.811	0.113	-0.024	0.00640	-1.05	0.295

[a]For each endpoint, a hypothesis test was performed to determine if the slope relating dose of diazepam and ln time to event was significantly different from zero. The test statistic was calculated by $\hat{\beta}/\sqrt{Var(\hat{\beta})}$. The null hypothesis of $\beta = 0$ was rejected at the 5 percent significance level for each endpoint with an observed significance level less than 0.05.

The estimated regression model (solid line) together with 95 percent confidence bounds (dashed curves) for the regression model are plotted against diazepam dose in Figures 1, 2, and 3 for time to onset of convulsions, duration of convulsions, and duration of prostration, respectively. Observed results are displayed in each figure with the symbol 1 used for uncensored values, and the symbol 0 used for right censored values.

The time during which 80 percent of the animals would be free from convulsions corresponds to the 20th percentile for the time to onset of convulsions. The regression model fitted to the onset of convulsions data was used to estimate at each dose of diazepam the 20th percentile for time to onset of convulsions. Figure 4 displays a plot of the estimated 20th percentiles for time to onset of convulsions (solid line) together with 95 percent confidence limits (dashed curves) for the 20th percentiles versus diazepam dose.

The dose of diazepam at which 80 percent of the monkeys would be free from convulsions during the first 90 minutes following GD challenge (diazepam 90 min ED_{80}) was calculated to be 74.8 μg/kg based on the model fitted to the times to onset of convulsion data. The corresponding 95 percent confidence interval for the diazepam 90 min ED_{80} was calculated to be 12.2 to 2110 μg/kg. The diazepam 4 hr ED_{80} and 95 percent confidence limits were calculated to be 193 μg/kg [44.1 to 83,400 μg/kg] based on the model fitted to the times to onset of convulsion data. These results are comparable to those presented in Tables 6 and 9 based on the probit models.

3.4 Pathology

As part of the study on the efficacy of diazepam in controlling GD-induced convulsive activity, 12 rhesus monkeys were necropsied to investigate cause of death. This included four of the ten animals used to approximate the 48-hr GD LD_{50}, and eight animals from diazepam efficacy groups. Selected tissues were saved from three of the efficacy group animals. Two of these (6MG, 6V5) were found dead. One (6PC) was in moribund condition and anesthetized and perfused with 10 percent neutral buffered formalin prior

FIGURE 1. ONSET OF CONVULSIONS PLOTTED AGAINST DIAZEPAM DOSE WITH FITTED LN-LN REGRESSION MODEL
Symbol Used is Censoring Indicator

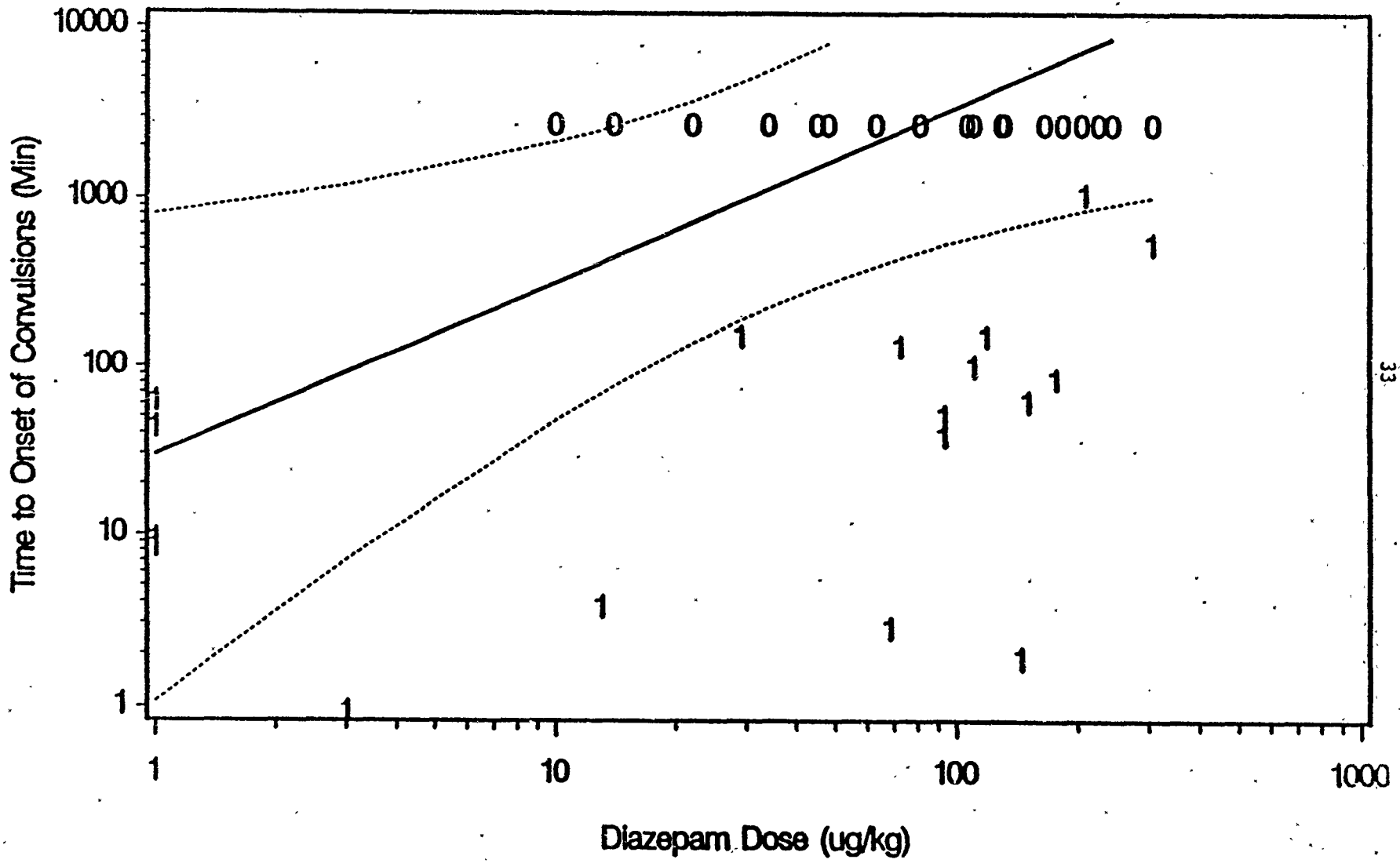
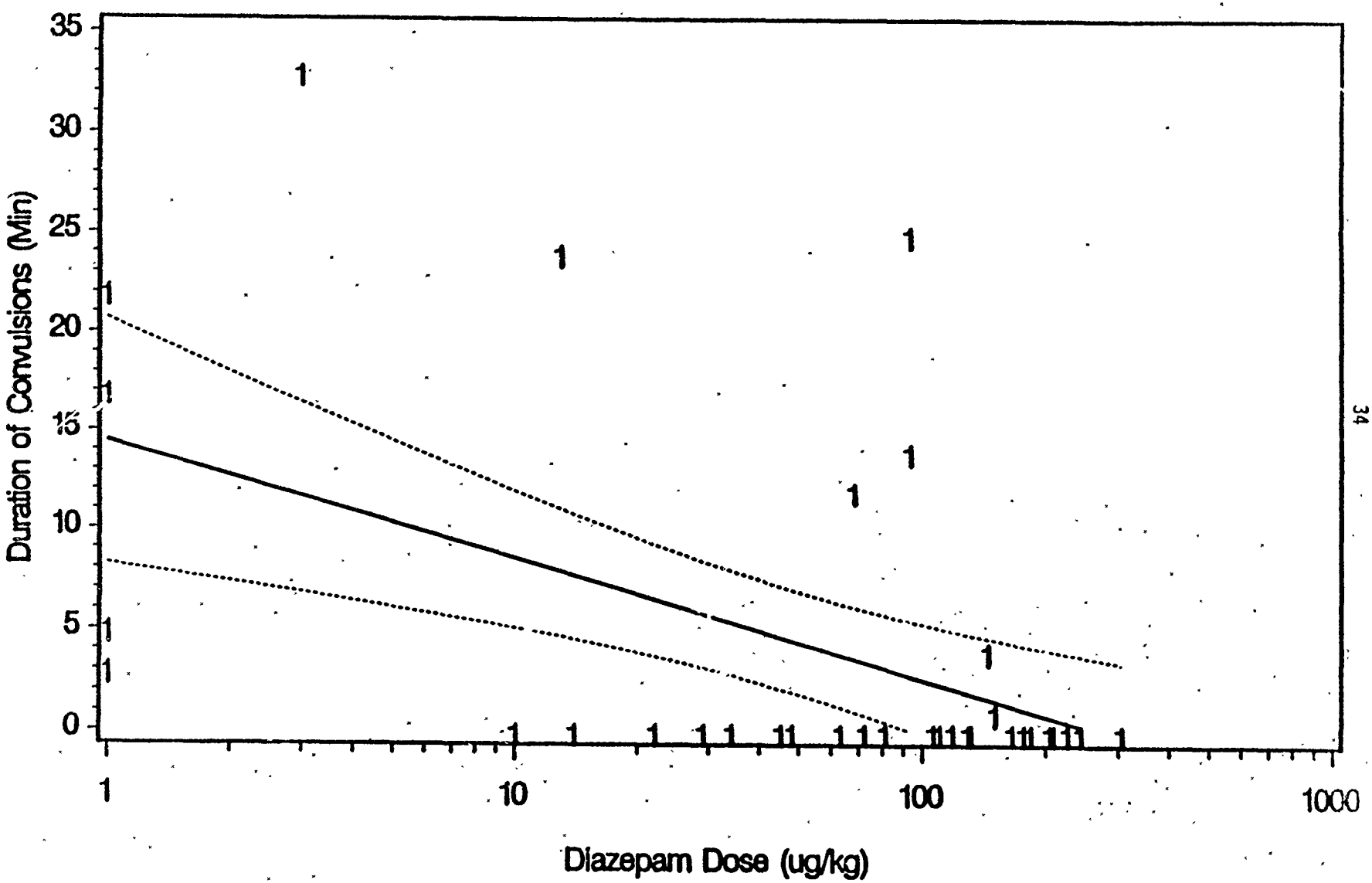


FIGURE 2. DURATION OF CONVULSIONS WITHIN FIRST 90 MIN AFTER GD CHALLENGE PLOTTED AGAINST DIAZEPAM DOSE WITH FITTED LN-LN REGRESSION MODEL



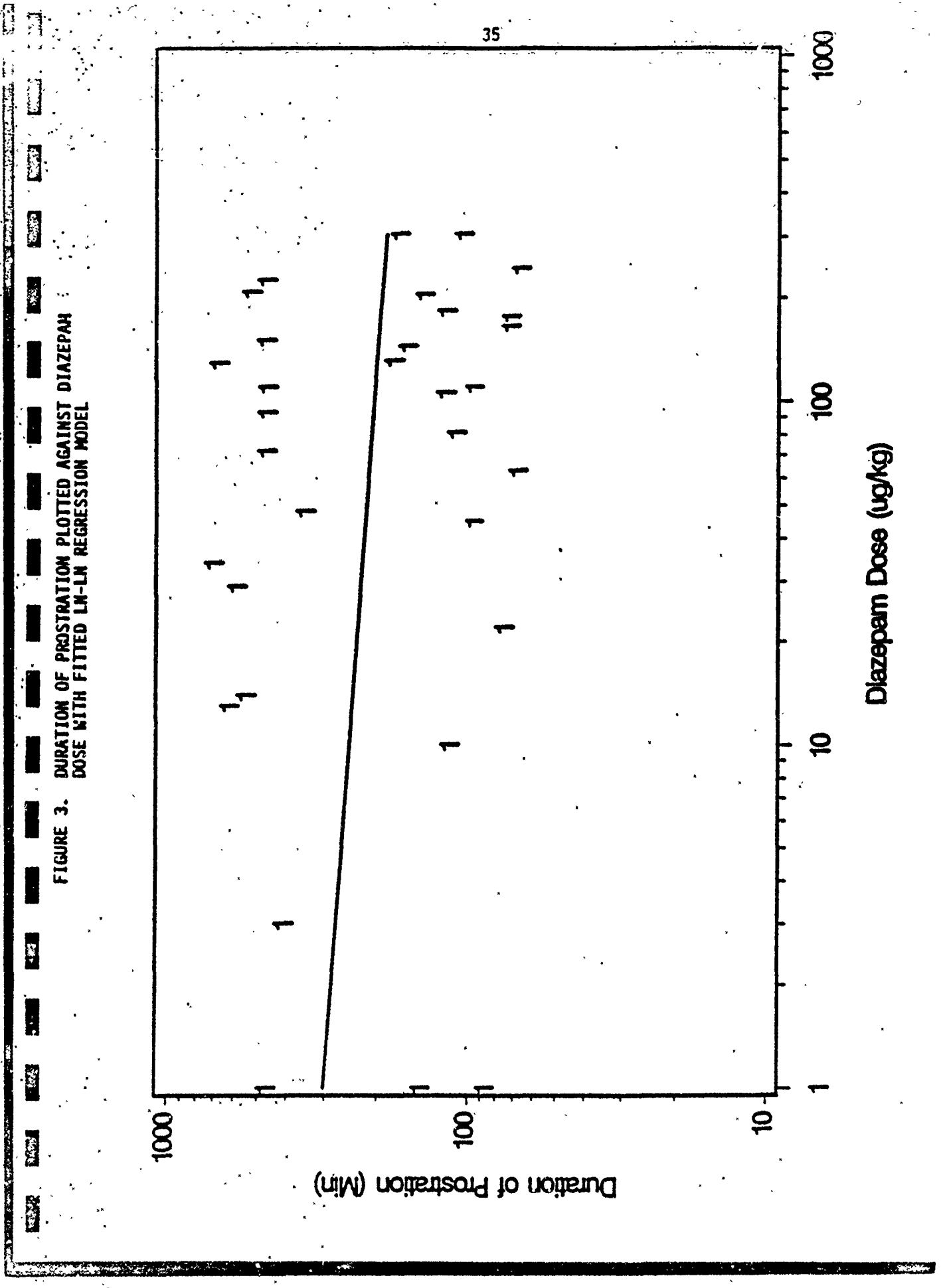
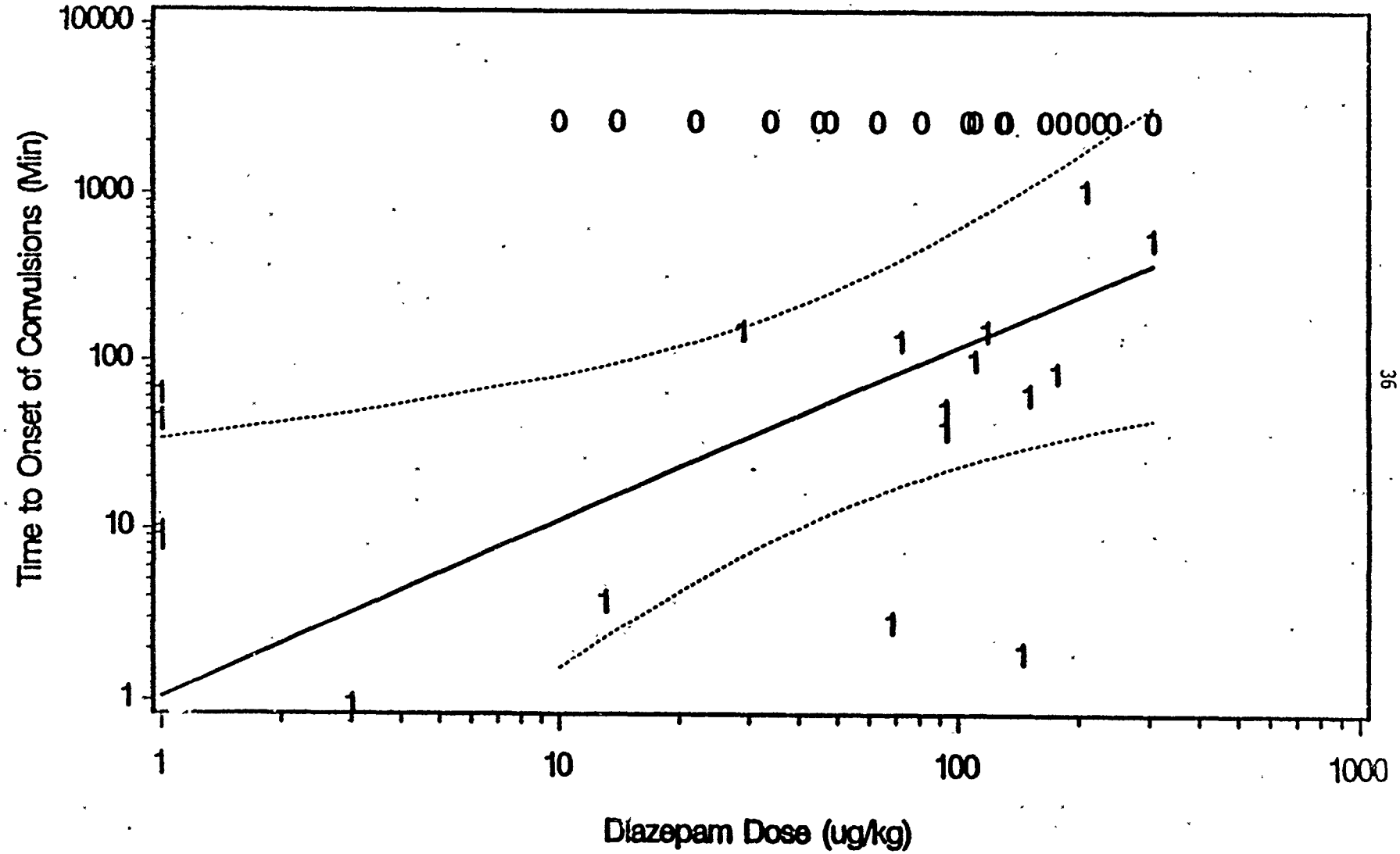


FIGURE 3. DURATION OF PROSTRATION PLOTTED AGAINST DIAZEPAM DOSE WITH FITTED LN-LN REGRESSION MODEL

FIGURE 4. 20TH PERCENTILES OF TIME TO ONSET OF CONVULSIONS PREDICTED FROM LN-LN REGRESSION MODEL PLOTTED AGAINST DIAZEPAM DOSE
Symbol Used is Censoring Indicator



to tissue collection. All tissues, except eyes, were immersion-fixed in 10 percent neutral buffered formalin. Eyes were fixed in Bouin's solution.

In all cases, necropsy results were compatible with death or moribund condition due to GD intoxication (nonspecific excessive cholinergic stimulation). In many cases, no gross morphologic alterations were found. This would be expected following exposure to a powerful anticholinesterase agent. Gross lesions which could be attributed to GD toxicity were not found in any of the 48-hr GD LD_{50} animals examined. Of the eight monkeys given some therapy, Table 15 lists gross changes which were interpreted to have been related to experimental treatment.

TABLE 15. GROSS PATHOLOGY RELATED TO EXPERIMENTAL TREATMENT

Animal ID	Necropsy Date	Gross Observations(s)/Comment
5VA	1-16-90	Lungs-diffuse edema Lungs-red discoloration Comment: Trachea/Bronchi plugged with white froth. Pulmonary edema/hemorrhage probably related to GD exposure.
6V5	2-09-90	Ileum-serosal hemorrhage Comment: Hemorrhage of terminal ileum frequently associated with pyridostigmine pretreatment.
6PC	2-22-90	Ileum-serosal hemorrhage Comment: Presumably related to pyridostigmine pretreatment.

Tissues saved from three monkeys (6PC, 6V5, and 6MG) were embedded, trimmed for targeted sites, cut at five 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, caudate nucleus; 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), and heart (SA node, left ventricle, ventricular septum, left atrium, bundle of His).

Significant neuropathology was not found in the two monkeys given diazepam (6PC - 62 μg/kg diazepam, 6V5 - 128 μg/kg diazepam) as part of a therapy to control convulsions. In monkey 6MG, however, no diazepam was given, and significant neuronal necrosis (ranging in distribution/severity from minimal to moderate) was found in most brain sections examined. Spinal cord and peripheral nerve sections were essentially normal in all three monkeys. The neuropathology attributed to GD-induced neuronal toxicity consisted essentially of "ischemic cell change" in individual neurons, and was presumably the cause of death.

Monkey 6PC had multifocal areas of cardiac myocyte degeneration, as well as subendocardial hemorrhage. These heart lesions are presumably related to the GD treatment, but whether they represent a cardiac-specific GD effect, or a secondary effect induced by excessive endogenous corticosteroid release (or some other secondary effect) cannot be ascertained from sections examined. Some vacuolar change was also noted in the heart sections of monkey 6V5, but, since this monkey died and was not perfused, the vacuolar change could not be unequivocally differentiated from artifact, and no "lesion" was recorded. The heart lesions may have contributed to the moribund state of 6PC, but no specific morphologic alteration was found to fully explain the death of monkey 6V5. Perhaps significant physiologic or metabolic alterations (such as pH changes resulting in arrhythmias or other disorders) contribute to GD-associated toxicity without causing significant morphologic alterations.

Two monkeys (6MG, 6V5) had evidence of hepatic fatty change suggestive of excessive release of free fatty acids from adipose tissue. This is not surprising considering the clinical state prior to death, and supports the possibility of metabolic alterations contributing to the cause of death/moribund condition. Pathologic findings in individual animals are tabulated in Appendix C.

3.5 Pharmacokinetic Study

Nine monkeys (6AS, 6AR, 61P, 6R1, 68W, 71M, 5V7, 6BJ, and 5WF) were randomly selected from the total number of animals available to form a group of monkeys weight homogenized with those used in the efficacy and the LD_{50} studies. Because body weights of these nine monkeys prior to the first day of the pharmacokinetic study ranged from 2.8 to 4.6 kg, animals were divided into three groups with the lightest three animals in one group, the middle three in another, and the heaviest in the last. Each monkey in each group of three was randomly assigned a sequence in which low, medium or high doses of diazepam were to be administered. Using this approach, body weight or sequence of dosing bias should be eliminated. Doses of diazepam to be administered, as requested by USAMRICD personnel, were 70, 110, and 220 μg/kg body weight. Nine monkeys were dosed per study day and approximately 1 month separated study days to allow time for monkeys to regain normal blood values and physiologic state.

On one day of analysis, using plasma samples from the second day of the pharmacokinetic study, teflon liners were inadvertently not placed in the caps of culture tubes used to mix benzene and plasma samples for extraction. This resulted in extraction of compounds from the caps which led to interfering peaks on GC analyses, and prevented accurate determination of diazepam concentrations in these samples. As a result, these three animals were again given the desired dose of diazepam approximately 1 month following the third study day.

Actual diazepam doses administered, based on weight differentials of loaded and spent syringes and body weights taken immediately prior to catheterization, ranged from 67 to 79 μg/kg with a mean of 72.3 μg/kg for the 70 μg/kg target dose group. For the 110 μg/kg target dose, actual doses ranged from 109 to 114 μg/kg with a mean of 111.1 μg/kg, and for the 220 μg/kg target dose, actual doses ranged from 220 to 229 μg/kg with a mean of 223.2 μg/kg. The plasma diazepam concentrations measured for each of the nine monkeys at each of the diazepam dose levels and the actual doses are presented in Table 16.

TABLE 16. PLASMA DIAZEPAM CONCENTRATIONS AT SAMPLING TIMES

Time* (min)	Animal No.	6AS	6R1	5V7	61P	68W	5WF	6AR	71M	6BJ	Mean	STD
	Dose (μg/kg)	67	71	79	71	74	71	72	75	71		
	Body Wt (kg)	2.8	3.4	4.3	3.2	3.9	4.1	3.4	3.8	4.8		
-10.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
2.5		19.3	6.1	19.7	0.0	0.0	7.9	0.0	0.0	6.6	6.62	7.97
5.0		33.9	12.8	18.9	5.8	8.4	12.9	9.9	6.7	14.1	13.71	8.59
10.0		49.3	23.9	29.6	18.9	11.6	19.8	20.1	12.6	22.2	23.10	11.22
15.0		51.8	26.9	32.9	37.1	15.7	21.1	22.3	14.6	21.2	27.07	11.86
25.0		45.4	26.4	37.9	32.7	13.4	19.4	22.1	13.1	23.1	25.95	10.94
40.0		33.4	24.7	28.2	22.5	17.1	14.9	18.7	15.3	22.1	21.87	6.18
60.0		20.8	16.0	22.6	18.6	10.9	10.6	14.9	19.1	19.5	16.99	4.21
90.0		11.4	12.0	11.3	14.7	9.2	9.4	4.2	11.1	13.2	10.72	2.98
120.0		9.6	9.4	5.0	9.2	6.2	5.9	0.0	11.4	9.4	7.35	3.47
180.0		0.0	5.0	0.0	6.4	0.0	0.0	0.0	4.2	9.7	2.81	3.65
240.0		0.0	4.9	0.0	0.0	0.0	0.0	0.0	2.1	0.0	0.78	1.69
480.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
1,440.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00

Time* (min)	Animal No.	6AS	6R1	5V7	61P	68W	5WF	6AR	71M	6BJ	Mean	STD
	Dose (μg/kg)	112	111	112	110	110	114	109	115	111		
	Body Wt (kg)	3.0	3.4	4.3	3.3	3.4	4.0	3.3	3.8	4.9		
-10.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
2.5		10.4	6.4	0.0	0.0	9.3	24.5	14.4	16.2	8.2	9.93	7.79
5.0		23.9	9.6	5.8	22.4	17.2	64.5	31.8	17.9	16.8	23.32	17.21
10.0		48.0	15.5	14.2	52.9	26.3	71.1	49.8	23.2	44.5	38.39	19.45
15.0		43.0	25.8	14.3	61.9	36.3	62.3	55.3	21.6	45.5	40.67	17.54
25.0		44.7	36.0	20.8	48.9	45.6	35.5	53.9	24.2	37.6	38.57	11.00
40.0		44.9	36.7	25.3	34.6	42.4	18.8	47.0	17.0	32.5	33.24	10.94
60.0		27.7	28.3	25.0	19.7	34.7	8.6	34.2	12.3	23.7	23.79	8.96
90.0		16.4	18.0	23.9	10.0	23.7	0.0	20.3	8.8	19.4	15.61	7.89
120.0		8.6	12.3	17.2	6.5	17.3	0.0	14.0	6.8	15.9	10.96	5.89
180.0		7.2	9.0	11.0	0.0	9.9	0.0	7.2	0.0	5.5	5.53	4.45
240.0		5.5	6.6	8.8	0.0	**	0.0	8.8	0.0	0.0	3.71	4.10
480.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
1,440.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00

Table 16.
(Continued)

Time* (min)	Animal No. / Dose (μg/kg) / Body Wt (kg)	6AS 224 3.0	6R1 221 3.3	5V7 224 4.3	61P 222 2.8	68W 225 3.7	5WF 229 3.8	6AR 220 3.3	71M 223 3.5	6BJ 221 4.6	Mean	STD
-10.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
2.5		10.5	6.0	12.4	11.8	8.8	68.1	15.2	23.1	6.8	18.08	19.44
5.0		33.9	16.4	19.9	63.6	16.1	104.2	29.2	35.7	13.6	36.96	29.58
10.0		53.1	28.2	37.3	102.0	38.7	90.3	54.1	52.1	36.3	54.68	25.29
15.0		54.9	26.5	44.4	124.7	47.6	77.2	67.1	54.6	49.6	60.73	27.87
25.0		62.3	36.0	39.8	117.5	50.7	69.5	83.0	56.0	62.4	64.13	24.66
40.0		60.5	36.2	33.1	80.9	***	53.1	66.1	66.9	57.1	56.74	15.97
60.0		45.1	28.6	34.0	50.2	34.3	41.0	47.3	55.4	42.8	42.08	8.58
90.0		28.7	18.8	29.3	34.3	49.0	25.0	34.8	35.1	27.7	31.41	8.43
120.0		15.6	12.3	20.2	27.5	16.2	15.8	22.2	27.2	20.5	19.73	5.29
180.0		9.6	9.1	13.6	15.6	10.1	8.9	12.8	19.2	9.9	12.09	3.53
240.0		6.3	4.5	12.5	13.9	6.7	0.0	9.5	13.9	8.0	8.36	4.63
480.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.6	0.0	0.62	1.87
1,440.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00

*Targeted blood collection times. Actual collection time may vary slightly (see Table 19).
**Irresolvable diazepam peak due to interference in gas chromatogram.
***Loss of plasma sample due to breakage of tube during centrifugation.

The plasma diazepam concentration as a function of time data best fit a single compartment, open pharmacokinetic model. For such a model, the plasma diazepam concentration at time t, C_{pt}, assuming availability of the total dose of diazepam administered IM, is represented by the following expression:[11]

$$C_{pt} = \frac{D}{V_d} \frac{k_a}{k_a - k_{el}} (e^{-k_{el}t} - e^{-k_a t})$$

where D is the dose of diazepam injected, V_d is the apparent volume of distribution, k_a is the first-order rate constant for diazepam absorption, k_{el} is the first-order rate constant for diazepam elimination. The mean diazepam

C_{pt} values for nine monkeys given a similar dose of diazepam were calculated for all time points at which blood was sampled. Using a Symphony® (Lotus Development Corporation, Cambridge, MA) program, these values were plotted on a ln plasma concentration versus time graph, and a best-fit curve drawn and feathered, and "seed" values of k_a, k_{el}, and V_d obtained. These "seeds" were then used as starting values for these parameters in a SAS (Statistical Analysis System, Cary, NC) NLIN program to fit, for individual animals, a non-linear regression model by least squares. This program used the Marquardt iterative method to regress residuals onto the model's partial derivatives with respect to the parameters until the iterations converged. The derivatives of k_a, k_{el}, and V_d were obtained to help model changes in direction for these parameters in the iterative process and to determine 95 percent confidence limits on these values. A listing of the computer program used in these analyses is provided in Appendix D.

From the pharmacokinetic models, the following parameters were estimated for each animal:

$AUC_{0-\infty}$ (Area under the curve of the plasma concentration versus time graph from time 0 to ∞; ng * min/mL)

$$AUC_{0-\infty} = \frac{D}{V_d \, k_{el}}$$

$AUC_{0-\infty}/D$ (Area under the curve divided by dose; kg * min/L)

t_{k_a} (Absorption phase half-life; min)

$$t_{k_a} = \ln 2/k_a$$

$t_{k_{el}}$ (Elimination phase half-life; min)

$$t_{k_{el}} = \ln 2/k_{el}$$

t_{max} (Time at which maximum plasma diazepam concentration occurs; min)

$$t_{max} = \frac{\ln (k_a/k_{el})}{k_a - k_{el}}$$

C_{pmax} (Maximum plasma diazepam concentration obtained; ng/mL)

$$C_{pmax} = \frac{D}{V_d} \frac{k_a}{k_a - k_{el}} (e^{-k_{el}t_{max}} - e^{-k_a t_{max}})$$

C_{pmax}/D (Maximum plasma diazepam concentration divided by dose; kg/L)

CL (Clearance rate of diazepam from the plasma; mL/min/kg)

$$CL = D/AUC$$

Model-derived pharmacokinetic parameters for individual animals at each diazepam dose level are given in Tables 17 and 18. AUC was also calculated for the raw data from each animal by using the trapezoidal rule and computing area for each half minute under the line connecting plasma diazepam concentration for each time point at which a blood sample was taken. These areas are presented in Table 19. Although blood sampling times were established, it was not always possible to draw blood samples, usually because of blood flow in the catheters, exactly at desired times. Times at which blood samples were actually obtained, to the nearest quarter of a minute, are recorded for each animal at each sampling time in Table 19. Pharmacokinetic parameters, as well as the AUCs for raw data, were estimated using the actual times of blood collection.

TABLE 17. SINGLE-COMPARTMENT MODEL-DERIVED DIAZEPAM PHARMACOKINETIC PARAMETERS FOR INDIVIDUAL ANIMALS AT EACH DOSE LEVEL

Animal	D (μg/kg)	C_{peax} (ng/mL)	C_{peax}/D (kg/L)	t_{max} (min)	V_d (L/kg)	K_a (min^{-1})	K_{el} (min^{-1})	t_{k_a} (min)	$t_{k_{el}}$ (min)	CL (mL/min/kg)	$AUC_{0-\infty}$ (ng•min/mL)	AUC_{0-480} (ng•min/mL)	$AUC_{0-\infty}/D$ (kg•min/L)
6AS	67	58.6	0.746	18.4	0.92	0.128	0.023	5.42	30.2	21.1	3,169	3,169	47
6R1	71	26.7	0.377	22.8	2.82	0.113	0.012	6.13	56.1	25.8	2,837	2,829	40
5V7	79	36.4	0.461	19.8	1.64	0.116	0.018	5.99	38.1	27.9	2,827	2,826	36
61P	71	29.8	0.419	25.8	1.84	0.084	0.015	8.23	46.5	24.6	2,901	2,898	41
68W	74	15.8	0.213	25.1	3.59	0.100	0.011	6.95	64.7	38.4	1,927	1,914	26
5WF	71	20.2	0.285	17.8	2.77	0.156	0.014	4.44	49.5	38.7	1,834	1,832	26
6AR	72	23.5	0.326	22.8	1.67	0.063	0.030	10.97	23.4	46.8	1,546	1,545	21
71M	75	17.3	0.231	38.4	3.89	0.061	0.009	11.36	74.9	28.8	2,619	2,583	35
6BJ	71	23.5	0.331	21.8	2.58	0.143	0.008	4.86	89.8	19.9	3,565	3,478	50

Animal	D (μg/kg)	C_{peax} (ng/mL)	C_{peax}/D (kg/L)	t_{max} (min)	V_d (L/kg)	K_a (min^{-1})	K_{el} (min^{-1})	t_{k_a} (min)	$t_{k_{el}}$ (min)	CL (mL/min/kg)	$AUC_{0-\infty}$ (ng•min/mL)	AUC_{0-480} (ng•min/mL)	$AUC_{0-\infty}/D$ (kg•min/L)
9AS	112	48.8	0.428	22.8	1.52	0.085	0.018	8.15	38.9	28.8	3,918	3,916	35
6R1	111	33.2	0.305	35.7	1.90	0.049	0.014	14.16	49.2	27.9	3,975	3,968	36
5V7	112	25.5	0.228	51.9	2.90	0.048	0.007	27.48	92.7	22.3	5,029	4,858	45
61P	110	55.3	0.503	17.8	1.10	0.089	0.034	7.76	20.8	37.8	2,972	2,972	27
68W	110	43.5	0.395	31.5	1.68	0.063	0.013	11.01	52.9	22.8	5,018	4,998	46
5WF	114	68.4	0.600	18.8	0.87	0.145	0.065	4.78	10.8	58.8	2,014	2,014	18
6AR	109	55.7	0.511	19.5	1.47	0.125	0.015	5.55	47.8	21.5	5,074	5,069	47
71M	111	23.8	0.213	12.3	4.81	0.255	0.013	2.72	53.5	51.8	2,138	2,134	19
6BJ	111	41.5	0.373	19.5	2.87	0.131	0.013	5.30	52.8	27.2	4,082	4,073	37

Animal	D (μg/kg)	C_{peax} (ng/mL)	C_{peax}/D (kg/L)	t_{max} (min)	V_d (L/kg)	K_a (min^{-1})	K_{el} (min^{-1})	t_{k_a} (min)	$t_{k_{el}}$ (min)	CL (mL/min/kg)	$AUC_{0-\infty}$ (ng•min/mL)	AUC_{0-480} (ng•min/mL)	$AUC_{0-\infty}/D$ (kg•min/L)
6AS	224	63.8	0.285	26.3	2.39	0.082	0.015	8.48	45.5	38.4	8,155	8,149	27
6R1	221	35.5	0.161	28.8	4.41	0.078	0.012	9.08	58.2	52.5	4,208	4,192	19
5V7	224	46.8	0.182	21.8	4.76	0.149	0.007	4.65	103.9	31.7	7,072	6,778	32
61P	222	115.8	0.521	19.1	1.30	0.107	0.021	6.48	33.6	28.7	8,301	8,300	37
68W	225	52.4	0.233	31.8	3.07	0.072	0.011	9.81	64.1	33.2	6,789	6,724	30
5WF	229	93.2	0.407	6.7	2.20	0.533	0.017	1.30	41.6	36.8	6,255	6,252	27
6AR	228	73.9	0.336	26.8	2.82	0.079	0.015	8.73	46.5	30.1	7,389	7,382	33
71M	223	82.8	0.282	24.1	2.92	0.120	0.008	5.78	86.1	23.5	9,471	9,257	42
6BJ	221	57.6	0.261	31.8	2.31	0.055	0.016	12.57	43.3	37.8	5,977	5,973	27

TABLE 18. SINGLE-COMPARTMENT MODEL-DERIVED PLASMA DIAZEPAM AUCs FOR INDIVIDUAL ANIMALS AT EACH DOSE LEVEL

78 μg/kg

Animal	$AUC_{0-2.5}$	AUC_{0-5}	AUC_{0-10}	AUC_{0-15}	AUC_{0-25}	AUC_{0-45}	AUC_{0-65}	AUC_{0-90}	AUC_{0-120}	AUC_{0-180}	AUC_{0-240}	AUC_{0-480}
6AS	28.8	83.2	288.8	521.8	1,018.8	1,823.7	2,196.7	2,677.9	2,922.5	3,186.9	3,153.3	3,186.9
6R1	9.8	36.8	129.8	248.3	512.5	892.3	1,315.8	1,787.8	2,112.4	2,492.8	2,672.7	2,828.8
5V7	13.5	54.5	188.3	357.5	718.3	1,284.8	1,895.4	2,178.7	2,448.4	2,698.9	2,783.8	2,826.1
61P	8.5	35.1	127.2	261.7	548.9	973.4	1,457.8	1,975.7	2,389.8	2,858.8	2,861.8	2,896.2
68W	4.7	19.5	69.8	138.8	298.4	528.8	788.9	1,181.8	1,327.8	1,611.8	1,781.8	1,914.8
5WF	8.8	34.9	115.7	213.1	412.9	679.4	981.1	1,268.5	1,457.2	1,671.4	1,763.9	1,821.7
6AR	6.8	28.2	102.5	203.1	433.5	788.3	1,081.5	1,348.7	1,482.8	1,531.4	1,543.1	1,546.5
71M	3.5	14.9	56.4	116.1	267.3	523.5	855.4	1,275.8	1,668.1	2,034.8	2,283.2	2,582.5
6BJ	8.9	35.5	121.1	228.5	481.4	799.8	1,197.8	1,691.8	2,081.7	2,836.7	2,982.8	3,476.8

118 μg/kg

Animal	$AUC_{0-2.5}$	AUC_{0-5}	AUC_{0-10}	AUC_{0-15}	AUC_{0-25}	AUC_{0-45}	AUC_{0-65}	AUC_{0-90}	AUC_{0-120}	AUC_{0-180}	AUC_{0-240}	AUC_{0-480}
6AS	14.5	66.8	215.5	423.8	894.3	1,672.3	2,287.5	2,985.9	3,336.4	3,744.5	3,868.5	3,915.4
6R1	6.5	27.9	106.3	221.1	514.9	1,018.3	1,655.8	2,418.5	2,948.5	3,631.4	3,784.2	3,968.8
5V7	3.8	15.6	61.8	129.6	318.3	868.3	1,173.4	1,894.8	2,587.4	3,413.4	3,998.9	4,857.8
61P	28.3	82.8	288.4	549.8	1,093.5	1,789.5	2,338.9	2,738.5	2,886.5	2,988.5	2,978.4	2,971.9
68W	9.8	41.3	154.6	315.3	712.7	1,359.8	2,148.9	3,083.8	3,693.5	4,418.8	4,738.8	4,998.2
5WF	48.8	155.4	476.7	818.8	1,332.8	1,748.1	1,948.8	2,083.5	2,012.4	2,013.9	2,013.9	2,013.9
6AR	28.8	84.8	288.6	548.7	1,099.2	1,859.9	2,869.4	3,529.9	4,071.8	4,865.7	4,899.8	5,868.9
71M	14.6	53.9	182.2	279.9	583.5	792.1	1,099.3	1,433.9	1,668.7	1,9[illegible]	2,837.4	2,133.9
6BJ	15.8	63.4	216.7	489.3	826.9	1,391.5	2,018.9	2,684.8	3,139.5	3,663.1	3,886.7	4,073.4

228 μg/kg

Animal	$AUC_{0-2.5}$	AUC_{0-5}	AUC_{0-10}	AUC_{0-15}	AUC_{0-25}	AUC_{0-45}	AUC_{0-65}	AUC_{0-90}	AUC_{0-120}	AUC_{0-180}	AUC_{0-240}	AUC_{0-480}
6AS	17.8	74.1	269.2	534.1	1,151.8	2,079.2	3,119.1	4,226.8	[illegible]2.5	5,664.2	5,957.8	6,149.5
6R1	9.8	37.5	138.2	277.5	612.4	1,139.7	1,768.7	2,496.8	[illegible]3.4	3,621.9	3,921.2	4,191.5
5V7	16.7	62.6	212.2	399.1	863.7	1,394.8	2,182.5	3,083.8	3,741.7	4,848.3	5,576.5	6,778.5
61P	41.7	169.4	589.1	1,124.8	2,289.8	3,868.8	5,387.3	6,887.5	7,432.1	8,848.8	8,227.5	8,299.8
68W	12.4	52.2	193.7	391.9	875.9	1,655.8	2,611.2	3,753.8	4,587.2	5,628.2	6,172.4	6,724.8
5WF	95.2	384.1	786.9	1,283.4	1,978.2	2,923.3	3,868.8	4,885.5	5,375.8	5,938.2	6,134.8	6,251.5
6AR	28.1	83.9	385.8	668.7	1,328.4	2,488.5	3,624.8	4,942.5	5,793.4	6,887.9	7,854.5	7,381.8
71M	28.8	84.7	296.4	571.4	1,187.8	2,184.7	3,195.8	4,541.9	5,599.5	7,882.8	7,997.5	9,257.4
6BJ	12.5	53.1	199.9	489.8	933.7	1,791.3	2,829.4	3,998.8	4,739.7	5,581.7	5,794.8	5,972.9

TABLE 19. PLASMA DIAZEPAM AUCs FOR UNMODELED DATA FROM INDIVIDUAL MONKEYS AT EACH DOSE LEVEL

70 µg/kg

Animal	Time	Conc. (ng/mL)	dAUC (ng•min/mL)	Cum. AUC (ng•min/mL)	Animal	Time	Conc. (ng/mL)	dAUC (ng•min/mL)	Cum. AUC (ng•min/mL)	Animal	Time	Conc. (ng/mL)	dAUC (ng•min/mL)	Cum. AUC (ng•min/mL)
6AS	0	0.0	0.0	0.0	61P	0	0.0	0.0	0.0	6AR	0	0.0	0.0	0.0
6AS	3	19.3	29.0	29.0	61P	2.5	0.0	0.0	0.0	6AR	2.5	0.0	0.0	0.0
6AS	6	33.9	79.8	108.8	61P	5	5.0	7.3	7.3	6AR	5	9.9	12.4	12.4
6AS	10	49.3	166.4	275.2	61P	10	10.9	61.8	69.0	6AR	10	20.1	76.0	87.4
6AS	15	61.8	252.6	527.9	61P	15	37.1	140.0	209.0	6AR	15	22.3	106.0	193.4
6AS	25	45.4	485.0	1,013.9	61P	25	32.7	349.0	558.0	6AR	25	22.1	222.0	415.4
6AS	40	33.4	591.0	1,604.9	61P	40	22.5	414.0	972.0	6AR	40	18.7	306.0	721.4
6AS	60	20	542.0	2,146.9	61P	60	18.0	411.0	1,385.0	6AR	60	14.9	338.0	1,057.4
6AS	90	11.4	483.0	2,629.9	61P	90	14.7	499.5	1,882.5	6AR	90	4.2	286.5	1,343.9
6AS	120	9.0	315.0	2,944.9	61P	120	9.2	358.5	2,241.0	6AR	120	0.0	63.0	1,406.9
6AS	180	0.0	288.0	3,232.9	61P	180	6.4	468.0	2,709.0	6AR	180	0.0	0.0	1,406.9
6AS	240	0.0	0.0	3,232.9	61P	240	0.0	192.0	2,901.0	6AR	240	0.0	0.0	1,406.9
6AS	480	0.0	0.0	3,232.9	61P	480	0.0	0.0	2,901.0	6AR	480	0.0	0.0	1,406.9
6AS	1,440	0.0	0.0	3,232.9	61P	1440	0.0	0.0	2,901.0	6AR	1440	0.0	0.0	1,406.9
6R1	0	0.0	0.0	0.0	68W	0	0.0	0.0	0.0	71M	0	0.0	0.0	0.0
6R1	2.5	6.1	7.6	7.6	68W	3	0.0	0.0	0.0	71M	2.75	0.0	0.0	0.0
6R1	5	12.8	23.8	31.3	68W	5	8.4	8.4	8.4	71M	5	6.7	7.5	7.5
6R1	10	23.9	91.8	123.0	68W	10	11.8	50.0	58.4	71M	10	12.0	48.3	55.8
6R1	15	26.0	127.0	250.0	68W	15	15.7	68.3	126.7	71M	15	14.0	68.0	123.8
6R1	25	26.4	266.5	516.5	68W	25	13.4	145.5	272.2	71M	25	13.1	138.5	262.3
6R1	40	24.7	383.3	899.8	68W	40	17.1	228.0	500.9	71M	40	15.3	213.0	475.3
6R1	60	16.0	487.0	1,386.8	68W	60	10.9	280.0	780.9	71M	60	19.1	344.0	819.3
6R1	90	12.0	420.0	1,726.8	68W	90	9.2	361.5	1,082.4	71M	90	11.1	453.0	1,272.3
6R1	120	9.4	321.0	2,047.8	68W	120	8.2	231.0	1,313.4	71M	120	11.4	337.5	1,609.8
6R1	180	5.0	432.0	2,479.8	68W	180	0.0	186.0	1,499.4	71M	180	4.2	468.0	2,077.8
6R1	240	4.9	297.0	2,776.8	68W	240	0.0	0.0	1,499.4	71M	240	2.1	189.0	2,266.8
6R1	480	0.0	588.0	3,364.8	68W	480	0.0	0.0	1,499.4	71M	480	0.0	252.0	2,518.8
6R1	1,440	0.0	0.0	3,364.8	68W	1440	0.0	0.0	1,499.4	71M	1,440	0.0	0.0	2,518.8
5V7	0	0.0	0.0	0.0	5WF	0	0.0	0.0	0.0	6BJ	0	0.0	0.0	0.0
5V7	2.5	19.7	24.6	24.6	5WF	3	7.9	11.9	11.9	6BJ	2.5	0.0	0.3	0.3
5V7	4.5	18.9	38.6	63.2	5WF	5	12.9	20.6	32.7	6BJ	5	14.1	25.9	34.1
5V7	10	29.6	133.4	196.6	5WF	10	19.8	81.0	114.4	6BJ	10	22.2	98.0	124.9
5V7	15	32.9	156.3	352.9	5WF	15	21.1	102.3	216.7	6BJ	15	21.2	108.5	233.4
5V7	25	37.9	354.0	706.9	5WF	25	19.4	202.5	419.2	6BJ	25	23.1	221.5	454.9
5V7	40	28.2	495.0	1,202.0	5WF	40	14.9	267.3	676.4	6BJ	40	22.1	339.0	793.9
5V7	60	22.5	508.0	1,710.0	5WF	60	10.4	255.0	931.4	6BJ	60	19.5	418.0	1,209.9
5V7	90	11.3	508.5	2,219.1	5WF	90	9.4	300.0	1,231.4	6BJ	90	13.2	498.5	1,708.4
5V7	120	5.0	244.5	2,463.6	5WF	120	5.9	229.5	1,460.9	6BJ	120	9.4	339.0	2,039.4
5V7	180	0.0	150.0	2,613.6	5WF	180	0.0	177.0	1,637.9	6BJ	180	9.7	573.0	2,612.4
5V7	240	0.0	0.0	2,613.6	5WF	240	0.0	0.0	1,637.9	6BJ	240	0.0	291.0	2,903.4
5V7	480	0.0	0.0	2,613.6	5WF	480	0.0	0.0	1,637.9	6BJ	480	0.0	0.0	2,903.4
5V7	1,440	0.0	0.0	2,613.6	5WF	1440	0.0	0.0	1,637.9	6BJ	1,440	0.0	0.0	2,903.4

TABLE 19.
(Continued)

118 µg/kg

Animal	Time	Conc. (ng/mL)	dAUC (ng•min/mL)	Cum. AUC (ng•min/mL)	Animal	Time	Conc. (ng/mL)	dAUC (ng•min/mL)	Cum. AUC (ng•min/mL)	Animal	Time	Conc. (ng/mL)	dAUC (ng•min/mL)	Cum. AUC (ng•min/mL)
6AR	0	0.0	0.0	0.0	6AS	0	0.0	0.0	0.0	61P	0	0.0	0.0	0.0
6AR	2	14.4	14.4	14.4	6AS	4	18.4	20.8	20.8	61P	2.25	0.0	0.0	0.0
6AR	5	31.8	69.3	83.7	6AS	5	23.9	17.2	38.0	61P	5	22.4	38.8	38.8
6AR	10	49.8	204.0	287.7	6AS	10	48.0	170.0	217.7	61P	10	52.9	188.3	219.1
6AR	15	55.3	282.0	550.5	6AS	15	43.0	227.5	445.2	61P	15	61.9	287.0	506.1
6AR	25	53.9	546.0	1,396.5	6AS	25	44.7	438.5	883.7	61P	25	48.9	554.0	1,060.1
6AR	40	47.0	760.0	1,863.2	6AS	40	44.9	872.0	1,555.7	61P	40	34.6	826.3	1,886.3
6AR	60	34.2	612.0	2,865.2	6AS	60	27.7	720.0	2,281.7	61P	60	19.7	543.0	2,229.3
6AR	90	28.3	817.5	3,482.7	6AS	90	18.4	681.5	2,943.2	61P	90	10.0	445.5	2,674.8
6AR	120	14.0	514.5	3,997.2	6AS	120	8.6	376.0	3,318.2	61P	120	6.5	247.5	2,922.3
6AR	180	7.2	636.0	4,633.2	6AS	180	7.2	474.3	3,792.2	61P	180	0.0	195.0	3,117.3
6AR	240	0.0	486.0	5,119.2	6AS	240	5.5	381.0	4,173.2	61P	240	0.0	0.0	3,117.3
6AR	480	0.0	1,056.0	6,169.2	6AS	480	0.0	660.0	4,833.2	61P	480	0.0	0.0	3,117.3
6AR	1,440	0.0	0.0	6,169.2	6AS	1,440	0.0	0.0	4,833.2	61P	1,440	0.0	0.0	3,117.3
68W	0	0.0	0.0	0.0	71M	0	0.0	0.0	0.0	6R1	0	0.0	0.0	0.0
68W	3	9.3	14.0	14.0	71M	3	16.2	24.3	24.3	6R1	2.5	6.4	0.0	0.0
68W	5	17.2	26.5	40.5	71M	5	17.9	34.1	58.4	6R1	5	0.0	28.0	28.0
68W	10	26.3	108.0	149.2	71M	10	23.2	102.0	101.2	6R1	10	15.5	62.0	90.0
68W	15	36.3	156.5	305.7	71M	15	21.0	112.0	273.2	6R1	15	25.0	103.3	194.0
68W	25	45.0	409.5	715.2	71M	25	24.2	229.0	502.2	6R1	25	36.0	309.0	503.0
68W	40	42.4	660.0	1,375.2	71M	40	17.0	309.0	811.2	6R1	40	30.7	545.3	1,048.3
68W	60	34.7	771.0	2,146.2	71M	60	12.3	293.0	1,104.2	6R1	60	20.3	650.0	1,698.3
68W	90	23.7	870.0	3,022.2	71M	90	8.0	316.5	1,420.7	6R1	90	10.0	694.5	2,392.8
68W	120	17.3	615.0	3,637.2	71M	120	0.0	234.0	1654.7	6R1	120	12.3	454.5	2,847.3
68W	180	9.9	816.0	4,453.2	71M	180	0.0	284.0	1858.7	6R1	180	0.0	639.0	3,486.3
68W	240	.			71M	240	0.0	0.0	1858.7	6R1	240	0.0	468.0	3,954.3
68W	480	0.0	1,485.0	5,938.2	71M	480	0.0	0.0	1858.7	6R1	480	0.0	792.0	4,746.3
68W	1,440	0.0	0.0	5,938.2	71M	1,440	0.0	0.0	1858.7	6R1	1440	0.0	0.0	4,746.3
6BJ	0	0.0	0.0	0.0	6V7	0	0.0	0.0	0.0	6WF	0	0.0	0.0	0.0
6BJ	2.25	0.2	0.2	0.2	6V7	2.5	0.0	0.0	0.0	6WF	2.5	24.5	38.8	38.8
6BJ	5	10.0	34.4	43.6	6V7	5	5.0	7.3	7.3	6WF	5	64.5	111.3	141.9
6BJ	10	44.5	153.3	196.0	6V7	10	14.2	50.0	57.3	6WF	10	71.1	339.0	480.9
6BJ	15	45.5	225.0	421.0	6V7	15	14.3	71.3	128.5	6WF	15	62.3	333.5	814.4
6BJ	25	37.0	415.5	837.4	6V7	25	20.0	175.5	304.0	6WF	25	35.5	489.0	1,303.4
6BJ	40	32.5	525.0	1,363.1	6V7	40	25.3	345.0	649.0	6WF	40	18.0	487.3	1,710.8
6BJ	60	23.7	562.0	1,925.1	6V7	60	25.0	503.0	1152.0	6WF	60	8.0	274.0	1,984.8
6BJ	90	19.4	646.5	2,571.6	6V7	90	23.9	733.5	1886.3	6WF	90	0.0	129.0	2,113.8
6BJ	120	15.9	529.5	3,101.1	6V7	120	17.2	610.5	2502.0	6WF	120	0.0	0.0	2,113.8
6BJ	180	5.5	642.0	3,743.1	6V7	180	11.0	840.0	3348.0	6WF	180	0.0	0.0	2,113.8
6BJ	240	0.0	165.0	3,908.1	6V7	243	0.0	623.7	3972.5	6WF	240	0.0	0.0	2,113.8
6BJ	480	0.0	0.0	3,908.1	6V7	480	0.0	1842.0	5815.3	6WF	480	0.0	0.0	2,113.8
6BJ	1,440	0.0	0.0	3,908.1	6V7	1,440	0.0	0.0	5815.3	6WF	1440	0.0	0.0	2,113.8

TABLE 19.
(Continued)

220 µg/kg

Animal	Time	Conc. (ng/mL)	dAUC (ng•min/mL)	Cum. AUC (ng•min/mL)	Animal	Time	Conc. (ng/mL)	dAUC (ng•min/mL)	Cum. AUC (ng•min/mL)	Animal	Time	Conc. (ng/mL)	dAUC (ng•min/mL)	Cum. AUC (ng•min/mL)
6AR	0	0.0	0.0	0.0	6AS	0	0.0	0.0	0.0	61P	0	0.0	0.0	0.0
6AR	2.5	15.2	19.0	19.0	6AS	2.5	18.5	13.1	13.1	61P	2.75	11.8	16.2	16.2
6AR	5	29.2	55.5	74.5	6AS	5.5	33.9	88.8	79.7	61P	4	63.8	47.1	63.4
6AR	10	54.1	208.3	282.8	6AS	10	53.1	195.8	275.5	61P	10	102.8	498.8	668.2
6AR	15	67.1	383.0	585.8	6AS	15	54.9	278.8	545.5	61P	15	124.7	568.8	1,126.9
6AR	25	83.8	758.5	1,336.3	6AS	25	82.3	586.8	1,131.5	61P	25	117.5	1,211.8	2,337.9
6AR	40	66.1	1,118.3	2,454.5	6AS	40	66.5	921.8	2,052.5	61P	40	88.9	1,486.8	3,826.9
6AR	60	47.3	1,134.8	3,588.5	6AS	60	45.1	1,056.8	3,108.5	61P	60	60.2	1,311.8	5,138.9
6AR	90	34.8	1,231.5	4,820.0	6AS	90	28.7	1,107.8	4,215.5	61P	90	34.3	1,267.5	6,404.4
6AR	120	22.2	355.0	5,676.0	6AS	120.5	15.0	676.8	4,891.1	61P	120	27.5	927.0	7,331.4
6AR	180	12.8	1,050.0	6,725.0	6AS	180	9.0	749.7	5,640.8	61P	181	15.6	1,314.0	8,646.0
6AR	240	9.5	669.0	7,394.0	6AS	240	6.3	477.0	6,117.0	61P	240	13.9	876.3	9,516.2
6AR	480	0.0	1,140.0	8,534.0	6AS	480	0.0	756.0	6,873.0	61P	480	0.0	1,668.0	11,184.2
6AR	1,440	0.0	0.0	8,534.0	6AS	1,440	0.0	0.0	6,873.0	61P	1,440	0.0	0.0	11,184.2
71M	0	0.0	0.0	0.0	6R1	0	0.0	0.0	0.0	68W	0	0.0	0.0	0.0
71M	2.5	23.1	28.9	28.9	6R1	2	0.0	0.0	0.0	68W	2.5	0.0	11.0	11.0
71M	5	35.7	73.5	102.4	6R1	5	18.4	33.8	38.8	68W	5	18.1	31.1	42.1
71M	10	52.1	219.5	321.9	6R1	10	28.2	111.5	151.1	68W	10	38.7	137.8	179.1
71M	15	54.8	268.8	588.8	6R1	15.75	26.5	157.3	308.4	68W	15	47.8	215.8	394.9
71M	25	56.8	553.8	1,141.8	6R1	25	38.8	289.1	597.4	68W	25	58.7	491.5	886.4
71M	40	66.9	921.8	2,063.4	6R1	40.25	36.2	558.5	1,148.8	68W	40			
71M	60	55.4	1,223.8	3,286.4	6R1	60	28.6	639.9	1,787.9	68W	60	34.3	1,487.5	2,373.9
71M	90	35.1	1,357.5	4,643.9	6R1	90	18.8	711.8	2,498.9	68W	90	49.8	1,249.5	3,623.4
71M	120	27.2	934.5	5,578.4	6R1	120	12.3	486.5	2,985.4	68W	120	16.2	978.8	4,601.4
71M	180	19.2	1,392.8	6,970.4	6R1	180.75	9.1	858.8	3,815.4	68W	180	18.1	789.8	5,390.4
71M	240	13.9	993.8	7,963.4	6R1	240.25	4.5	484.8	4,020.8	68W	240	8.7	504.8	5,894.4
71M	480	5.8	2,340.8	10,303.4	6R1	480	0.0	539.4	4,559.4	68W	480	0.0	804.8	8,698.4
71M	1,440	3.0	2,888.8	12,991.4	6R1	1,440	0.0	0.0	4,559.4	68W	1,440	0.0	0.0	8,698.4
6WF	0	0.0	0.0	0.0	6BJ	0	0.0	0.0	0.0	6V7	0	0.0	0.0	0.0
6WF	2.5	68.1	85.1	85.1	6BJ	2.5	0.0	0.5	0.5	6V7	2.25	12.4	14.0	14.0
6WF	5	184.2	215.4	300.5	6BJ	5	13.6	25.5	34.0	6V7	5	19.9	44.4	53.4
6WF	10	90.3	486.3	786.8	6BJ	10	30.3	124.8	158.8	6V7	10	37.3	143.8	201.4
6WF	15	77.2	418.8	1,205.5	6BJ	15	49.6	214.8	373.5	6V7	15	44.4	204.3	405.8
6WF	25	69.5	733.5	1,939.0	6BJ	25	62.4	588.8	933.5	6V7	25	39.8	421.8	826.8
6WF	40	53.1	919.5	2,858.5	6BJ	40	57.1	898.3	1,829.8	6V7	40	33.1	548.8	1,373.4
6WF	60	41.8	941.8	3,799.5	6BJ	60	42.8	999.8	2,828.8	6V7	60	34.8	871.8	2,844.4
6WF	90	25.8	998.8	4,789.5	6BJ	90	27.7	1,067.5	3,886.3	6V7	90	29.3	949.5	2,993.9
6WF	120	15.8	812.8	5,601.5	6BJ	120	20.5	723.8	4,609.3	6V7	120	28.2	742.5	3,736.4
6WF	180	8.9	741.8	6,142.5	6BJ	180	9.9	912.8	5,521.3	6V7	180	13.6	1,014.8	4,750.4
6WF	240	0.5	267.8	6,409.5	6BJ	240	0.8	537.8	6,058.3	6V7	240	12.5	783.8	5,533.4
6WF	480	0.8	0.0	6,409.5	6BJ	480	0.8	960.0	7,018.3	6V7	480	0.0	1,500.0	7,033.4
6WF	1,440	0.0	0.0	6,409.5	6BJ	1,440	0.0	0.0	7,018.3	6V7	1,440	0.0	0.0	7,033.4

Plasma samples were taken from each of the nine monkeys prior to their use in the pharmacokinetic study and sent to Dr. David J. Greenblatt, for determination of the percent of free diazepam as opposed to diazepam bound to plasma proteins. The diazepam free fractions, as determined by Dr. Greenblatt, are presented in Table 20. The AUCs for free diazepam, based on these percentages and the unmodeled total plasma diazepam concentrations at each time for each monkey, were calculated and are presented in Table 21.

Mean pharmacokinetic parameter values, standard deviations, minimum and maximum values, and standard errors of the means were calculated from the data for nine monkeys at each dose level of diazepam. This information is presented in Table 22. Figure 5 is a graph of the mean plasma diazepam concentrations as a function of time for the three dose levels. Figures 6, 7, and 8 demonstrate the variability from the mean of individual values for the three diazepam dose levels. Graphs of plasma diazepam concentrations as a function of time for the three dose levels in individual animals are presented in Appendix D.

Experimental designs in which the same animals are tested on multiple occasions using different dose levels on different testing days are called cross-over designs. By using a cross-over design, comparisons among the pharmacokinetic parameters across the dose levels of diazepam can be made on an individual animal basis. Controlling for the animal to animal variability by using each animal as its own control provides more precise comparisons across the dose levels of diazepam. However, the effect of the diazepam dose administered one day of testing may carry over into the next day of testing with the same animal. Therefore, a dose of diazepam may have a direct effect on the results of the day in which the diazepam was injected and a residual effect on the succeeding day of experimentation. The pharmacokinetic study was designed using Latin squares balanced for dose effects, day of testing effects, and carryover effects. The sequence in which individual monkeys received doses of diazepam is given in Table 23. A relatively long washout and recovery period was used to allow return to normal blood values and physiologic state and to reduce any possible residual effects.

TABLE 20. PLASMA DIAZEPAM FREE FRACTION FOR INDIVIDUAL ANIMALS

Animal ID	Free Fraction (percent unbound)
5V7	3.05
5WF	3.48
61P	3.44
68W	3.56
6AR	2.72
6AS	3.16
6BJ	3.38
6R1	2.85
71M	3.40

TABLE 21. AUCs OF PLASMA FREE DIAZEPAM FOR INDIVIDUAL ANIMALS BASED ON UNMODELED AUCs

LOW DOSE (~70 μg/kg)

Animal	Time	Total DZ Conc. (ng/mL)	Free DZ Conc. (ng/mL)	Free DZ dAUC (ngmin/mL)	Free DZ Cum. AUC (ngmin/mL)	Animal	Time	Total DZ Conc. (ng/mL)	Free DZ Conc. (ng/mL)	Free DZ dAUC (ngmin/mL)	Free DZ Cum. AUC (ngmin/mL)	Animal	Time	Total DZ Conc. (ng/mL)	Free DZ Conc. (ng/mL)	Free DZ dAUC (ngmin/mL)	FreeDZ Cum.AUC (ngmin/mL)
6AS	0	0.0	0.000	0.0	0.0	61P	0	0.0	0.000	0.0	0.0	6AR	0	0.0	0.000	0.0	0.0
6AS	3	19.3	0.618	0.9	0.9	61P	2.5	0.0	0.000	0.0	0.0	6AR	2.5	0.0	0.000	0.0	0.0
6AS	6	33.9	1.071	2.5	3.4	61P	5	5.8	0.200	0.2	0.2	6AR	5	9.9	0.269	0.3	0.3
6AS	10	49.3	1.556	5.3	8.7	61P	10	10.9	0.650	2.1	2.4	6AR	10	20.1	0.547	2.8	2.4
6AS	15	51.8	1.637	8.0	16.7	61P	15	37.1	1.278	4.8	7.2	6AR	15	22.3	0.607	2.9	5.3
6AS	25	45.4	1.436	15.4	32.0	61P	25	32.7	1.125	12.8	19.2	6AR	25	22.1	0.601	8.8	11.3
6AS	40	33.4	1.055	18.7	50.7	61P	40	22.5	0.774	14.2	33.4	6AR	40	18.7	0.509	8.3	19.6
6AS	60	28.8	0.857	17.1	67.8	61P	60	18.6	0.640	14.1	47.6	6AR	60	14.9	0.405	9.1	28.8
6AS	90	11.4	0.368	15.3	83.1	61P	90	14.7	0.506	17.2	64.8	6AR	90	4.2	0.114	7.8	36.6
6AS	120	9.8	0.303	10.8	93.1	61P	120	9.2	0.316	12.3	77.1	6AR	120	0.0	0.000	1.7	38.3
6AS	180	0.0	0.000	9.1	102.2	61P	180	6.4	0.220	16.1	93.2	6AR	180	0.0	0.000	0.0	38.3
6AS	240	0.0	0.000	0.0	102.2	61P	240	0.0	0.000	0.0	99.8	6AR	240	0.0	0.000	0.0	38.3
6AS	480	0.0	0.000	0.0	102.2	61P	480	0.0	0.000	0.0	99.8	6AR	480	0.0	0.000	0.0	38.3
6AS	1,440	0.0	0.000	0.0	102.2	61P	1,440	0.0	0.000	0.0	99.8	6AR	1,440	0.0	0.000	0.0	38.3
6R1	0	0.0	0.000	0.0	0.0	68W	0	0.0	0.000	0.0	0.0	71M	0	0.0	0.000	0.0	0.0
6R1	2.5	6.1	0.174	0.2	0.2	68W	3	0.0	0.000	0.0	0.0	71M	2.75	0.0	0.000	0.0	0.0
6R1	5	12.8	0.365	0.7	0.9	68W	5	8.4	0.299	0.3	0.3	71M	5	8.7	0.228	0.3	0.3
6R1	10	23.9	0.681	2.0	3.5	68W	10	11.6	0.413	1.8	2.1	71M	10	12.8	0.428	1.6	1.9
6R1	15	26.9	0.767	3.8	7.1	68W	15	15.7	0.559	2.4	4.5	71M	15	14.8	0.496	2.3	4.2
6R1	25	26.4	0.752	7.8	14.7	68W	25	13.4	0.477	5.2	9.7	71M	25	13.1	0.445	4.7	8.9
6R1	40	24.7	0.704	10.9	25.6	68W	40	17.1	0.609	8.1	17.8	71M	40	15.3	0.520	7.2	16.2
6R1	60	16.0	0.456	11.6	37.2	68W	60	10.9	0.388	10.0	27.8	71M	60	10.1	0.849	11.7	27.9
6R1	90	12.0	0.342	12.5	49.2	68W	90	9.2	0.328	10.7	38.5	71M	90	11.1	0.377	15.4	43.3
6R1	120	9.4	0.268	9.1	58.4	68W	120	6.2	0.221	8.2	46.8	71M	120	11.4	0.388	11.5	54.7
6R1	180	5.0	0.143	12.3	70.7	68W	180	0.0	0.000	6.6	53.4	71M	180	4.2	0.143	15.9	70.6
6R1	240	4.9	0.140	8.5	79.1	68W	240	0.0	0.000	0.0	53.4	71M	240	2.1	0.071	8.4	77.1
6R1	480	0.0	0.000	16.8	95.9	68W	480	0.0	0.000	0.0	53.4	71M	480	0.0	0.000	0.0	85.6
6R1	1,440	0.0	0.000	0.0	95.9	68W	1,440	0.0	0.000	0.0	53.4	71M	1,440	0.0	0.000	0.0	85.6
5V7	0	0.0	0.000	0.0	0.0	5WF	0	0.0	0.000	0.0	0.0	6BJ	0	0.0	0.000	0.0	0.0
5V7	2.5	19.7	0.681	0.8	0.8	5WF	3	7.9	0.275	0.4	0.4	6BJ	2.5	0.0	0.223	0.3	0.3
5V7	4.5	18.9	0.678	1.2	1.9	5WF	5	12.9	0.449	0.7	1.1	6BJ	5	14.1	0.477	0.9	1.2
5V7	10	29.8	0.983	4.1	8.8	5WF	10	19.8	0.689	2.8	4.0	6BJ	10	22.2	0.758	3.1	4.2
5V7	15	32.9	1.083	4.8	18.8	5WF	15	21.1	0.734	3.6	7.5	6BJ	15	21.2	0.717	3.7	7.9
5V7	25	37.9	1.156	10.8	21.6	5WF	25	19.4	0.675	7.8	14.6	6BJ	25	23.1	0.781	7.5	15.4
5V7	40	28.2	0.868	15.1	36.7	5WF	40	14.9	0.519	9.8	23.5	6BJ	40	22.1	0.747	11.6	26.8
5V7	60	22.6	0.689	15.C	52.2	5WF	60	10.6	0.369	8.9	32.4	6BJ	60	19.5	0.659	14.1	40.9
5V7	90	11.3	0.345	15.5	67.7	5WF	90	9.4	0.327	10.4	42.9	6BJ	90	13.2	0.446	10.8	57.5
5V7	120	5.8	0.153	7.5	75.1	5WF	120	5.9	0.205	8.8	50.8	6BJ	120	9.4	0.318	11.5	68.9
5V7	180	0.0	0.000	4.6	79.7	5WF	180	0.0	0.000	6.2	57.0	6BJ	180	9.7	0.328	19.4	88.3
5V7	240	0.0	0.000	0.0	79.7	5WF	240	0.0	0.000	0.0	57.0	6BJ	240	0.0	0.000	9.8	98.1
5V7	480	0.0	0.000	0.0	79.7	5WF	480	0.0	0.000	0.0	57.0	6BJ	480	0.0	0.000	0.0	98.1
5V7	1,440	0.0	0.000	0.0	79.7	5WF	1,440	0.0	0.000	0.0	57.0	6BJ	1,440	0.0	0.000	0.0	98.1

TABLE 21.
(Continued)

MIDDLE DOSE (~110 µg/kg)

Animal	Time	Total DZ Conc. (ng/mL)	Free DZ Conc. (ng/mL)	Free DZ dAUC (ngmin/mL)	Free DZ Cum. AUC (ngmin/mL)	Animal	Time	Total DZ Conc. (ng/mL)	Free DZ Conc. (ng/mL)	Free DZ dAUC (ngmin/mL)	Free DZ Cum. AUC (ngmin/mL)	Animal	Time	Total DZ Conc. (ng/mL)	Free DZ Conc. (ng/mL)	Free DZ dAUC (ngmin/mL)	FreeDZ Cum.AUC (ngmin/mL)
6AR	0	0.0	0.000	0.0	0.0	6AS	0	0.0	0.000	0.0	0.0	61P	0	0.0	0.000	0.0	0.0
6AR	2	14.4	0.392	0.4	0.4	6AS	4	10.4	0.329	0.7	0.7	61P	2.25	0.0	0.000	0.0	0.0
6AR	5	31.0	0.865	1.9	2.3	6AS	5	23.9	0.765	0.5	1.2	61P	5	22.4	0.771	1.1	1.1
6AR	10	49.0	1.356	5.5	7.0	6AS	10	40.0	1.517	5.7	6.9	61P	10	52.9	1.820	6.5	7.5
6AR	15	55.3	1.504	7.1	15.0	6AS	15	43.0	1.359	7.2	14.1	61P	15	61.9	2.129	9.9	17.4
6AR	25	53.9	1.466	14.9	29.0	6AS	25	44.7	1.413	13.9	27.9	61P	25	48.9	1.682	19.1	36.5
6AR	40	47.0	1.278	20.6	50.4	6AS	40	44.9	1.419	21.2	49.2	61P	40	34.6	1.190	21.5	58.0
6AR	60	34.2	0.930	22.1	72.5	6AS	60	27.7	0.875	22.9	72.1	61P	60	19.7	0.678	18.7	76.7
6AR	90	20.3	0.552	22.2	94.7	6AS	90	16.4	0.518	20.9	93.0	61P	90	10.0	0.344	15.3	92.0
6AR	120	14.0	0.381	14.0	108.7	6AS	120	8.6	0.272	11.9	104.9	61P	120	6.5	0.224	8.5	100.5
6AR	180	7.2	0.196	17.3	126.0	6AS	180	7.2	0.228	15.0	119.8	61P	180	0.0	0.000	0.7	107.2
6AR	240	0.0	0.239	13.1	139.1	6AS	240	5.5	0.174	12.0	131.9	61P	240	0.0	0.000	0.0	107.2
6AR	480	0.0	0.000	28.7	167.8	6AS	480	0.0	0.000	20.9	152.7	61P	480	0.0	0.000	0.0	107.2
6AR	1,440	0.0	0.000	0.0	167.8	6AS	1,440	0.0	0.000	0.0	152.7	61P	1,440	0.0	0.000	0.0	107.2
68W	0	0.0	0.000	0.0	0.0	71M	0	0.0	0.000	0.0	0.0	6R1	0	0.0	0.000	0.0	0.0
68W	3	9.3	0.331	0.5	0.5	71M	3	16.2	0.551	0.0	0.0	6R1	2.5	0.4	0.102	0.2	0.2
68W	5	17.2	0.612	0.9	1.4	71M	5	17.0	0.609	1.2	2.0	6R1	5	0.0	0.274	0.0	0.0
68W	10	26.3	0.938	3.9	5.3	71M	10	23.2	0.789	3.5	5.5	6R1	10	15.5	0.442	1.0	2.0
68W	15	36.3	1.292	5.0	10.0	71M	15	21.0	0.734	3.0	8.3	6R1	15	25.0	0.735	2.9	5.5
68W	25	45.6	1.623	14.6	25.5	71M	25	24.2	0.823	7.0	17.1	6R1	25	36.0	1.020	8.0	14.3
68W	40	42.4	1.509	23.5	49.0	71M	40	17.0	0.578	10.5	27.0	6R1	40	30.7	1.040	15.5	29.9
68W	60	34.7	1.235	27.4	76.4	71M	60	12.3	0.418	10.0	37.5	6R1	60	20.3	0.007	10.5	40.4
68W	90	23.7	0.844	31.2	107.6	71M	90	8.0	0.299	10.0	48.3	6R1	90	10.0	0.513	19.0	60.2
68W	120	17.3	0.616	21.9	129.5	71M	120	6.0	0.231	0.0	50.3	6R1	120	12.3	0.351	13.0	01.1
68W	180	0.0	0.352	29.1	150.5	71M	180	0.0	0.000	0.0	03.2	6R1	180	0.0	0.257	10.2	99.4
68W	240					71M	240	0.0	0.000	0.0	03.2	6R1	240	0.0	0.100	13.3	112.7
68W	480	0.0	0.000	52.9	211.4	71M	480	0.0	0.000	0.0	03.2	6R1	480	0.0	0.000	22.0	135.3
68W	1,440	0.0	0.000	0.0	211.4	71M	1,440	0.0	0.000	0.0	03.2	6R1	1,440	0.0	0.000	0.0	135.3
6BJ	0	0.0	0.000	0.0	0.0	6V7	0	0.0	0.000	0.0	0.0	6WF	0	0.0	0.000	0.0	0.0
6BJ	2.25	0.2	0.277	0.3	0.3	6V7	2.5	0.0	0.000	0.0	0.0	6WF	2.5	24.5	0.053	1.1	1.1
6BJ	5	10.0	0.500	1.2	1.5	6V7	5	5.0	0.177	0.2	0.2	6WF	5	04.5	2.245	3.9	4.9
6BJ	10	44.5	1.504	5.2	0.7	6V7	10	14.2	0.433	1.5	1.7	6WF	10	71.1	2.474	11.0	10.7
6BJ	15	45.5	1.530	7.0	14.3	6V7	15	14.3	0.436	2.2	3.9	6WF	15	02.3	2.150	11.0	20.3
6BJ	25	37.0	1.271	14.0	20.3	6V7	25	20.0	0.034	5.4	9.3	6WF	25	35.5	1.235	17.0	45.4
6BJ	40	32.5	1.099	17.0	40.1	6V7	40	25.3	0.772	10.5	19.0	6WF	40	10.0	0.054	14.2	59.5
6BJ	60	23.7	0.001	19.0	05.1	6V7	60	25.0	0.703	15.3	35.2	6WF	60	0.0	0.299	9.5	09.1
6BJ	90	19.4	0.050	21.9	00.9	6V7	90	23.9	0.729	22.4	57.5	6WF	90	0.0	0.000	4.5	73.0
6BJ	120	15.9	0.537	17.9	104.0	6V7	120	17.2	0.525	10.0	70.3	6WF	120	0.0	0.000	0.0	73.0
6BJ	100	5.5	0.100	21.7	120.5	6V7	100	11.0	0.330	25.0	102.1	6WF	100	0.0	0.000	0.0	73.0
6BJ	240	0.0	0.000	5.0	132.1	6V7	240	0.0	0.200	19.0	121.2	6WF	240	0.0	0.000	0.0	73.0
6BJ	480	0.0	0.000	0.0	132.1	6V7	480	0.0	0.000	31.0	153.0	6WF	480	0.0	0.000	0.0	73.0
6BJ	1,440	0.0	0.000	0.0	132.1	6V7	1,440	0.0	0.000	0.0	153.0	6WF	1,440	0.0	0.000	0.0	73.0

TABLE 21.
(Continued)

HIGH DOSE (~220 μg/kg)

Animal	Time	Total DZ Conc. (ng/mL)	Free DZ Conc. (ng/mL)	Free DZ dAUC (ng•min/mL)	Free DZ Cum. AUC (ng•min/mL)	Animal	Time	Total DZ Conc. (ng/mL)	Free DZ Conc. (ng/mL)	Free DZ dAUC (ng•min/mL)	Free DZ Cum. AUC (ng•min/mL)	Animal	Time	Total DZ Conc. (ng/mL)	Free DZ Conc. (ng/mL)	Free DZ dAUC (ng•min/mL)	FreeDZ Cum.AUC (ng•min/mL)
61P	0	0.0	0.000	0.0	0.0	6AR	0	0.0	0.000	0.0	0.0	6AS	0	0.0	0.000	0.0	0.0
61P	2.75	11.8	0.486	0.6	0.6	6AR	2.5	15.2	0.413	0.5	0.5	6AS	2.5	18.5	0.332	0.4	0.4
61P	4	63.8	2.188	1.6	2.2	6AR	5	29.2	0.794	1.5	2.0	6AS	5.5	33.9	1.071	2.1	2.5
61P	10	102.0	3.509	17.1	19.3	6AR	10	54.1	1.472	5.7	7.7	6AS	10	53.1	1.670	6.2	8.7
61P	15	124.7	4.290	19.5	38.8	6AR	15	67.1	1.825	8.2	15.9	6AS	15	54.9	1.736	8.5	17.2
61P	25	117.5	4.042	41.7	80.4	6AR	25	83.0	2.258	20.4	36.3	6AS	25	62.3	1.969	18.5	35.8
61P	40	80.9	2.783	51.2	131.6	6AR	40	66.1	1.798	30.4	66.8	6AS	40	60.5	1.912	29.1	64.9
61P	60	50.2	1.727	45.1	176.7	6AR	60	47.3	1.287	36.8	97.6	6AS	60	45.1	1.425	33.4	98.2
61P	90	34.3	1.180	43.6	220.3	6AR	90	34.8	0.947	33.5	131.1	6AS	90	28.7	0.907	35.0	133.2
61P	120	27.5	0.946	31.9	252.2	6AR	120	22.2	0.604	23.3	154.4	6AS	120.5	15.6	0.493	21.3	154.6
61P	181	15.6	0.537	45.2	297.4	6AR	180	12.6	0.348	28.6	182.9	6AS	180	9.6	0.303	23.7	178.3
61P	240	13.9	0.478	29.9	327.4	6AR	240	9.5	0.258	18.2	201.1	6AS	240	6.3	0.199	15.1	193.3
61P	480	0.0	0.000	57.4	384.7	6AR	480	0.0	0.000	31.8	232.1	6AS	480	0.0	0.000	23.9	217.2
61P	1,440	0.0	0.000	0.0	384.7	6AR	1,440	0.0	0.000	0.0	232.1	6AS	1,440	0.0	0.000	0.0	217.2
71M	0	0.0	0.000	0.0	0.0	6R1	0	0.0	0.000	0.0	0.0	6BW	0	0.0	0.000	0.0	0.0
71M	2.5	23.1	0.795	1.6	1.6	6R1	2	5.0	0.171	0.2	0.2	6BW	2.5	8.8	0.313	0.4	0.4
71M	5	35.7	1.214	2.5	3.5	6R1	5	16.4	0.487	1.0	1.1	6BW	5	16.1	0.573	1.1	1.5
71M	10	52.1	1.771	7.5	10.9	6R1	10	28.2	0.884	3.2	4.3	6BW	10	36.7	1.378	4.9	6.4
71M	15	54.6	1.856	9.1	20.0	6R1	15.75	26.5	0.755	4.5	8.8	6BW	15	47.6	1.695	7.7	14.1
71M	25	58.8	1.994	18.8	38.8	6R1	25	36.8	1.026	8.2	17.0	6BW	25	58.7	1.885	17.5	31.6
71M	40	66.9	2.275	31.3	70.2	6R1	40.25	36.2	1.032	15.7	32.7	6BW	40				
71M	60	55.4	1.884	41.8	111.7	6R1	60	28.6	0.815	18.2	51.0	6BW	60	34.3	1.221	53.6	84.5
71M	90	35.1	1.193	46.2	157.9	6R1	90	18.8	0.536	20.3	71.2	6BW	90	49.8	1.744	44.5	129.0
71M	120	27.2	0.925	31.8	189.7	6R1	120	12.3	0.351	13.3	84.5	6BW	120	16.2	0.577	34.0	163.0
71M	180	19.2	0.653	47.3	237.0	6R1	180.75	9.1	0.259	10.5	103.0	6BW	180	10.1	0.360	28.1	191.0
71M	240	13.9	0.473	33.8	270.8	6R1	240.25	4.6	0.128	11.5	114.6	6BW	240	6.7	0.239	17.9	209.0
71M	480	5.6	0.190	79.6	350.3	6R1	480	0.0	0.000	15.4	129.9	6BW	480	0.0	0.000	28.6	238.5
71M	1,440	0.0	0.000	91.4	441.7	6R1	1,440	0.0	0.000	0.0	129.9	6BW	1,440	0.0	0.000	0.0	238.5
6WF	0	0.0	0.000	0.0	0.0	6BJ	0	0.0	0.000	0.0	0.0	6V7	0	0.0	0.000	0.0	0.0
6WF	2.5	68.1	2.378	3.0	3.0	6BJ	2.5	6.8	0.238	0.3	0.3	6V7	2.25	12.4	0.378	0.4	0.4
6WF	5	184.2	3.620	7.5	10.5	6BJ	5	13.6	0.488	0.9	1.1	6V7	5	19.9	0.687	1.4	1.8
6WF	10	90.3	3.142	16.9	27.4	6BJ	10	36.3	1.227	4.2	5.4	6V7	10	37.3	1.138	4.4	6.1
6WF	15	77.2	2.687	14.8	42.0	6BJ	15	49.6	1.676	7.3	12.6	6V7	15	44.4	1.364	8.2	12.4
6WF	25	69.6	2.419	25.5	67.5	6BJ	25	62.4	2.109	18.9	31.6	6V7	25	39.8	1.214	12.8	25.2
6WF	40	53.1	1.848	32.8	99.5	6BJ	40	57.1	1.938	36.3	81.8	6V7	40	33.1	1.018	16.7	41.9
6WF	60	41.0	1.427	32.7	132.2	6BJ	60	42.8	1.447	33.8	96.6	6V7	60	34.8	1.037	28.5	62.4
6WF	90	25.8	0.878	34.5	166.7	6BJ	90	27.7	0.938	35.7	131.4	6V7	90	29.3	0.894	29.8	91.3
6WF	120	15.8	0.550	21.3	188.0	6BJ	120	20.5	0.693	24.4	155.8	6V7	120	20.2	0.618	22.8	114.0
6WF	180	8.9	0.310	25.8	213.8	6BJ	180	9.9	0.335	30.8	186.6	6V7	180	13.6	0.415	30.9	144.9
6WF	240	8.0	0.000	9.3	223.1	6BJ	240	8.0	0.270	10.2	204.8	6V7	240	12.5	0.381	23.9	168.8
6WF	480	0.0	0.000	0.0	223.1	6BJ	480	0.0	0.000	32.4	237.2	6V7	480	0.0	0.000	45.8	214.5
6WF	1,440	0.0	0.000	0.0	223.1	6BJ	1,440	0.0	0.000	0.0	237.2	6V7	1,440	0.0	0.000	0.0	214.5

TABLE 22. DIAZEPAM PHARMACOKINETIC PARAMETERS FROM SINGLE-COMPARTMENT MODEL STATISTICS FOR NINE ANIMALS GIVEN EACH DOSE LEVEL

LOW DOSE

Variable	Mean	Standard Deviation	Minimum Value	Maximum Value	Standard Error of Mean
D (μg/kg)	72.3	3.35	67	79	1.12
C_{pmax}	27.0	10.7	15.8	50.0	3.57
C_{pmax}/D	0.377	0.161	0.213	0.746	0.054
t_{max}	22.8	6.00	16.4	36.4	2.00
V_d (L/kg)	2.19	0.865	0.920	3.59	0.288
k_a	0.107	0.033	0.061	0.156	0.011
k_{el}	0.016	0.007	0.008	0.030	0.002
t_{k_a}	7.15	2.53	4.44	11.3	0.845
$t_{k_{el}}$	52.5	21.2	23.4	89.0	7.07
CL	30.1	9.10	19.9	46.6	3.04
$AUC_{0-\infty}$	2,581	671	1,546	3,565	224
AUC_{0-480}	2,563	656	1,545	3,476	219
$AUC_{0-\infty}/D$	35.8	9.90	21.5	50.2	3.30

Variable	Mean	Standard Deviation	Minimum Value	Maximum Value	Standard Error of Mean
$AUC_{0-2.5}$	9.4	5.2	3.5	20.8	1.7
AUC_{0-5}	38.1	20.4	14.9	83.2	6.8
AUC_{0-10}	132.3	67.2	56.4	280.6	22.4
AUC_{0-15}	253.0	122.5	116.1	521.6	40.8
AUC_{0-25}	516.4	229.0	267.3	1,010.8	76.3
AUC_{0-48}	886.3	350.7	520.8	1,623.7	116.9
AUC_{0-60}	1,282.7	447.8	788.9	2,190.7	149.3
AUC_{0-90}	1,698.4	514.6	1,101.0	2,677.9	171.5
AUC_{0-120}	1,968.8	542.2	1,327.8	2,922.5	180.7
AUC_{0-150}	2,271.2	571.4	1,531.4	3,106.9	190.5
AUC_{0-240}	2,416.2	596.8	1,543.1	3,153.3	198.9
AUC_{0-480}	2,563.4	656.1	1,545.5	3,475.6	218.7

TABLE 22.
(Continued)

MEDIUM DOSE

Variable	Mean	Standard Deviation	Minimum Value	Maximum Value	Standard Error of Mean
D (μg/kg)	111	1.45	109	114	0.484
C_{pmax}	43.9	14.8	23.6	68.4	4.92
C_{pmax}/D	0.395	0.131	0.213	0.600	0.044
t_{max}	24.5	13.1	10.0	51.9	4.38
V_d (L/kg)	1.96	0.980	0.866	4.01	0.327
k_a	0.109	0.066	0.040	0.255	0.022
k_{el}	0.021	0.018	0.007	0.065	0.006
t_{k_a}	8.55	4.82	2.72	17.5	1.61
$t_{k_{el}}$	46.3	23.2	10.6	92.7	7.75
CL	32.8	13.1	21.5	56.6	4.38
$AUC_{0-\infty}$	3,801	1,192	2,014	5,074	397
AUC_{0-48}	3,778	1,169	2,014	5,069	390
$AUC_{0-\infty}/D$	34.3	10.9	17.7	46.6	3.64

Variable	Mean	Standard Deviation	Minimum Value	Maximum Value	Standard Error of Mean
$AUC_{0-2.5}$	16.3	10.9	3.6	40.8	3.6
AUC_{0-5}	64.9	40.9	15.6	155.4	13.6
AUC_{0-10}	218.9	123.0	61.0	476.7	41.0
AUC_{0-15}	409.3	205.6	129.6	810.0	68.5
AUC_{0-25}	809.7	331.3	316.3	1,332.0	110.4
AUC_{0-48}	1,352.6	437.9	666.3	1,859.9	146.0
AUC_{0-60}	1,925.1	528.5	1,099.3	2,669.4	176.2
AUC_{0-96}	2,527.0	655.4	1,433.9	3,520.9	218.5
AUC_{0-120}	2,922.7	772.2	1,660.7	4,071.0	257.4
AUC_{0-168}	3,366.8	941.8	1,918.8	4,655.7	313.9
AUC_{0-240}	3,576.3	1,041.1	2,013.9	4,899.6	347.0
AUC_{0-480}	3,777.9	1,168.8	2,013.9	5,068.9	389.6

TABLE 22.
(Continued)

HIGH DOSE

Variable	Mean	Standard Deviation	Minimum Value	Maximum Value	Standard Error of Mean
D (μg/kg)	223	2.73	220	229	0.909
C_{peak}	66.2	25.2	35.6	115.6	8.41
C_{peak}/D	0.296	0.112	0.161	0.521	0.037
t_{peak}	23.8	7.61	6.71	31.6	2.54
V_d (L/kg)	2.82	1.12	1.30	4.75	0.375
k_a	0.141	0.149	0.055	0.533	0.050
k_{el}	0.013	0.004	0.007	0.021	0.001
t_{k_a}	7.41	3.27	1.30	12.6	1.09
$t_{k_{el}}$	58.1	23.2	33.6	103.9	7.73
CL	34.2	8.27	23.5	52.5	2.76
$AUC_{0-\infty}$	6,835	1,492	4,208	9,471	497
AUC_{0-480}	6,769	1,447	4,192	9,257	482
$AUC_{0-\infty}/D$	30.6	6.73	19.0	42.5	2.24

Variable	Mean	Standard Deviation	Minimum Value	Maximum Value	Standard Error of Mean
$AUC_{0-2.5}$	27.2	27.2	9.0	95.2	9.1
AUC_{0-5}	102.4	84.7	37.5	304.1	28.2
AUC_{0-10}	330.1	208.8	138.2	765.9	69.6
AUC_{0-15}	613.3	329.0	277.5	1,203.4	109.7
AUC_{0-25}	1,237.0	551.4	612.4	2,269.6	183.8
AUC_{0-40}	2,143.2	818.3	1,139.7	3,800.6	272.8
AUC_{0-60}	3,158.4	1,048.4	1,768.7	5,307.3	349.5
AUC_{0-90}	4,271.9	1,212.0	2,496.8	6,687.5	404.0
AUC_{0-120}	5,023.5	1,266.1	3,010.4	7,432.1	422.0
AUC_{0-180}	5,889.5	1,286.3	3,621.9	8,048.8	428.8
AUC_{0-240}	6,315.2	1,311.6	3,921.2	8,227.5	437.2
AUC_{0-480}	6,768.8	1,447.0	4,191.5	9,257.4	482.3

FIGURE 5. PHARMACOKINETICS OF DIAZEPAM ADMINISTERED AT THREE DOSE LEVELS - MODEL-PREDICTED VALUES USING DATA FROM NINE RHESUS MONKEYS AT EACH DOSE LEVEL

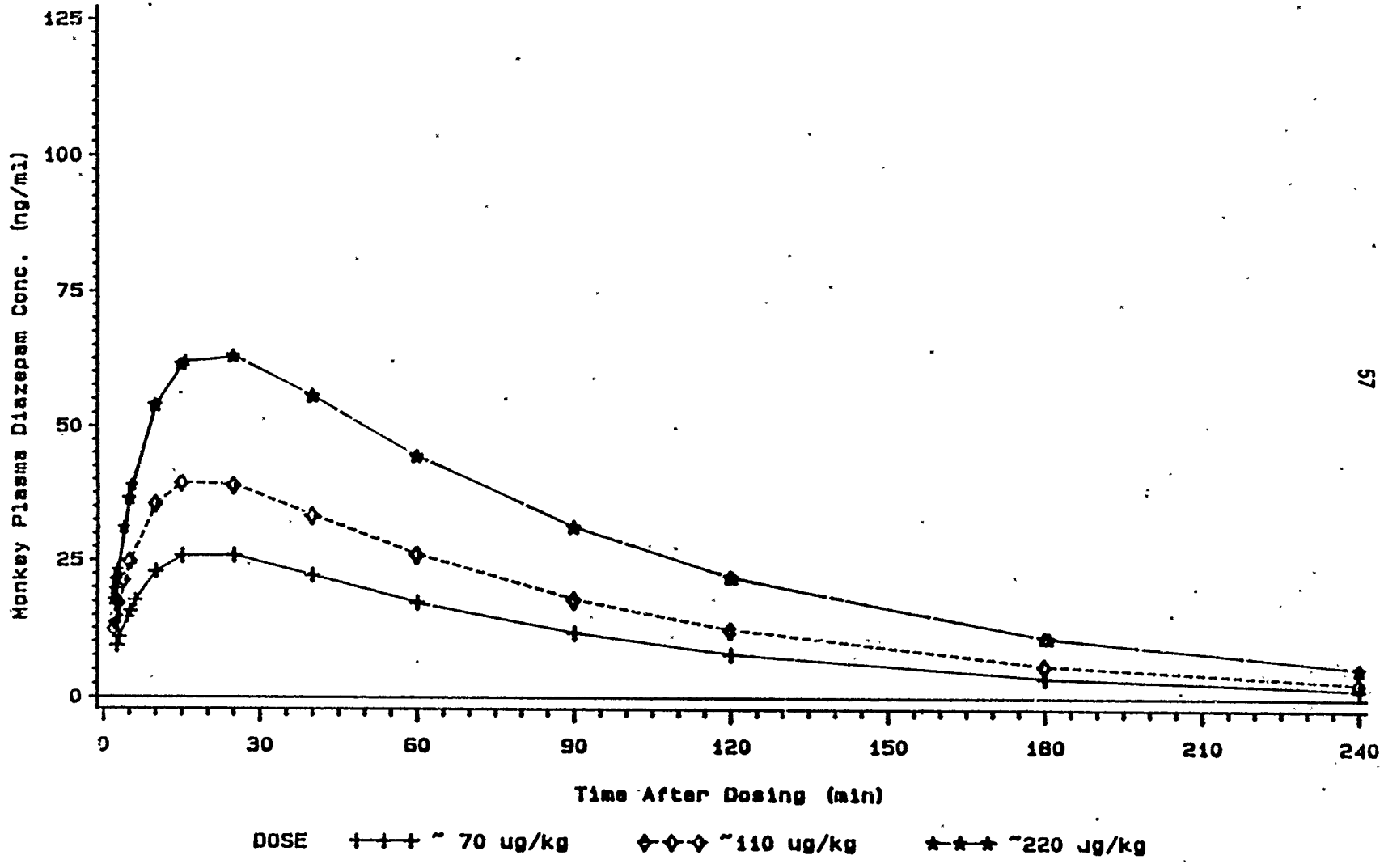


FIGURE 6. PHARMACOKINETICS OF DIAZEPAM ADMINISTERED IN NINE RHESUS MONKEYS: MODEL-PREDICTED VALUES (_____) WITH LOWER AND UPPER 95 PERCENT CONFIDENCE LIMITS (– – –)
Dose = ~70 µg/kg

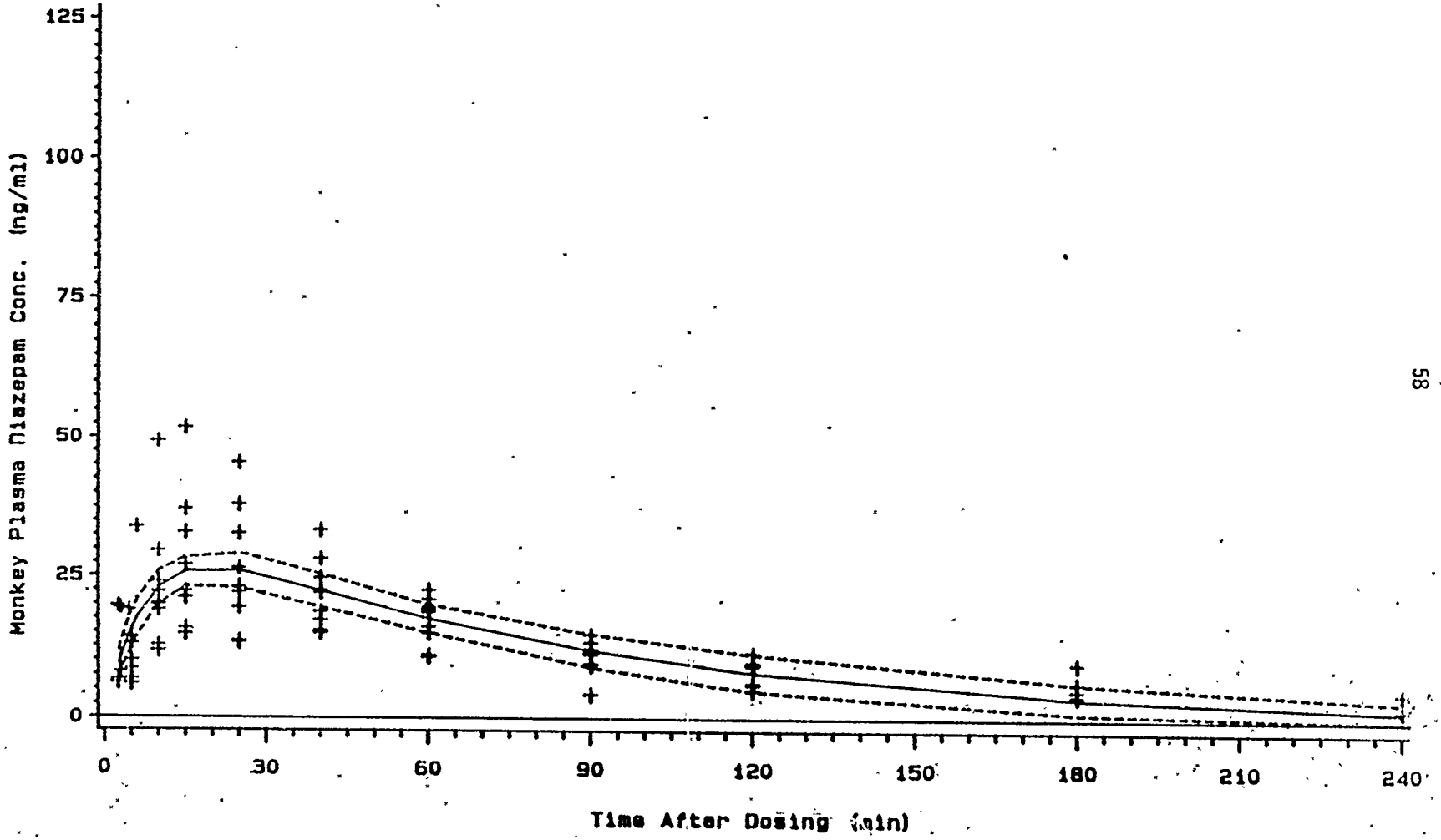


FIGURE 7. PHARMACOKINETICS OF DIAZEPAM ADMINISTERED IN NINE RHESUS MONKEYS: MODEL-PREDICTED VALUES (____) WITH LOWER AND UPPER 95 PERCENT CONFIDENCE LIMITS (- - -)
Dose= ~110 µg/kg

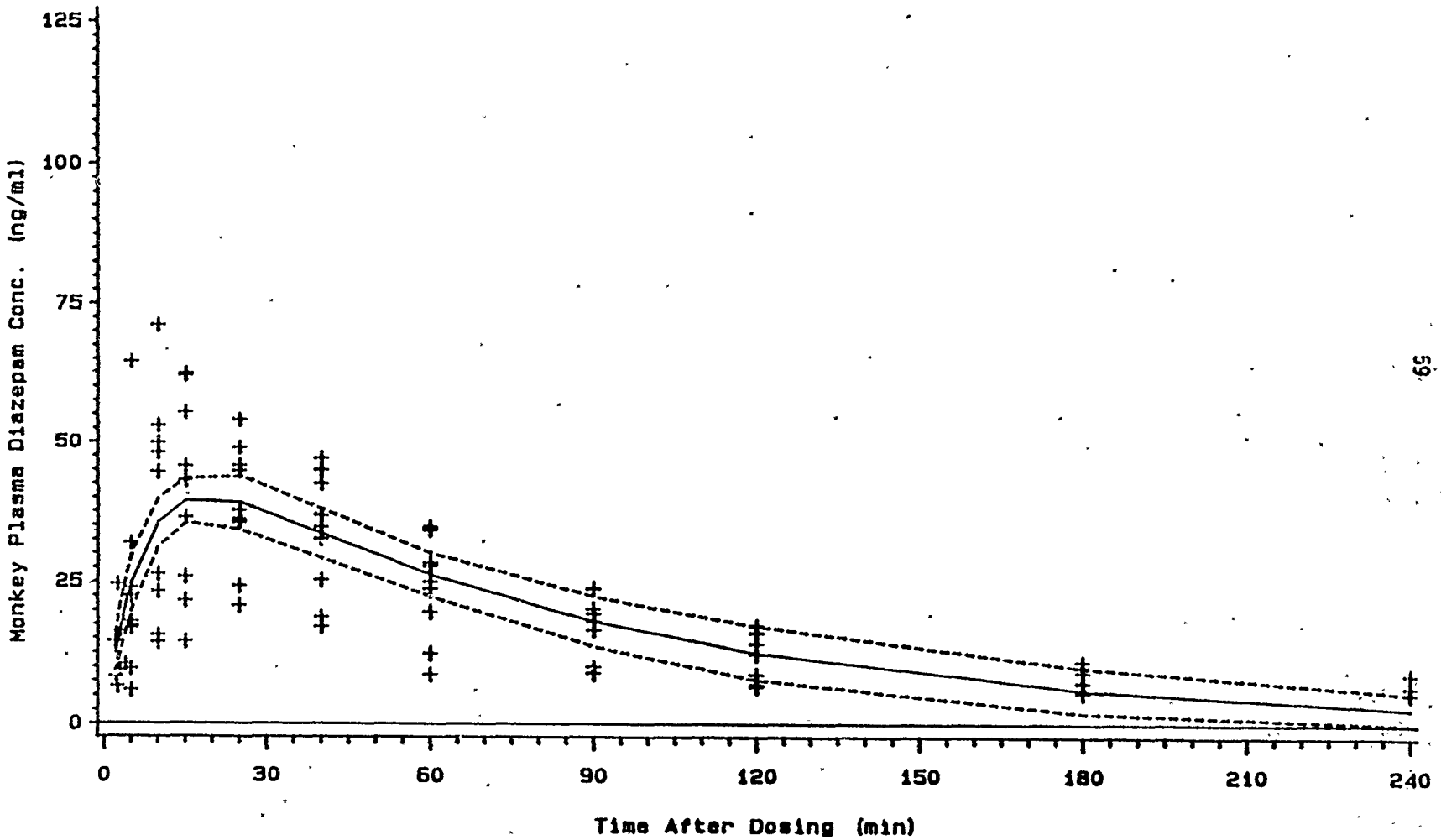


FIGURE 8. PHARMACOKINETICS OF DIAZEPAM ADMINISTERED IN NINE RHESUS MONKEYS: MODEL-PREDICTED VALUES (____) WITH LOWER AND UPPER 96 PERCENT CONFIDENCE LIMITS (---)
Dose = ~220 µg/kg

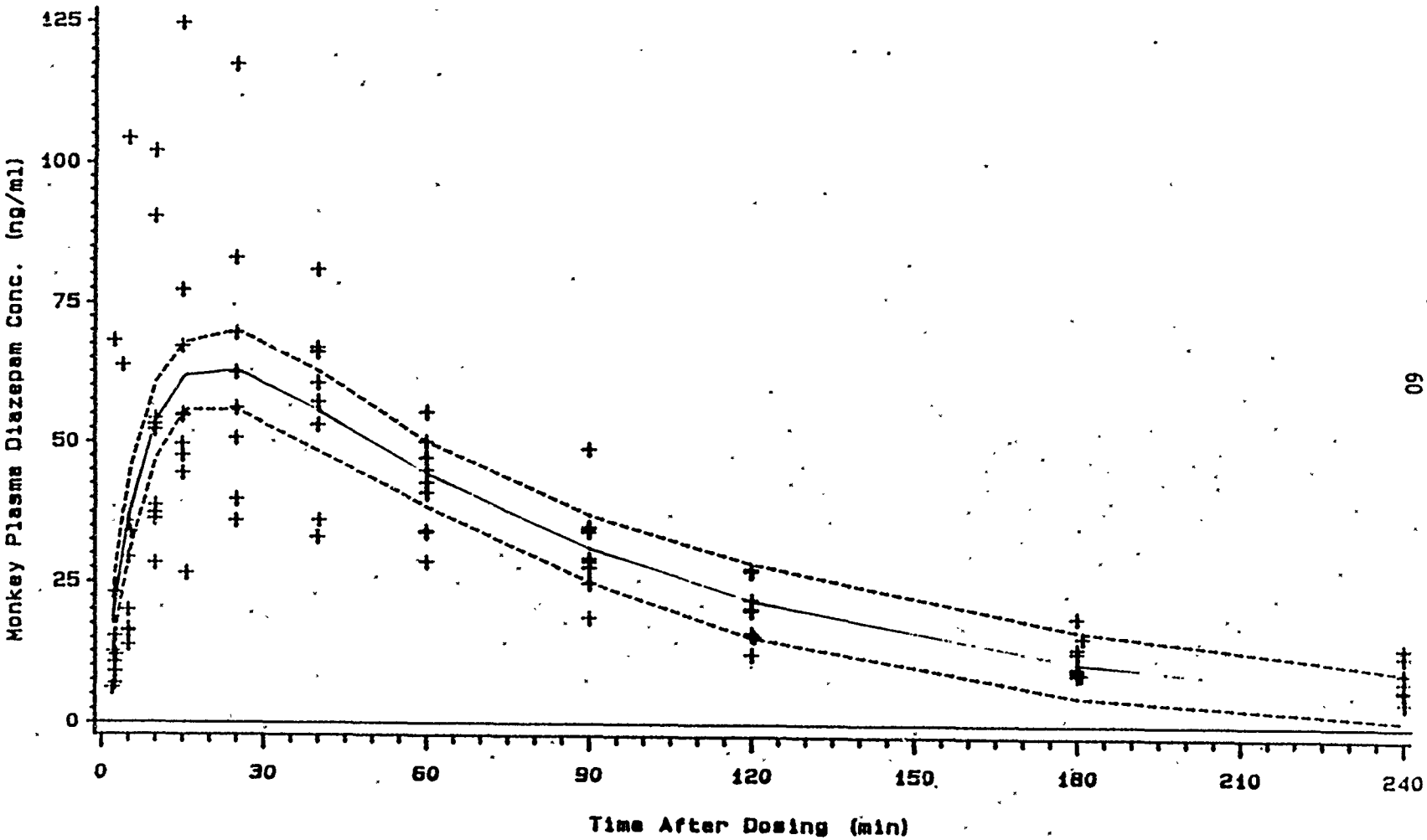


TABLE 23. EXPERIMENTAL DESIGN FOR PHARMACOKINETICS

Animal ID	Dose Level of Diazepam		
	Day 1 of Dosing	Day 2 of Dosing	Day 3 of Dosing
6AS	Low	Medium	High
6RI	Low	High[a]	Medium
5V7	Low	Medium	High
619	High	Low	Medium
68W	Medium	Low[a]	High
5WF	High	Low	Medium
6AR	Medium	High	Low
71M	High	Medium[a]	Low
6BJ	Medium	High	Low

[a]Difficulty in chemical analyses resulted in these animals being dosed again on a fourth day.

An analysis of variance appropriate for cross-over designs was carried out for each pharmacokinetic parameter to assess statistical significance of any residual effects. The effects included in the analysis of variance are given in the following equation for a generic pharmacokinetic parameter Y:

$$Y = \mu + \text{square} + \beta(\text{square}) + \gamma(\text{square}) + \tau + \rho + \epsilon$$

where

μ = average value of the pharmacokinetic parameter,

square = effect of block subset within the Latin square,

β = effect of animal within a square,

γ = effect of day of testing within a square,

τ = direct effect of the dose level applied that day,

ρ = residual effect of the dose level applied in the preceding day of testing, and

ϵ = uncontrolled variation within an animal.

A statistical hypothesis test was performed for each pharmacokinetic parameter to determine if residual effects were statistically significant. Residual effects were determined to be statistically insignificant for all of the pharmacokinetic parameters.

A second analysis of variance was carried out to assess the effects of the dose level of diazepam, day of dosing, and animal to animal variability. Table 24 summarizes these results. The model-predicted average values of the pharmacokinetic parameters calculated from the analysis of variance model are shown in the second, third, and fourth columns of the table for the 70, 110, and 220 μg/kg doses, respectively. The average values of the pharmacokinetic parameters predicted at each dose level from the analysis of variance model are similar but not identical to the observed averages presented in Table 22. The difference between the two sets of values is a result of the analysis of variance model adjusting the predicted averages for the effects of day, animal, and Latin square. Because equal numbers of animals were tested at each dose level of diazepam, the standard errors of the averages are identical for each of the doses. The standard error of the average pharmacokinetic parameter for each dose group is displayed in the fifth column of the table. For each pharmacokinetic parameter, a statistical hypothesis test was performed to determine if the effect of dose level of diazepam was statistically significant. The value of the F tests and their observed significance levels are given in the next two columns of the table.

The component of variation due to the effects of the different animals was estimated for each pharmacokinetic parameter. The estimates of the between animal variance components (σ_A^2) are displayed in column eight of Table 24. To assess the magnitude of the animal to animal variability, the between animal variance components were statistically compared to the variance component estimated for the variability within animals (σ_e^2). Ratios of the two variance components, and statistical significance levels for the between animal variance component are contained in the ninth and tenth columns of the table. For each pharmacokinetic parameter, a statistical hypothesis test was performed to determine if the effect of day of testing was statistically significant. The value of the F tests and their observed significance levels are displayed in the last two columns of the table.

The effects of diazepam dose were determined to be statistically significant (at the 5 percent significance level) for V_d, $AUC_{0-\infty}$, and C_{pmax}. Both the AUC and C_{pmax} were increased with higher doses of diazepam. The V_d

TABLE 24. SUMMARY OF STATISTICAL ANALYSIS OF DIAZEPAM DOSE, ANIMAL TO ANIMAL VARIABILITY, AND DAY OF TESTING FOR EACH PHARMACOKINETIC PARAMETER

Pharmacokinetic Parameter (units)	Effects of Diazepam Dose: Model Predicted Average per Dose			SE[a] of Avg.	F-Value	P-Value[b]	Animal Variability			Day of Testing	
	Low	Medium	High				σ_A^{2}[c]	σ_A^2/σ_e^{2}[d]	P-Value[e]	F-Value	P-Value
V_d (L/kg)	2.35	2.13	2.98	(0.22)	4.35	0.038	0.173	0.428	0.181	4.18	0.025
k_a (min-1)	0.18	0.18	0.14	(0.03)	0.68	0.019	0.002	0.383	0.155	1.74	0.208
k_{el} (min-1)	0.014	0.020	0.012	(0.004)	1.34	0.290	0.000	0.036	0.417	1.00	0.448
$AUC_{0-\infty}$ (ng • min/mL)	2581	3782	6016	(293)	58.32	<.001	71500	0.097	0.333	5.78	0.008
$AUC_{0-\infty}/D$ (kg • min/L)	35.57	34.03	30.37	(2.01)	1.09	0.360	1.30	0.022	0.439	3.99	0.020
t_{ka} (min)	7.30	8.75	7.02	(1.05)	0.52	0.606	0.507	0.053	0.391	3.22	0.052
t_{kel} (min)	54.90	40.70	60.50	(6.05)	0.77	0.403	120	0.317	0.140	0.31	0.004
t_{max} (min)	23.70	25.40	24.75	(2.33)	0.15	0.001	15.5	0.331	0.140	3.92	0.029
C_{pmax} (ng/mL)	24.25	41.15	53.40	(3.74)	20.70	<.001	07.1	0.550	0.005	4.90	0.014
C_{pmax}/D (kg/L)	0.35	0.37	0.27	(0.03)	3.01	0.007	0.002	0.250	0.103	4.10	0.024
Cl (mL/min/kg)	30.43	33.10	34.53	(2.43)	0.77	0.405	12.0	0.250	0.100	0.53	0.005

[a] Standard error of the estimated average value of the pharmacokinetic parameter for each dose grouping. Because equal numbers of animals were dosed at each level, the standard errors are the same for each dose grouping.
[b] Observed significance level. The effects of diazepam dose were determined to be statistically significant (at the 0.05 significance level) for those pharmacokinetic parameters with p-values less than 0.05.
[c] Estimate of the animal to animal variance component.
[d] Ratio of the variance components estimated for animals to the variance component estimated for uncontrolled error.
[e] Observed significance level for the animal to animal variance component.

was higher at the high dose of diazepam than at the lower and medium doses. The between animal variance component was not determined to be statistically significant (at the 5 percent level) for any of the pharmacokinetic parameters. The variation in the pharmacokinetic parameters over the three days of testing, was determined to be statistically significant for V_d, AUC_{∞}, AUC_{∞}/D, t_{ka}, t_{max}, C_{peak}, C_{peak}/D and Cl.

4.0 CONCLUSIONS

The incidence of convulsions in monkeys pretreated with four doses of 1.2 mg/kg pyridostigmine bromide intragastrically q8h, injected IM with 5 X 48-hr GD LD_{50} of untreated animals at four hr after the last pyridostigmine dose, and treated IM with 0.4 mg/kg atropine free base and 25.71 mg/kg 2-PAM at one minute following the GD injection was too low to determine the dose of diazepam needed to reduce the incidence of convulsions to 20 percent of the animals so treated. To determine an ED_{80} of diazepam, the GD dose was increased to 10 X the 48-hr LD_{50} of untreated animals and the dose of atropine was reduced to 0.2 mg/kg. With this treatment regimen, one monkey given 2 μg/kg diazepam and four given none convulsed within 90 min after challenge with GD. None of seven monkeys dosed with more than 180 μg/kg diazepam convulsed within 4 hr. The occurrence of convulsions in animals given diazepam doses between these levels was unpredictable, and the slope of the diazepam dose-absence of convulsions curve was very shallow, approximately 1.34. The 90 min diazepam ED_{80} was estimated to be 112 μg/kg with 95 percent confidence limits of 47 to 588 μg/kg. The estimated 4 hr diazepam ED_{80} is 230 μg/kg with 95 percent confidence limits of 94 to 3,160 μg/kg.

Clinical observations were made on each animal treated. For each animal, time to onset and duration of tremors and/or convulsions, and time to onset and duration of prostration as a function of diazepam dose were statistically analyzed. Only onset and duration of convulsions were determined to be statistically significantly associated with diazepam dose. As the diazepam dose was increased, the time to onset of convulsions increased and the duration of convulsions decreased. Diazepam does appear to be effective in reducing or preventing convulsions in animals pretreated with

pyridostigmine bromide, challenged with GD, and treated with atropine and 2-PAM in conjunction with the diazepam. This is in agreement with other research.[2,9,12]

Findings of gross necropsies on animals dying following GD exposure were consistent with death being due to GD intoxication. Significant microscopic neuropathology was not found in the central nervous systems of two monkeys given diazepam, but neuronal necrosis was seen in the brain of one monkey which did not receive diazepam. This is consistent with findings of other investigators.[1,2]

Pharmacokinetic parameters of diazepam injected IM in nine monkeys at levels of 70, 110, and 220 μg/kg in a cross-over study were estimated. Statistical analyses of these parameters demonstrated a significant difference (at the 5 percent level) in apparent volume of distribution, area under the plasma concentration versus time curve, and maximum plasma concentration due to dose of diazepam injected. Day of injection was responsible for differences in apparent volume of distribution, area under the plasma concentration versus time curve, area under the plasma concentration versus time curve divided by dose, absorption phase half life, time at which maximum plasma diazepam concentration occurs, maximum plasma diazepam concentration, maximum plasma diazepam concentration divided by dose, and clearance rate of diazepam from the plasma. These variations in pharmacokinetic parameters may explain why the diazepam dose versus absence of convulsions response curve slope was so shallow in the efficacy phase of the task.

5.0 RECORD ARCHIVES

Forty monkeys arrived at the MREF on June 2, 1989 and 30 more arrived on July 7, 1989. LD_{50} studies were run from October 23 to November 4, 1989. Efficacy study dosing occurred from November 6, 1989 to February 20, 1990. Pharmacokinetic studies were accomplished on March 15, April 18, May 17, and June 21, 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 dosing solutions are unstable under prolonged storage and have been destroyed. Plasma samples have been used. Samples of diazepam, pyridostigmine bromide, atropine, and 2-PAM dosing solutions will be maintained at the MREF. 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:

Name	Title	Degree
Dr. Garrett S. Dill	Principal Investigator	D.V.M.
Dr. Carl T. Olson	Study Director	D.V.M., Ph.D.
Dr. Ronald G. Menton	Study Statistician	Ph.D.
Ms. Robyn C. Kiser	Study Supervisor	B.S.
Mr. Timothy L. Hayes	Study Chemist	B.A.
Mr. Thomas H. Snider	Pharmacokinetics Modeler	B.S.
Dr. Allen W. Singer	Study Pathologist	D.V.M.
Dr. Peter L. Jepsen	Study Veterinarian	D.V.M.

There are a number of people who made the performance of this task possible. Their invaluable assistance is gratefully acknowledged by the authors. Among the many are: Claire Matthews for statistical analyses; James Arp, Malcolm Reeves, and Sheri Moore for chemical analyses; Michael Hingson for supervision, and preparation for, coordination and performance of numerous tasks; Kandy Audet, Linda Adams, Karen Brown, Pamela Cooley, William Hart, Pamela Kinney, Jonathon Kohne, Harold Nitz, Jean Ostovich, Cynthia Pelley, and Jack Waugh for outstanding performance of technical tasks; Rebekah Starner and Mary Lou Briner for AChE activity determinations; Anthony Stuart for pathology support; and Tami Kay and Pamela Wimer for secretarial tasks and report preparation.

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APPENDIX A

Efficacy of Diazepam in Reducing the Incidence of Convulsions in Indian Rhesus Monkeys Pretreated with Pyridostigmine Bromide, Challenged with Soman, and Treated with Atropine and Pralidoxime Chloride in Conjunction with the Diazepam

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

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5. Study Veterinarian: Peter L. Jepsen, D.V.M.
6. Sponsor: United States Army Medical Research and Development Command (USAMRDC)
7. Sponsor Monitor: LTC J. Bruce Johnson, D.V.M., United States Army Medical Research Institute of Chemical Defense (USAMRICD)
8. Introduction: Current standard therapy in research involving non-human primates exposed to Soman (pinacolyl methylphosphono-fluoridate; GD) is pretreatment with pyridostigmine bromide and treatment with atropine and pralidoxime chloride (2-PAM). GD-induced convulsions often occur during these studies. Because GD-induced convulsions have been shown to increase the incidence of brain lesions in non-human primates,(1) it is likely that similar lesions could occur in man. It would be desirable to add an anticonvulsant to the treatment regimen for nerve agent poisoning to prevent convulsions and increase the chance of survival. Investigations with diazepam are currently being performed at USAMRICD and additional studies are needed at Battelle. This study will be conducted under the requirements of the U.S. Food and Drug Administration's (FDA) Good Laboratory Practices (GLP) regulations.
9. Objective: The objective of this study is to determine the smallest dose of diazepam which results in no more than a 20 percent incidence of convulsions in monkeys pretreated with pyridostigmine bromide, exposed to a 5X LD_{50} dose of GD, and given a standard treatment regimen of atropine and 2-PAM in conjunction with diazepam.

The study is conducted in two phases:

Phase I - Determine the approximate 24 hr LD_{50} for GD in monkeys given no therapy. This is done in an up-down manner, using as few animals as possible, but no more than ten.

Phase II - Assuming that at 5X LD_{50} of GD a diazepam dose-convulsion incidence response exists, a stagewise design experiment, using different doses of diazepam, is used to determine the minimum dose of diazepam that results in no more than a 20 percent incidence of convulsions in monkeys given a 5X LD_{50} dose of GD. Monkeys are given pyridostigmine prior to challenge and atropine/2-PAM in conjunction with the diazepam after challenge with GD. The number of monkeys required is dependent upon the slope of the diazepam dose-convulsion response curve and the degree of accuracy required in the estimate. The study will cease when a 10 percent or less standard error in the estimate of the required diazepam dose is reached or when a maximum of 50 monkeys has been tested.

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. Male rhesus monkeys exhibit pyridostigmine, atropine, and 2-PAM pharmacokinetics similar to that of humans.(2) 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.(3) 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 48 hr 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 a sodium pentobarbital overdose. Anesthetics, analgesics, or tranquilizers, other than the diazepam test solution, 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 2.0 and 4.0 kg.

(3) Quarantine - All primates received at Battelle will undergo at least a 1 month quarantine period at the Medical Research and Evaluation Facility (MREF). All animals are examined by the Study Veterinarian within 1 week of arrival at Battelle. Blood samples are taken for hematology and serum chemistries and erythrocyte (RBC) acetylcholinesterase (AChE) values. 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 approximately 2 week 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, as possible, across phases and stages of the experiment.

(5) Animal Identification - Animals are received with tattoos either on their chest or inner thigh. 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 the study, 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 for obtaining body weights and blood samples (femoral venipuncture) and for restraint when pretreating with pyridostigmine (nasogastric tube) or challenging with GD.

(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. Chemical analyses of certified feeds are available from Purina. No contaminants that would interfere with the results of the study are known to be present in the feed.

(12) Water - Water is supplied from the Battelle water system and given ad libitum 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 water.

(13) Battelle's Animal Resources Facilities have been registered with the U.S. Department of Agriculture (USDA) as a Research Facility (Number 31-21) 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 (NIH) 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 (P.L. 89-544 and P.L. 91-579).

(14) On January 31, 1978, Battelle Columbus Division 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 - The treatment compounds, atropine and 2-PAM, are provided by USAMRICD. Pyridostigmine bromide in a syrup base (Mestinon®) is purchased locally. Diazepam is purchased from Roche Laboratories. Drug identities and concentrations are confirmed by Battelle using chemical analytical techniques.

(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 materiel (CSM), and in Standard Operating Procedures (SOPs) for storage and use of CSM.

C. Test Groups

(1) Initial Tests to Establish an LD_{50} - No more than ten monkeys are used to approximate the 24 hr GD LD_{50} in animals given no pretreatment or therapy. This is accomplished in a modified up-down type experiment, challenging one or two monkeys per day. If after three or more monkeys have been challenged, the estimated LD_{50} of this study falls within the 95 percent confidence limits of the historic GD LD_{50} in Indian rhesus monkeys, the historic LD_{50} will be accepted as the LD_{50} in this group of animals.

(2) Establishing a Minimum Diazepam Dose That Will Result in No More Than a 20 Percent Incidence of Convulsions in Monkeys Given 5X GD LD_{50} and Standard Therapy - Groups of monkeys will be given doses of 1.2-mg pyridostigmine bromide/kg body weight by gastric intubation every 8 hr a total of four times. Blood samples will be taken immediately prior to the first dose and again just prior to challenge with GD at 4 hr following the fourth and final dose of pyridostigmine to determine the RBC AChE inhibition level. Monkeys are fasted for 12 hr prior to the start of pyridostigmine dosing and fed 3 to 4 hr after dosing with pyridostigmine to minimize differences in the gastrointestinal absorption of the compound. Monkeys are fasted for the 4 hr between the last pyridostigmine dose and challenge with GD. Monkeys will be challenged with 5X GD LD_{50} using exempt concentrations of GD

given intramuscularly (IM) in the calf of the right leg in the region of the gastrocnemius muscle. GD dosing will be accomplished with monkeys restrained on a platform and within a hood approved for the use of highly hazardous material.

Syringes used for dosing pyridostigmine, atropine, 2-PAM, and GD 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 daily 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, 2-PAM, and diazepam. 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.

After decontamination of the skin surrounding the IM GD dosing site, using a hypochlorite solution and then water, monkeys are removed from the hood, and, at 1 min following agent challenge, are given 0.4-mg atropine per kg body weight IM in the anterior lateral area of the left thigh in the region of the Vastus lateralis head of the Quadriceps femoris muscle. Immediately after atropine injection, 2-PAM, at a dose of 25.71 mg/kg body weight, will be given IM in the same area of the thigh, but separated by at least 1 inch from the atropine injection site. This will be immediately followed by a dose of diazepam given in the same area, but separated from the two other injection sites. If the dose of diazepam becomes too large to be injected in one site, consultation with USAMRICD point of contact will be required.

Monkeys are returned to their individual cages after treatment and are closely and continuously observed for the first 2 hr following dosing and at intervals thereafter with observations annotated at 4, 6, 8, 12, 24, 36, and 48 hr. Signs specifically monitored include muscle fasciculations, tremors, convulsions, prostration, salivation/bronchial discharge, miosis/mydriasis, and death. Video recordings of monkeys during the post-agent challenge period when convulsions most often occur will be made, in order to assist in the classification of any convulsions. Such recordings are stored under tight security.

The number of monkeys required to determine the minimum dose of diazepam necessary to result in no more than a 20 percent incidence of convulsions in monkeys pretreated with pyridostigmine bromide, challenged with 5X GD LD_{50}, and treated with atropine/2-PAM in conjunction with the diazepam is dependent upon the slope of the diazepam dose-convulsion response curve and the required degree of accuracy of the estimate. The study will cease when a 10 percent or less standard error in the estimate of the necessary diazepam dose is obtained or when a maximum of 50 monkeys has been challenged. If it does not appear that the diazepam dose can be estimated with a 10 percent standard error, USAMRICD personnel will be notified as soon as possible after the beginning of the experiment.

D. Study Preparations - Prior to challenge and IM dosing, hair over the anterior lateral aspect of the left thigh and over the posterior calf of the right leg are clipped using an Oster Model A-2 animal clipper, or equivalent, and a No. 40 blade. Monkeys are weighed prior to the start of pyridostigmine administration and doses of this compound administered on a body weight basis. Each monkey is again weighed within 24 hr of challenge and this weight is used to calculate the volumes of agent and treatment compounds. Anesthesia is not used since it would affect the occurrence of clinical signs of organophosphate intoxication.

E. Disposition of Experimental Animals - Monkeys on study are euthanatized with an overdose of pentobarbital sodium if, in the opinion of the Study Veterinarian or Study Director, conditions exist such that continuation on study would be inhumane. All animals that die on study or are sacrificed in moribund condition receive a necropsy and gross tissue examination by a qualified veterinary pathologist. The examining pathologist determines whether the necropsy findings are consistent with those seen in other animals exposed to organophosphates. At the discretion of the pathologist, representative tissues, especially central nervous system tissues, may be harvested, preserved in neutral buffered 10 percent formalin, processed at Battelle, and evaluated microscopically by a qualified veterinary pathologist. After necropsy, animal remains are incinerated. Surviving animals, which should be the large majority, are returned to USAMRICD.

11. Statistical Approach: A modified up-down approach is used to estimate the untreated 24 hr GD LD_{50} in monkeys of Indian origin. Monkeys will be dosed with GD one or two at a time, starting at a dose approximating the historic LD_{50}. 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 statisticians in order to most efficiently estimate the 24 hr GD LD_{50} in the present population of monkeys. If, after a minimum of three monkeys have been challenged, the estimated LD_{50} falls within the 95 percent confidence limits of the historic Indian rhesus monkey 24 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 further experimentation for determination of the LD_{50} will not be accomplished.

Assuming that at 5X LD_{50} of GD a diazepam dose-convulsion incidence response exists, a stagewise design experiment, using different doses of diazepam, is used to determine the minimum dose of diazepam that results in no more than a 20 percent incidence of convulsions in monkeys given a 5X LD_{50} dose of GD. Monkeys are given pyridostigmine prior to challenge and atropine/2-PAM in conjunction with the diazepam after challenge with GD. Initial doses of diazepam to be tested are in the region of 0.100 mg/kg, but multiple doses, selected by statisticians based upon predictions of dose-response curve slope and estimated percentiles of response based on probit analysis, are used. As data are obtained, all available information is used to select new doses to be tested. The number of monkeys required is dependent upon the slope of the diazepam dose-convulsion response curve and the degree of accuracy required in the estimate. The study will cease when a 10 percent or less standard error in the estimate of the required diazepam dose is reached or when a maximum of 50 monkeys has been tested. If it becomes evident during the performance of this study that an estimate of the minimum effective dose with a 10 percent or less standard error cannot be obtained, USAMRICD personnel will be notified.

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 evaluations

F. Necropsy and histopathology records

G. Decontamination, monitoring, and disposal records

13. Reports:

A. A letter report stating the smallest dose of diazepam which results in no more than a 20 percent incidence of convulsions will be sent as soon as the dose is known.

B. A Draft Final Report is prepared within 90 days after completion of the study. The Draft Final report includes:

(1) List 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:

1. Wall H. G., Jaax, N. K., and Hayward, I. J. Brain Lesions in Rhesus Monkeys After Acute Soman Intoxication, Proceedings of the Sixth Medical Chemical Defense Biosciences Review, Commander, USAMRICD, Aberdeen Proving Ground, MD, 1987.

2. Joiner, R. L. and Kluwe, W. M. Task 85-18: Conduct of Pralidoxime Chloride, Atropine in Citrate Buffer and Pyridostigmine Bromide Pharmacokinetics Studies, and Comparative Evaluation of the Efficacy of Pyridostigmine Plus Atropine and Pralidoxime Versus Atropine and Pralidoxime Alone Against Acute Soman Poisoning in Male Rhesus Monkeys, Final Report to U.S. Army Medical Research and Development Command, Institute of Chemical Defense, August 1988.

3. Contract No. DAMD17-83-C-3129. Letter dated 25 July 1988, from Battelle to Commander, U.S. Army Medical Research Acquisition Activity, Ft. Detrick, MD, regarding results of Task 87-34: "The Effect of Treatment Regimens of Variable Concentrations of Atropine Sulfate in Combination with Pralidoxime Chloride on the Survival of Soman-Challenged Rhesus Monkeys Pretreated with Pyridostigmine Bromide".

15. Approval Signatures:

Carl T. Olson, D.V.M., Ph.D.
Study Director — Date: 5-11-89

Garrett S. Dill, D.V.M.
Principal Investigator — Date: 5/12/89

Paul I. Feder, Ph.D.
Statistician — Date: 5/15/89

Allen W. Singer, D.V.M.
Pathologist — Date: 5-16 89

Peter L. Jepsen, D.V.M.
Study Veterinarian — Date: 5-16-89

Ramona A. Mayer, Manager
Quality Assurance Unit — Date: 5/24/89

Anna D. Barker, Ph.D.
Group Vice President and
General Manager
Health and Environment

5/24/89
Date

LTC D. Bruce Johnson, D.V.M.
USAMRICD COR

9 June 89
Date

MAJ(P) Richard P. Solana
Chief, Pharmacology
Division, USAMRICD

5 Jun 89

MREF Protocol 52
Medical Research and
Evaluation Facility
November 3, 1989

Efficacy of Diazepam in Reducing the Incidence of Convulsions in Indian Rhesus Monkeys Pretreated with Pyridostigmine Bromide, Challenged with Soman, and Treated with Atropine and Pralidoxime Chloride in Conjunction with the Diazepam

Protocol Amendment No. 1

Change: Page 3, Section 9.
Page 5, Section 10.C.(1).
Page 7, Section 11.

"24 hr LD_{50}" is changed to read "48 hr LD_{50}".

Reason: Most data analyses in previous experiments with primates using Soman have been based on a 48 hr rather than a 24 hr LD_{50}. To retain consistency, USAMRICD personnel have requested that the 48 hr LD_{50} value be used.

Impact on Study: The 24 hr LD_{50} and 48 hr LD_{50} are expected to be quite similar and a challenge dose of 5X either LD_{50} should likewise be similar. This change should not impact the results of the study and should make comparisons with earlier studies more valid.

Carl T. Olson

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

3 Nov 89
Date

James R. Stewart

MAJ James R. Stewart
USAMRICD COR

3 Nov 89
Date

MREF Protocol 52
Medical Research and
Evaluation Facility
November 30, 1989

Efficacy of Diazepam in Reducing the Incidence of Convulsions in Indian Rhesus Monkeys Pretreated with Pyridostigmine Bromide, Challenged with Soman, and Treated with Atropine and Pralidoxime Chloride in Conjunction with the Diazepam

Protocol Amendment No. 2

Change: Page 6, Section 10.C.(2), 3rd paragraph.

The "0.4-mg" atropine per kg body weight should be changed to "0.2-mg".

Reason: Pyridostigmine-pretreated monkeys given 0.4 mg/kg atropine and 25.71 mg/kg 2-PAM 1 min after challenge with 5X LD_{50} GD do not demonstrate a high incidence of convulsions. This prevents the evaluation of diazepam as an anticonvulsant. Reducing the amount of atropine administered may increase the incidence of convulsions sufficiently to evaluate the use of diazepam as an anticonvulsant.

Impact on Study: Reducing the atropine dose may allow the evaluation of diazepam's ability to prevent GD-induced convulsions.

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

11-30-89
Date

MAJ James R. Stewart
USAMRICD COR

12-4-89
Date

MREF Protocol 52
Medical Research and
Evaluation Facility
December 11, 1989

Efficacy of Diazepam in Reducing the Incidence of Convulsions in Indian Rhesus Monkeys Pretreated with Pyridostigmine Bromide, Challenged with Soman, and Treated with Atropine and Pralidoxime Chloride in Conjunction with the Diazepam

Protocol Amendment No. 3

Change: Pages 1 and 2, Section 9.
Pages 5 and 7, Section 10.C.(2).
Page 8, Section 11.

The challenge dose of GD should be changed from "5X LD_{50}" to "10X LD_{50}".

Reason: Pyridostigmine-pretreated monkeys given 0.2 mg/kg atropine and 25.71 mg/kg 2-PAM 1 min after challenge with 5X LD_{50} GD do not demonstrate a high incidence of convulsions. This prevents the evaluation of diazepam as an anticonvulsant. Increasing the dose of GD should increase the incidence of convulsions and allow the evaluation of diazepam as an anticonvulsant for GD-induced seizures.

Impact on Study: As originally written, the protocol does not allow the evaluation of diazepam as an anticonvulsant for GD-induced convulsions. This amendment should allow that evaluation to be made.

Carl T. Olson

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

12-11-89
Date

James R. Stewart

FOR James R. Stewart
USAMRICD COR

12-11-89
Date

Efficacy of Diazepam in Reducing the Incidence of Convulsions in Indian Rhesus Monkeys Pretreated with Pyridostigmine Bromide, Challenged with Soman, and Treated with Atropine and Pralidoxime Chloride in Conjunction with the Diazepam

Deviation: This protocol specifies monkeys will be held in rooms with a temperature range of 72-82 F and a relative humidity of 40-60 percent. Conditions in animal rooms are recorded twice daily using a hand-held combination thermometer/hygrometer. The temperatures actually recorded in rooms in which monkeys were held between arrival and termination of the efficacy phase of the experiment ranged from 69 to 84 F and relative humidity ranged from 30 to 77 percent. Occurrence of excursions outside temperature or relative humidity ranges specified in the protocol were reported to a maintenance engineer and necessary adjustments made.

Impact on Study: Temperature and relative humidity ranges recommended for rhesus monkeys by the National Institutes of Health in their Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, Revised 1985) are 64.4-84.2 F and 30-70 percent. During the period when the LD_{50} determination and efficacy studies were actually conducted, temperature ranges were within 70 to 82 F and relative humidity 30 to 68 percent. The short-lived excursions outside temperature and relative humidity specifications stated in the protocol should have no impact on the validity of the study.

Carl T. Olson — 10-1-90

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

Date

Don W. Korte — 5OCT90

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

Date

Pharmacokinetic Evaluation of Diazepam in 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. Pharmacokineticists: Thomas H. Snider, B.S., Ronald G. Menton, Ph.D.

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

5. Sponsor: U.S. Army Medical Research and Development Command (USAMRDC)

6. Sponsor Monitor: MAJ James R. Stewart, D.V.M., U.S. Army Medical Research Institute of Chemical Defense (USAMRICD)

7. Introduction: Current standard therapy in research involving non-human primates exposed to pinacolyl methylphosphono-fluoridate (Soman; GD) is pretreatment with pyridostigmine bromide and treatment with atropine and pralidoxime chloride (2-PAM). GD-induced convulsions often occur during these studies. Because GD-induced convulsions have been shown to increase the incidence of brain lesions in non-human primates[1], it is likely that similar lesions could occur in man. It would be desirable to add an anticonvulsant to the treatment regimen for nerve agent poisoning to prevent convulsions and increase the chance of survival. Investigations with diazepam are currently being performed at USAMRICD and additional studies are needed at Battelle.

8. Objective:

The objective of this study is to determine the pharmacokinetics of the smallest dose of diazepam which results in no more than a 20 percent incidence of convulsions in monkeys pretreated with pyridostigmine bromide, exposed to a 5X LD_{50} dose of GD, and given a standard treatment regimen of atropine and 2-PAM in conjunction with diazepam. The pharmacokinetics of three doses of diazepam, including that dose described above, will be evaluated.

9. 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. Male rhesus monkeys exhibit pyridostigmine, atropine, and 2-PAM pharmacokinetics similar to that in humans.[2] 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.[3] Monkeys for use in this study are provided by USAMRICD.

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 3.0 and 5.0 kg.

(3) Quarantine - All primates received at Battelle will undergo at least a 1 month quarantine period at the Medical Research and Evaluation Facility (MREF). All animals are examined by the Study Veterinarian within one week of arrival at Battelle. Blood samples are taken for hematology and serum chemistries and erythrocyte (RBC) acetylcholinesterase (AChE) values. 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.

(4) Animal Selection - Based on physical examinations and clinical laboratory findings, acceptable animals are identified by the Study Director and Study Veterinarian.

(5) Animal Identification - Animals are received with tattoos either on their chest or inner thigh. 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 inch wide, 34 inch high, and 26 inch deep, with automatic watering systems.

(7) Acclimation - Prior to the start of the study, monkeys are acclimated to placement in a restraint chair.

(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 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 are available from Purina.

(12) Water - Water is supplied from the Battelle West Jefferson 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 for potability on a quarterly basis and for contaminants on an annual basis.

(13) Battelle's Animal Resources Facilities have been registered with the U.S. Department of Agriculture (USDA) as a Research Facility (Number 31-21) 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 (P.L. 89-544 and P.L. 91-579).

(14) On January 31, 1978, Battelle Memorial Institute 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

The diazepam used in this pharmacokinetic study is obtained from Roche Laboratories. Drug identity and concentrations are confirmed by Battelle using chemical analytical techniques.

C. Test Groups

A total of nine monkeys will be given each dose of diazepam with a minimum 3 week washout and recovery period between dosing. On each day of experimentation, an equal number of monkeys will be given each dose of diazepam. The three doses to be tested will be specified, based on results of efficacy studies performed in accordance with MREF Protocol 52, by USAMRICD personnel. Blood samples are obtained prior to dosing and at 2.5, 5, 10, 15, 25, 40, 60, 90, 120, 180, 240, 480, and 1,440 min after dosing. Blood is collected in heparinized containers, centrifuged, and the plasma frozen at approximately -70 C until analyzed for diazepam. Both free and total diazepam plasma levels are estimated using chemical analytical techniques at the MREF. Extrapolation of free diazepam levels is based on in vitro analyses of percent bound versus total diazepam levels in monkey blood spiked with radioactive-labeled diazepam.

D. Study Preparations

Prior to challenge and intramuscular (IM) dosing, hair over the anterior lateral aspect of the thighs and over the calves is clipped using an Oster Model A-2 animal clipper, or equivalent, and a No. 40 blade. Monkeys are weighed prior to the start of dosing and diazepam doses administered on a body weight basis intramuscularly in the area of the Vastus lateralis. Animals are restrained in primate chairs and catheters placed in saphenous veins. Blood samples for diazepam analyses are drawn from the leg opposite to the one injected with diazepam. After the 240 min blood draw, catheters will be removed and animals may be removed from restraint chairs and placed in individual cages. Subsequent blood samples are obtained, using blood collection needles and heparinized containers, from the femoral vein.

E. Disposition of Experimental Animals

When pharmacokinetic studies are completed, monkeys will be returned to USAMRICD.

10. Pharmacokinetic Approach:

When diazepam analyses are completed, plasma concentrations as a function of time, maximum concentrations, time to maximum concentrations, area under the plasma concentration-time curves, absorption and elimination rate constants, and apparent volumes of distribution are estimated.

11. Records to be Maintained:

A. Diazepam dosing solution analyses

B. Animal data

C. Diazepam plasma concentration data

D. Pharmacokinetic analysis data

12. Reports:

A. A draft final report is prepared within 60 days after completion of the study. 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 and analyses

(5) Pharmacokinetic analyses of data

(6) Discussions and conclusions.

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

13. References:

(1) Wall H. G., Jaax, N. K., and Hayward, I. J. Brain Lesions in Rhesus Monkeys After Acute Soman Intoxication, Proceedings of the Sixth Medical Chemical Defense Biosciences Review, Commander, USAMRICD, Aberdeen Proving Ground, MD, 1987.

(2) Joiner, R. L. and Kluwe, W. M. Task 85-18: Conduct of Pralidoxime Chloride, Atropine in Citrate Buffer and Pyridostigmine Bromide Pharmacokinetics Studies, and Comparative Evaluation of the Efficacy of Pyridostigmine Plus Atropine and Pralidoxime Versus Atropine and Pralidoxime Alone Against Acute Soman Poisoning in Male Rhesus Monkeys, Final Report to U.S. Army Medical Research and Development Command, Institute of Chemical Defense, August 1988.

(3) Contract No. DAMD17-83-C-3129. Letter dated 25 July 1988, from Battelle to Commander, U.S. Army Medical Research Acquisition Activity, Ft. Detrick, MD, regarding results of Task 87-34: "The Effect of Treatment Regimens of Variable Concentrations of Atropine Sulfate in Combination with Pralidoxime Chloride on the Survival of Soman-Challenged Rhesus Monkeys Pretreated with Pyridostigmine Bromide".

14. Approval Signatures:

Carl T. Olson, D.V.M., Ph.D.
Study Director — Date: 10-25-89

Garrett S. Dill, D.V.M.
Principal Investigator — Date: 10/25/89

Thomas H. Snider, B.S.
Pharmacokineticist — Date: 10/25/89

Timothy Hayes, B.A.
Chemist — Date: 10/27/89

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

10/27/89
Date

Ramona A. Mayer, Manager
Quality Assurance

10/30/89
Date

Anna D. Barker, Ph.D.
Vice President
Health and Environment Group

10/30/89
Date

MAJ James R. Stewart, D.V.M.
USAMRICD COR

26 Oct 89
Date

APPENDIX B

STANDARD OPERATING PROCEDURE
MREF SOP-88-31

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

LABORATORY: MREF SOP APPROVAL DATE: February 28, 1990

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 2/19/90
Signature/Date

Timothy L. Hayes, Principal Research Scientist
Printed Name/Title

Approved By: [signature] 2/22/90
Signature/Date

Garrett S. Dill, D.V.M., Manager
Printed Name/Title

Approved By: David L Stitcher 2/22/90
Signature/Date

David L. Stitcher, CIH, Safety/Surety Officer
Printed Name/Title

Revised February 19, 1990

Approved By:

Sandra M. Anderson 03/07/90
Signature/Date

Quality Assurance Unit
Health and Environment Group
Printed Name/Title

Chas K. Burdick
Signature/Date

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

SIGNATURES

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

Signature	Date	Signature	Date
Sheri D. Moore	3/30/90		
[illegible]	5/11/90		
[illegible]	5/14/90		
[illegible]	5/23/90		
[illegible]	6-8-90		

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 materiel (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 materiel (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 CSM/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 deluge 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 insure that the following are observed:

   a. Only authorized personnel meeting requirements set forth in Section B.1 are allowed in the room during XCSM operations.

   b. XCSM control and accountability are maintained.

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 (RDTE) dilute solutions of CSM. The XCSM solutions that can be used following this SOP are those prepared from the following CSM.

a. Tabun (CAS 77-81-6): GA, ethyl N,N-dimethylphosphoramidocyanidate.

b. Sarin (CAS 107-44-8 or 50642-23-4): GB, isopropyl methylphosphonofluoridate.

c. Soman (CAS 96-64-0 or 50642-24-5): 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 XHD, XL, XHDL, 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 on

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 Routes of Entry: Inadvertent skin contact with these XCSM is the most common cause of laboratory accidents/incidents. The XCSM absorption rate will likely be accelerated through unprotected cuts and abrasions.

Signs and Symptoms: The first indication of exposure of anticholinesterase XCSM to the skin is likely to be a reaction at the point of exposure, i.e., localized sweating and/or twitching. If exposed to vapor from some type of vapor generating system, pinpointed pupils (miosis), muscular 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.

(4) Muscle twitching.

(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

Sheet (MSDS) 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 60 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 hypocholride 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 lfpm. 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.

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 eye-wash 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 magnehelic 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 MREF SOP-83-5. The procedures used within this SOP are described in MREF 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 CSM: All XCSM samples generated must be clearly identified with the following information being regarded as the minimum requirements.

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 Samp. A1).

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 perfcrmed 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 permissable 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:

[(10.0-µL neat VX) (1.0083 mg/µL density of VX)] = 10.083-mg VX
[(10.083-mg VX)(0.95)] = 9.58-mg VX
[9.58-mg VX/10 mL] = .958 mg/mL dilute concentration

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

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-mm I.D. SE-54 with .3-μm film thickness
Carrier Gas:	Helium
Velocity :	30 L 5 cm/sec for Helium
Make-up Gas:	30 L 5 mL/min
Detector :	Flame Ionization Detector (FID)
Detector Gases:	H_2 = 400 mL/min L 10 mL/min Air = 40 mL/min L 5 mL/min
Injector Temperature:	275 L 10 C

Detector Temperature:	250 L 10 C
Oven Program:	Initial temperature = 60 C Initial time = 1.0 min Level 1 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 GD 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 :	FPD with 525-nm phosphorous selective filter
Detector Gases:	H_2 = 135 mL/min L 10 mL/min Air = 120 mL/min L 5 mL/min O_2 = 15 mL/min L 2 mL/min
Injector Temperature:	140 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 Post value = 215 C Post time = 2.0 min

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:	H_2 = 135 mL/min L 10 mL/min Air = 120 mL/min L 5 mL/min

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	O_2 = 15 mL/min L 2 mL/min
Injector Temperature:	140 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 = 0.5 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:	Splitless
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: The viscosity of multisol prohibits 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 .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:	H_2 = 135 mL/min L 10 mL/min Air = 120 mL/min L 5 mL/min O_2 = 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

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 3 C/min. The column can then be left at upper temperature overnight. This 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 G.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:

$$\frac{\text{Column Length in cm}}{\text{Retention Time of Methane or Acetone in sec}} = \text{Carrier Velocity in cm/sec}$$

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:

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

$$\frac{15 \text{ sec}}{60 \text{ sec/min}} = .25 \text{ min}$$

$$\frac{10 \text{ mL}}{.25 \text{ min}} = 40 \text{ mL/min (flow rate)}$$

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.

l. 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:

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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.

11. 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.

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c. For weekend shut-down, follow the same procedure but also extinguish the detector flame if appropriate by shutting off the detector gas valves.

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 XCSM, date of bagging, bag identification number, and name of person packaging the contaminated materials in accordance with MREF SOP-83-3, Section H. The double-contained materials can then be incinerated.

I. Emergency Procedures: If an XCSM 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 area 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.

TLH:cah

Revised February 19, 1990

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STANDARD OPERATING PROCEDURE
MREF SOP-88-39

TITLE: Analysis and Structural Verification of Pralidoxime Chloride

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

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 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 job site whenever the operation is being performed.

Submitted By: Timothy L Hayes 2/20/90
Signature/Date

Timothy L. Hayes, Principal Research Scientist
Printed Name/Title

Approved By: David L Dill 2/22/90
Signature/Date

Garrett S. Dill, D.V.M., Manager
Printed Name/Title

Approved By: David L Stitcher 2/26/90
Signature/Date

David L. Stitcher, CIH, Safety/Surety Officer
Printed Name/Title

Revised February 20, 1990

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Approved By: Richard A. Shanks 2.27.90
Signature/Date

Quality Assurance Unit
Health and Environment Group
Printed Name/Title

Approved By: Ronald F. Lamm for 2/27/90
Signature/Date

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

Revised February 20, 1990

SIGNATURES

I have read and understand the contents of MREF SOP-88-39.

Signature	Date	Signature	Date
James C. Chapman	5-14-90		
Sherri D. Moore	5/30/90		
Malcolm L. Reeves	4-9-90		
Carl T. Olson	4-20-90		
Randy K. Audet	5/16/90		
Melissa B. Myers	5/14/90		
Raymond L. Cunningham	6/25-90		

Revised February 20, 1990

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STANDARD OPERATING PROCEDURE 88-39

Analysis and Structural Verification of Pralidoxime Chloride

A. Statement of Work: This SOP describes the procedures for verification of identity and quantitative measurement of pralidoxime chloride (2-PAM Cl) by high performance liquid chromatography (HPLC). The procedures for structural verification by nuclear magnetic resonance (NMR) of 2-PAM Cl 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: Technical staff will consist of individuals designated by the Chemistry Coordinator to perform structural verification of the drug used in this task; i.e., 2-PAM Cl.

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 B.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 FSSP are followed when work is conducted at the MREF.

d. Each MREF or 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.

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.

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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 43201-2693.

C. Materials To Be Used: The 2-PAM Cl used on this program will be provided by the U.S. Army Medical Research and Development Command (USAMRDC) or purchased from a traceable source of purity. Upon receipt, the 2-PAM Cl will be stored in a desiccator at -10 C or as directed by the supplier. NMR spectra will be obtained on dilute solutions of the drug dissolved in > 99.8 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 (Burdick and Jackson HPLC grade or equivalent), deionized water or millipore water, acetic acid, glacial (Baker reagent grade Cat. No. 9508-03), tetrabutylammonium chloride (Aldrich 28,888-8), benzophenone (Aldrich 23,985-2), tetrabutylammonium nitrate (Kodak 9664), sodium lauryl sulfate (dodecyl sulfide, sodium salt) (Aldrich 86-201-0), helium gas, and nitrogen gas.

D. Equipment: 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 ultraviolet (UV) detector, HPLC injection system (autosampler), HPLC reverse-phase column, strip-chart recorder (optional), and electronic data system. Any equivalent system may be used once confirmation of performance has been established.

Other equipment includes: glass bottles, glass vials, Teflon cap liners, microsyringes, pipettes, volumetric flasks, graduated cylinders, autosampler vials, refrigerator, Teflon wash bottles, gas tight syringes, filter flask system, Pasteur pipettes, dropper bulbs, chart paper, spherisorb ODS 2 analytical HPLC column or equivalent, recorder pens, weighing paper, pipettes, pipette bulbs, and spatula.

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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 in the administrative area of the MREF or through Battelle's Safety Office at 505 King 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 8-hr permissible exposure limit (PEL) = 1 ppm, Action Level = 0.5 ppm.

F. Safety Requirements:

1. Hoods: Hood face velocity must average 100 ± 20 lfpm. 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.

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2. Protective Equipment: When working in the MREF 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, respirators, and other protective equipment.

3. First Aid: The location of the nearest eye-wash 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-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 magnehelic 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 8-inch line in the hood.

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

3. Sample Preparation: The drug formulation samples provided by the USAMRDC are 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.

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

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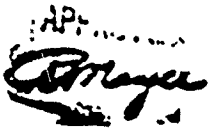

a. Analytical Reference Standard: 2-PAM Cl solid reference standard supplied by the USAMRDC is dried at 100 C, 0.4-mm Hg for 3 hr prior to use. This is performed by placing the solid material contained in its original container which has had its cap removed into a pre-heated oven. The oven is sealed and the vacuum adjusted to 0.4 mm Hg.

b. NMR Analysis: Approximately 2.0 mL of the 2-PAM Cl formulation is transferred to a 9.5 dram vial and frozen therein by partially immersing in dry ice/acetone after the vial is capped. This vial is placed in a chamber of a lyophilization apparatus and subjected to high vacuum until the sample reaches a state of dryness.

NMR samples are prepared by dissolution of several mg of the dried samples in deuterium oxide and are transferred into an NMR tube (tube capped after transfer) for NMR analysis.

c. HPLC Analysis: Samples are diluted with mobile phase so that the expected concentration range is between 0.01 and 0.10 mg/mL. Samples are refrigerated until analysis.

4. Preparation of Standard Solutions: Standard solutions of 2-PAM Cl are prepared for an NMR reference spectrum and HPLC standard curve determinations.

a. NMR: Within a glove bag thoroughly flushed with dry nitrogen or argon, weigh 10 mg ± 0.1 of 2-PAM Cl onto weighing table. 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: Accurately weigh 50 mg ± 0.1 mg of 2-PAM Cl onto weighing paper. Quantitatively transfer the 2-PAM Cl into a 50-mL volumetric flask containing approximately 40 mL of mobile phase (see Section G.5.b.). Mix the solution thoroughly. Dilute to 50 mL with water and remix the solution. The resulting concentration of the 2-PAM Cl stock will be approximately 1 mg/mL.

Weigh out 10 g ± 0.1 g of benzophenone, the internal standard (IS), and quantitatively transfer the material into a 25-mL volumetric flask containing approximately 20 mL of acetonitrile. Mix well until dissolved. Dilute to 25.0 mL with acetonitrile and remix the solution.

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The resulting concentration of the benzophenone internal standard stock is 400 mg/mL.

Mix and dilute the 2-PAM Cl stock solution with mobile phase (see Section G.4.b) in 10 mL volumetric flask as follows:

1.0-mL stock + 9.0-mL mobile phase
0.50-mL stock + 9.5-mL mobile phase
0.25-mL stock + 9.75-mL mobile phase
0.10-mL stock + 9.90-mL mobile phase
0.0-mL stock + 10.0-mL mobile phase

After the standards have been prepared, each level is the spiked 5 µL of the internal standard solution. The final standard concentrations are 0.10, 0.050, 0.025, 0.010, and 0.0 mg per mL.

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

5. Analysis Start-Up: NMR is performed to verify the structure of the 2-PAM Cl. HPLC is performed to quantitatively determine the concentration of 2-PAM in the samples and identity confirmation of 2-PAM in the dosing solution by retention indices comparison.

   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.

   b. Quantitative HPLC: Prepare HPLC mobile phase buffer for quantitative analysis by dissolving 2.7 g of tetramethylammonium chloride in approximately 900 mL of deionized water. Add 1.0 mL of glacial acetic acid and dilute to 1 L and mix. Store in a clean, 1-L glass bottle. Use within 30 days.

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

   Insure the appropriate analytical column has been installed in the analytical system and that the injector is equipped with at least a 20-µL sample injection loop.

All mobile phase must be 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 1.2 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 1.2 ± 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 about 30 min, adjust the detector zero with the balance control with the detector set at 0.064 AUFS. 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 buffer for the initial identity confirmation using a Supelco LC-1 column by dissolving 6.0 g of sodium lauryl sulfate and 1.0 g of tetrabutylammonium nitrate in 1,000 mL of deionized water. Add 20 mL of glacial acetic acid to the solution and mix. 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 the appropriate analytical column has been connected to the injector and detector, and that the injector is equipped with a 20-µL sample injection loop.

All mobile phase must be 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

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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 about 30 min, adjust the detector zero with the balance control with the detector set at 0.064 AUFS. 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 identify 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 interpretation.

b. Quantitative HPLC: The following is a set of HPLC conditions that have been found to be satisfactory for quantitative analysis of 2-PAM Cl:

Column: Alltech Spherisorb-ODS 2 (Stock No. 8736) and Supelco LC-18 Guard Column (Stock No. 5-8232).

Mobile Phase: See Section G.4.b

Detector: UV @ 298 nm

Flow Rate: 1.2 mL/min

Injection Volume: 20 µL

For quantitative analysis of 2-PAM Cl samples, transfer 1-mL duplicate aliquots of each 2-PAM Cl 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 data system 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, at least one blank sample and one standard must be analyzed. All samples must be analyzed under the same conditions used for the standards.

c. HPLC Identity Confirmation: For confirmation of the identity of 2-PAM Cl 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 2-PAM Cl:

Column: Supelco LC-1 (Stock No. 5-8296) 250 x 4.6 mm, 5 micron and Supelco LC-1 guard column (Stock No. 5-9551).

Mobile Phase: See Section G.4.c

Detector: UV @ 254 nm

Flow Rate: 1.0 mL/min

Injection Volume: 20 µL

For confirmation purposes, analyze a 2-PAM Cl standard and a formulation sample 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 spectrum obtained in Section G.5.a is compared with the reference spectrum to verify structural identity. HPLC samples analyzed in Section G.5.b are compared with results obtained from standards to determine concentration.

a. NMR: Compare the NMR spectrum for the sample with the spectrum obtained for the 2-PAM Cl reference standard. Verify correspondence of chemical shifts, multiplicities, and intensities for structural verification in conjunction with HPLC findings.

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b. Quantitative HPLC: Obtain printouts of the peak area ratios for each standard and sample as described in the instruction manual. Prepare a standard curve from the peak area ratios versus concentration of the standards.

Determine the 2-PAM Cl concentration in the samples and control standards using the standard curve. If necessary, correct for 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: Compare the retention times and relative responses of the 2-PAM Cl standard and sample peak for structural confirmation.

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.

TLH:cah

Revised February 20, 1990

STANDARD OPERATING PROCEDURE
MREF SOP-88-49

TITLE: Determination of Erythrocyte Acetylcholinesterase Activity in Laboratory Animal Blood with the COBAS/FARA Centrifugal Analyzer

LABORATORY: MREF SOP Approval Date: July 12, 1989

EXPIRATION DATE: August 10, 1991

PLACE OF OPERATION OR TEST: Throughout the MREF laboratory

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 trained properly and instructed in its provisions.

A copy of this SOP will be posted at the job site at all times.

Approved by: David Stitcher 10 Aug 90
Signature/Date

David L. Stitcher, CIH, Safety and Surety Officer
Printed Name/Title

STANDARD OPERATING PROCEDURE
MREF SOP-88-49

TITLE: Determination of Erythrocyte Acetylcholinesterase Activity in Laboratory Animal Blood with the COBAS/FARA Centrifugal Analyzer

LABORATORY: MREF SOP APPROVAL DATE: July 12, 1989

PLACE OF OPERATION OR TEST: Throughout the MREF laboratory

This Standard Operating Procedure (SOP) has been prepared as prescribed by Contract DAMD17-83-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.

Revised By: David W. Hobson 10 Jul 89
Signature/Date

David W. Hobson, Associate Manager
Printed Name/Title

Approved By: 7/10/89
Signature/Date

Garrett S. Dill, D.V.M., MREF Manager
Printed Name/Title

Approved By: David Stitel 7/12/89
Signature/Date

/Donald W. Cagle, CIH, Safety/Surety Officer
Printed Name/Title

Revised July 10, 1989

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Approved By:

Signature/Date 7/13/89

Ramona A. Mayer, Manager, QA Unit
Printed Name/Title

Approved By:

Signature/Date 7/16/89

Anna D. Barker, Ph.D.
Group Vice President and General Manager
Health and Environment
Printed Name/Title

Revised July 10, 1989

APPROVED

SIGNATURES

I have read and understand the contents of MREF SOP-88-49.

Signature	Date
Harold W. Nitz	9-12-89
Carole Wickline	9-12-89
Marie Moore	9-13-89
Werner L Wangaard	9-15-89
Wilbur E. Waist	9-26-89
Pamela H. Kinney	9-26-89
JACK WAUGH	9-26-89
Tracy Lenzini	9-26-89
James C Clay	9-27-89
Timothy L Clay	9-27-89
Kevin Braun	9-27-89
Gwen Stanek	9-28-89
Priscilla Parker	10-02-89
Cindy Pelley	100489
Carl T Olson	10-16-89
Randy K Guidet	10/17/89
Ruby C Kiser	10-17-89
Mary Jo Biever	10-17-89
Michael Hagen	10:8 89

Signature	Date
James A. Black	10/19/89
Deborah L. Rogers	10-20-89
Thomas A. Linder	10-24-89
Rebecca Lewis	10-25-89
Linda Adams	10-25-89
Michele Kinnes	10-27-89
Rebekah A. Starner	10-30-89
Pamela L. Cooley	11-3-89
Melissa Myers	11-6-89

STANDARD OPERATING PROCEDURE 88-49

Determination of Erythrocyte Acetylcholinesterase Activity in Laboratory Animal Blood with the COBAS/FARA Centrifugal Analyzer

A. Statement of Work: This SOP is to be used for analyzing erythrocyte (RBC) acetylcholinesterase (AChE) activity in laboratory animal blood in the range of 0.1 to 13.0 U/mℓ using the COBAS/FARA. The method described in this SOP using the COBAS/FARA centrifugal analyzer is also a cost-effective alternative to MREF SOP-85-18, "Determination of Erythrocyte Acetylcholinesterase Activity in Normal and Pyridostigmine Inhibited Rhesus Monkey Blood" and MREF SOP-86-21; "Determination of Erythrocyte Acetylcholinesterase Activity in Normal and Organophosphate Treated Rabbit Blood," which utilize the Technicon autoanalyzer. Previous work has shown the COBAS/FARA and Technicon methods to be essentially equivalent within the stated range.[1,2] Both methods of analysis are based on the following reactions:

AChE + Acetylthiocholine → Thiocholine + Acetic acid

Thiocholine + 5,5'-Dithiobis (2-nitrobenzoic acid) → Colored Anion

The analysis procedure is an adaptation of that described for erythrocyte AChE by Ellman, et al.[3]

B. Responsibility:

1. Personnel Qualifications: All technical staff will be familiar with handling hazardous materials within the MREF laboratory. They must know the requirements of the Buddy System. 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 B.1 are allowed in the room during operations. An escort is required for all others.

   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 Facility Safety and Surety Plan (FSSP) are followed.

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d. Each 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.

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 B.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 43201-2693.

C. Solvents and Chemicals To Be Used (and Suggested Sources):

1. Purchased from Sigma Chemical Company, St. Louis, MO:

a. Acetylthiocholine iodide (ATChI), Cat. No. A5751

b. 5,5-Dithiobis(2-nitrobenzoic) acid (DTNB), Cat. No. D8130

c. Triton X-100, Cat. No. Triton X-100

d. Acetylcholinesterase (eel) type VI-S, Cat. No. C3389

e. Bovine serum albumin, Cat. No. A6918

f. Tris (hydroxymethyl) aminomethane (TRIS), Cat. No. T1503

B. Purchased from Baker Chemical Company, Phillipsburg, NJ:

a. Hydrochloric acid (HCl), technical grade, Cat. No. 9535-3

b. Sodium chloride (NaCl), reagent grade, Cat. No. 3624-1

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C. Deionized water (dd H_2O) is prepared using a Milli-Q water purification system in conjunction with the Milli-RO4 water purification system. This set-up requires:

a. 1 particulate filter (5 micron)

b. 1 reverse osmosis filter

c. 1 charcoal polish filter

d. 2 ion exchange resin filters

D. Equipment and Supplies: Freezer, refrigerator, labels, first-aid kit, weighing paper, squirt bottles, wiping tissues, beakers, volumetric flasks, bottles, Eppendorf microfuge 5414, microcentrifuge tubes, positive displacement pipettes, pipette tips, Pasteur and volumetric pipettes, pipette bulbs, tissue paper, laboratory coat, safety shoes, safety glasses, spatula, latex gloves, Roche COBAS/FARA Serial No. 232185, sample cups for COBAS/FARA, cuvette rotors for COBAS/FARA, printer paper, reagent rack, Corning pH meter 140, SMI C digital adjust micro/pettor (5.0 to 30.0 µℓ) Catalog No. 12008 with siliconized glass capillaries S-1095-C, Eppendorf digital pipette (100 - 1,000 µℓ) with pipette tips, Mettler AE 100 balance, and Thermolyne 1,000 stir plate.

E. Hazards Involved: Blood samples may contain infectious agents, care should be taken in handling samples and in the proper wearing and use of required safety equipment.

F. Safety Requirements:

1. Protective Equipment: The following clothing and protective gear are required as a minimum for all personnel.

lab coat or scrub suit
safety shoes
latex gloves
safety glasses

All provisions of the MREF FSSP apply to the checking and testing of gloves and other protective equipment. In addition, if chemical surety materiel (CSM) is in use in the facility, a combination air-line/escape respirator will be readily available.

2. First Aid: The location of the nearest eye-wash fountain, deluge shower, and fire extinguisher will be known to all workers before work begins.

Revised July 10, 1989

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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 magnehelic gauge on any hoods in the room. If inspection reveals that any 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. Work Area and Instrument Set-Up: The work area for sample-handling is a clean and dry standard laboratory benchtop. The COBAS/FARA is turned on and allowed to warm up for at least 15 min prior to data collection. Select the appropriate analysis program and rack type. The COBAS/FARA quality control (QC) log book is checked prior to data collection to ensure that the instrument met the routine QC requirements at the last scheduled performance check.

3. Reagent Preparation:

   a. TRIS Buffer (pH 8.2, 0.05M): Dissolve 6.64 g of NaCl and 6.05 g of TRIS in approximately 900 mL of dd H_2O. Adjust pH to 8.2 with 6 N HCl. Dilute to 1,000 mL with dd H_2O before using. The shelf life of this preparation is 8 weeks.

   b. Saline (0.9 percent): Dissolve 0.9 g of NaCl in approximately 90 mL of dd H_2O. Bring to a final volume of 100 mL with dd H_2O before using. The shelf life of this preparation is 8 weeks. Pre-prepared, USP-grade 0.9 percent saline solution for injection may also be used alternatively.

   c. 5,5'-Dithio-bis-2-nitrobenzoic Acid (DTNB) Color Reagent (4.2×10^{-4} M): Dissolve 0.0832 g of DTNB in approximately 400 mL of TRIS buffer. Adjust pH to 8.2 with 1.0 M NaOH, if necessary, and bring total volume to 500 mL with TRIS buffer. The shelf life of this preparation is 4 weeks.

   d. Acetylthiocholine Iodide (ATChI) Substrate (5×10^{-2} M): Dissolve 5.784 g of ATChI in 90 mL of saline. Dilute to a final volume of 100 mL with saline prior to preparation for storage. Aliquot into 2.5-mL in-use quantities and store at 0 C. Thaw and dilute frozen aliquots with 7.5-mL saline prior to assay use. The shelf life of this preparation is 6 months.

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e. TRIS + Albumin Diluent: Dissolve 1.0 g of bovine serum albumin in 90 mℓ of TRIS buffer. Dilute to 50 mℓ with TRIS. Use immediately.

f. Control Eel AChE Sample: Weigh out eel AChE lyophilized powder equivalent to 25 U and dissolve in 100-mℓ TRIS + albumin diluent. Pipette 1-mℓ aliquots into 10-mℓ capacity glass vials and store at 0 C. The shelf life of this preparation is 6 months. On the day of the assay, thaw and dilute with 4-mℓ TRIS buffer per vial. The resulting activity is approximately 0.05 U/mℓ (or approximately 2.5 U/mℓ when multiplied by the 50-fold dilution factor used for RBC AChE assays).

4. COBAS/FARA Programming: The COBAS/FARA program parameters for the low (0.1 to 3.0 U/mℓ) and high (3.0 to 13.0 U/mℓ) range RBC cholinesterase assays are as follows (the parameters given are for both ranges unless indicated otherwise below):

a. Measurement Mode: ABSORB
b. Reaction Mode: P-IO-SR1-A1
c. Calibration Mode: FACTOR
d. Reagent Blank: REAG/DIL
e. Wavelength: 410 nm
f. Temperature: 37.0 C
g. Decimal Position: 2
h. Unit: U/mℓ
i. Sample: 7 µℓ Diluent: 20 µℓ
j. Reagent: 200 µℓ
k. Incubation: 120 s
l. Start Reagent: 10 µℓ Diluent: 20 µℓ
m. First: 60.0 s Number: 7
n. Interval:
   (1) low range: 120 s
   (2) high range: 15 s
o. M1: 55.0 s
p. Conversion Factor: 131.300
q. Offset: 0.0
r. Reaction Direction: INCREAS
s. Check: ON
t. Sample Limit: NO
u. Test Range:
   (1) low range: LO: 0.05 HI: 3.50 U/mℓ
   (2) high range: LO: 2.50 HI: 13.50 U/mℓ
v. Normal Range: LO: NO HI: NO U/mℓ
w. Calc. Steps: 1
x. Calc. Step A: KINETIC
y. Readings First: 1 Last: 7
z. React. Limit: NO
aa. Calib. Interval: EACH RUN
bb. Factor: 1.0

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cc. Reagent Range: LO: NO HI: NO
dd. Blank Range: LO: NO HI: NO
ee. Control (CS) Position: 1 to 10 (as required)
LO: mean value minus 2 standard deviations
HI: mean value plus 2 standard deviations
ff. Control Value Assigned: mean value for each control sample used.

5. Sample Collection and Preparation: Heparinized whole blood samples of at least 0.1 mℓ are obtained from laboratory animals. These blood samples are the source of RBCs throughout the study. The following procedure is used to process each sample immediately following collection:

a. Transfer a 50 to 500-µℓ aliquot (approximate) of the whole blood sample into a microcentrifuge tube. Centrifuge whole blood samples at approximately 15,000 revolutions per minute (rpm) for 2 min, then remove plasma from RBCs using a Pasteur pipette.

b. Transfer 10 µℓ of packed RBCs into a COBAS/FARA sample cup containing 490 mℓ of 1 percent Triton X-100 in 0.9 percent saline to solubilize RBC membranes. Mix by gently shaking the sample cup (or rack containing several sample cups) back and forth several times (10 times should be sufficient).

c. Check the samples to ensure that they are completely lysed (they will be bright red and clear if held up to a light with no visible RBCs or fragments remaining). If not completely lysed, continue to gently rock the samples back and forth a few more times. Place the samples in a COBAS/FARA sample rack to run.

d. Place reagents in a COBAS/FARA dual-reagent rack and the eel AChE controls in the CS positions of the control rack. Enter the analysis routine for the AChE assay (LACHE = low range assay, HACHE = high range assay), select the samples to be analyzed, and start the analyzer.

e. Results for the control and each sample (in AChE U/mℓ erythocytes) are printed by the COBAS/FARA following the analysis cycle.

6. Internal Controls:

a. Confirmation: Based on the accuracy in obtaining an established value for AChE activity eel AChE control samples for each analysis.

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b. QC: A QC range is established for each specified AChE control concentration level prior to routine use in the analysis procedure. The default control concentration level is 2.5 U/mL, approximately, unless otherwise specified and is prepared as described in Section G.3.f. All other control levels to be used are similarly prepared or may be prepared as a dilution of the Section G.3.f frozen control stock. More than one control level may be specified for the analysis. Controls for each concentration level to be used in the analysis are assayed five times from each of four different vials of each control preparation. The mean and standard deviation is calculated for each control level, and a QC range is established (mean ± 2 standard deviations). Each time new control stock solution is prepared, a new QC range must be established. Once established, data obtained from any assay for which the control sample value(s) used is/are not within the QC range specified for that control preparation are to be considered invalid and must be reanalyzed. All control samples to be included in an assay must be placed in the positions specified as "CS" positions in the COBAS/FARA control rack, and an established set of QC parameters must be entered into the analysis routine for each "CS" position used.

c. Linearity: This method has been determined to correlate positively over the range of 0.1 to 13 U/mL with the method of Ellman, et al. as performed on a Technicon autoanalyzer.[1,2,3]

d. Accuracy: Factors that influence the accuracy of the RBC AChE activity assay are:

(1) The volume of intercellular plasma remaining in the packed RBC sample.

(2) Number and volume of RBCs in the sample.

(3) Deviations in sample preparation timing, due to the degree of decay in AChE activity for that sample type with time.

(4) Storage conditions of the sample. Samples should be kept on ice or refrigerated until analysis.

H. References:

(1) MREF Pre-Task Pilot Study 87-10 Report, "Technicon and COBAS/FARA Analytical Method Comparison for the Determination of Erythrocyte Acetylcholinesterase in the Primate." USAMRDC Contract No. DAMD17-83-C-3129, November 1988.

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(2) MREF Pre-Task Pilot Study 87-10 Report, "Technicon and COBAS/FARA Analytical Method Comparison for the Determination of Erythrocyte Acetylcholinesterase in the Rabbit." USAMRDC Contract No. DAMD7-83-C-3129, November 1988.

(3) Ellman, G.L., Courtney, D. K., Andres, V., and Featherstone, R. M. A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity. Biochem. Pharmacol., 7, 88, 1961.

DWH:tsh

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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-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 job site whenever the operation is being performed.

Submitted By: Timothy L Hayes 2/20/90
Signature/Date

Timothy L. Hayes, Research Scientist
Printed Name/Title

Approved By: [signature] 2/20/90
Signature/Date

Garrett S. Dill, D.V.M., Manager
Printed Name/Title

Approved By: David Stitcher 2/26/90
Signature/Date

David L. Stitcher, CIH, Safety/Surety Officer
Printed Name/Title

Revised February 20, 1990

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-9050 and will be effective for one year from date of approval unless sooner rescinded or superseded.

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Submitted By: Timothy L Hayes 2/20/90
Signature/Date

Timothy L. Hayes, Research Scientist
Printed Name/Title

Approved By: [signature] 2/20/90
Signature/Date

Garrett S. Dill, D.V.M., Manager
Printed Name/Title

Approved By: David Stitcher 2/25/90
Signature/Date

David L. Stitcher, CIH, Safety/Surety Officer
Printed Name/Title

Revised February 20, 1990

SIGNATURES

I have read and understand the contents of MREF SOP-89-55.

Signature	Date	Signature	Date
Helen L. Reese	3-14-90		
Melissa A. Myers	3/19/90		
Sheri D. Moore	3/30/90		
James R. Beck	4/2/90		
James Cisar	4/2/90		
Raymond J. Cunningham	6/26/90		

Revised February 20, 1990

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STANDARD OPERATING PROCEDURE 89-55

Analysis and Structural Verification of
Atropine Base in Citrate Buffer

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 effor 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 B.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.

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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 B.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 43201-2693.

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 > 99.8 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 (Burdick and Jackson HPLC Grade), methanol (Burdick and Jackson HPLC Grade), benzene (Burdick 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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D. 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 King 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 8-hr permissible exposure limit (PEL) = 1 ppm, Action Level = 0.5 ppm.

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F. Safety Requirements:

1. Hoods: Hood face velocity must average 100 ± 20 lfpm. 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 eye-wash 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-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 magnehelic 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 8-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 lps 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 D_2SO_4 in D_2O 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 1.0 mg/mL.

4. Preparation of Standard Solutions: Standard solutions of atropine sulfate are prepared for NMR reference spectrum and HPLC standard curve determinations.

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 200 mL of acetonitrile and mix. Once the buffer has been prepared, it must be filtered and used within 30 days.

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Insure that the appropriate analytical column has been installed in the analytical system, and that the injector is equipped with at least a 20 µL 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.

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.

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 @ 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.

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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:

1. "Assay of Formulated Atropine Solution, WR-6241AK, B107753, Lot No. RU7144," Report No. 527, Contract No. DAMD17-85-C-5141, SRI International Project No. 8504, December 10, 1985.

TLH:cah

Revised February 20, 1990

ANALYST

STANDARD OPERATING PROCEDURE
MREF SOP-89-58

TITLE: Analysis and Structural Verification of Pyridostigmine Bromide

LABORATORY: MREF SOP APPROVAL DATE: October 26, 1989

PLACE OF OPERATION OR TEST: Throughout the MREF laboratory and King Ave.

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 SOF will be posted at the Medical Research and Evaluation Facility (MREF) job site at all times.

Submitted By: Timothy L Hayes 10/26/89
Signature/Date

Timothy L. Hayes, Principal Research Scientist
Printed Name/Title

Approved By: Garrett S. Dill 10/26/89
Signature/Date

Garrett S. Dill, C.V.M., Manager
Printed Name/Title

Approved By: Donald W. Cagle 10/26/89
Signature/Date

Donald W. Cagle, CIH, Safety/Surety Officer
Printed Name/Title

Revised October 26, 1989

APPROVED
[signature]

Approved By: Sue Harsh 10/26/89
Signature/Date

Sue Harsh, Researcher
Printed Name/Title

Approved By: Ramona Mayer 10/26/89
Signature/Date

Ramona A. Mayer, Manager, QA Unit
Printed Name/Title

Approved By: [signature] 10/26/89
Signature/Date

Anna O. Barker, Ph.D.
Group Vice President and General Manager
Health and Environment
Printed Name/Title

Revised October 25, 1989

APPROVED
[signature]

SIGNATURES

I have read and understand the contents of MREF SOP-89-58.

Signature	Date	Signature	Date
[signature]	10/26/89		
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STANDARD OPERATING PROCEDURE 89-58

Analysis and Structural Verification of Pyridostigmine Bromide

A. Statement of Work: The purpose of this work is to verify the structural identity of pyridostigmine bromide and to analyze quantitatively for the amount of pyridostigmine bromide present in drug formulations.

B. Responsibility:

1. Personnel Qualifications: All technical staff will be familiar with handling hazardous materials within the MREF laboratory. They must know the requirements of the Buddy System. 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 B.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 Facility Safety and Surety Plan (FSSP) are followed.

d. Each 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.

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 B.2. In addition, they must use

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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, OH 43201-2693.

C. Materials To Be Used:

1. Solvents and Chemicals: Pyridostigmine bromide - Prior to analysis, the pyridostigmine bromide will be stored in subdued lighting at room temperature. Nuclear magnetic resonance (NMR) spectra will be obtained on dilute solutions of the drug dissolved in > 99.8 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 (Burdick and Jackson High Performance Liquid Chromatography [HPLC] Grade), deionized water, glacial acetic acid (Baker Reagent Grade), tetrabutylammonium chloride (Aldrich 98+ percent), tetrabutylammonium nitrate (Eastman 99 percent), sodium lauryl sulfate (Aldrich 98 percent), p-aminobenzoic acid (Chem Services Inc., 99 percent), hydrobromic acid (Mallinckrodt 48 percent Reagent Grade or equivalent), Amberlite® IR-120 (plus) ion exchange resin (Aldrich or equivalent), and helium gas.

D. Tools and Equipment: Proton NMR spectra will be obtained on Battelle's Varian CFT-20 Fourier transform NMR spectrometer located in Room 7238A of the King Avenue facility.

The HPLC analytical system to be used consists of the following: HPLC pump, HPLC ultraviolet (UV) detector, HPLC autosampler, analytical column, strip-chart recorder, and electronic data system.

Other equipment includes glass bottles, labels, HPLC mobile phase filter system, wiping tissues, beakers, pipette bulbs, spatula, forceps, weighing paper, glass vials, Teflon cap liners, microsyringes, pipettes, volumetric flasks, graduated cylinders, autosampler vials, refrigerator, pH meter, Teflon wash bottles, Eppendorf pipettor, pipettor tips, Pasteur pipettes, chart paper, and recorder pens.

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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) are available in the MREF office files or through Battelle's Safety Office at 505 King Avenue.

2. 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 ± 10 lfpm. 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: The following clothing and protective gear are required as a minimum for all personnel. This equipment must be used as directed in the FSSP.

laboratory coat
latex gloves
safety glasses

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

3. First Aid: The location of the nearest eye-wash fountain, deluge 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 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 magnehelic gauge on the hood. If inspection reveals that the hood has failed, is marginal in flow, or operates outside the

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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 8-inch line in the hood.

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

   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. All sample preparation will be conducted in a hood.

   (a) Analytical Reference Standard: Pyridostigmine bromide solid reference standard is dried over P_2O_5 at 100 C, 0.4-mm Hg for 4 hr or dried at 100 C, 0.4-mm Hg for 4 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 preheated oven. The oven is sealed and the vacuum adjusted to 0.4-mm Hg.

   (b) NMR: For the NMR sample preparation, 2.0 mL of the pyridostigmine bromide test sample is dissolved in 48 mL of water and the solution slowly passed through a cation-exchange resin bed (Amberlite® IR-120 (plus) ion exchange resin, 1 x 4.5 cm). The column is washed with 50 mL of deionized water and the pyridostigmine bromide eluted with 200 mL of 1 N HBr prepared by diluting 22.6 mL of 48 percent HBr with 177.4 mL of deicnized water. The eluate is evaporated to dryness under reduced pressure at 50 C.

   NMR samples are prepared to be approximately 10 mg/mL concentration by dissolving a known amount of sample in the appropriate amount of deuterium oxide and transferring the solution into an NMR tube (tube capped after transfer) for NMR analysis.

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(c) HPLC: Samples are diluted with deionized water so that the deionized water range is between 0.02 and 0.08 mg/mL.

4. Preparation of Standard Solutions: Standard solutions of pyridostigmine bromide are prepared for NMR reference spectrum and HPLC standard curve determination.

(a) NMR: Accurately weigh to within 0.1 mg 10 mg of pyridostigmine bromide reference standard. Transfer the sample into a screw-capped bottle and close tightly. Dissolve the sample in approximately 1.0 mL of deuterium oxide and recap the bottle to minimize the contamination of the sample with undeuterated moisture.

(b) HPLC:

Pyridostigmine Bromide Stock Solution: Accurately weigh to within 0.1 mg 50 mg of pyridostigmine bromide. Dissolve the sample in approximately 40 mL of deionized water. Dilute to 50.0 mL with deionized water.

Internal Standard Stock Solution: Accurately weigh to within 0.1 mg 10 mg of p-aminobenzoic acid, the internal standard (IS), and dissolve in approximately 40 mL of methanol. Dilute to 100 ml with methanol.

Mix and dilute the pyridostigmine bromide stock solution with deionized water as follows:

1.0-mL stock + 4.0-mL water
0.50-mL stock + 4.5-mL water
0.25-mL stock + 4.75-mL water
0.10-mL stock + 4.90-mL water
0.0-mL stock + 5.0-mL water

Working standards are prepared by diluting 1.0-mL aliquots of each of these pyridostigmine bromide solutions with 1.0-mL aliquots of IS solution to give the following pyridostigmine bromide concentrations of 0.10, 0.050, 0.025, 0.010, and 0.0 mg/mL.

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

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5. Analysis Start Up: NMR is performed to verify the structure of pyridostigmine bromide. HPLC is performed to quantitatively determine the concentration of pyridostigmine bromide in the samples.
   (a) NMR: NMR analysis is carried out after the sample being analyzed is placed in the magnet and the response for the particular sample has been maximized.

   (b) Quantitative HPLC: Prepare HPLC mobile phase for quantitative analysis by dissolving 3.2 g of tetramethylammonium chloride and 6.9 g of KH_2PO_4 in approximately 900 mL of deionized water. Dilute to 1 L and mix. Adjust the pH of the solution to 3.0 with H_3PO_4. To 800 mL of this solution, add 200 mL of acetonitrile and mix. Store in a clean 1-L glass bottle. Filter the mobile phase and degas before using. Use within 30 days of preparation.

   If necessary, connect the appropriate column to the injector and detector. Connect a 20-µL sample loop to the injector. Degas the mobile phase for approximately 5 min with helium or nitrogen immediately prior to use. Turn on the detector and the pump with the pump set for 1.5 mL/min flow. After approximately 15 min, measure the flow for 5 min with a 10-mL graduated cylinder. The flow rate should be 1.5 ± 0.1 mL/min. Adjust the flow rate if necessary to obtain a flow rate within these limits.

   (c) HPLC Identity Confirmation: Prepare HPLC mobile phase for the initial identity confirmation using a Supelco LC-1 column by dissolving 6.0 g of sodium lauryl sulfate and 1.0 g of tetrabutylammonium nitrate in 1,000 mL of deionized water. Add 20 mL of glacial acetic acid to the solution and mix. Store in a clean glass bottle. Filter the mobile phase and degas before using. Use within 30 days of preparation.

   If necessary, connect column to the injector and detector. Connect a 20-µL sample loop to the injector. Degas the mobile phase for approximately 5 min with helium or nitrogen immediately prior to use. Turn on the detector and the pump with the pump set for 1.0 mL/min flow. 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 if necessary to obtain a flow rate within these limits.

   After the pump has been on for about 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.

6. Analysis of Samples: NMR is performed for structural confirmation. HPLC is performed to quantitatively determine the concentration of pyridostigmine bromide and confirm the identity of the pyridostigmine bromide in the samples.

(a) NMR: When the response for the sample being analyzed has been maximized, proceed with the analysis. 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 (DSS). A listing of shifts and parameters used will be obtained.

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

Column: Dupont Zorbax C8 or equivalent, 250-mm long x 4.6-mm inner diameter (I.D.) with 5 micron particle size.

Mobile Phase: 80 percent 0.05 M KH_2PO_4 with 3.0 mM tetramethylammonium chloride, pH 3.0, 20 percent acetonitrile (see Section G.5.b).

Detector: UV @ 269 nm.

Flow Rate: 1.5 mL/min.

Injection Volume: 20 µL.

For quantitative analysis of pyridostigmine bromide samples, transfer 1-mL duplicate aliquots of each pyridostigmine bromide 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 data system instruction manual. Transfer 1-mL duplicate aliquots of each sample to autosampler vials and place the vials in the autosampler. For every ten samples to be analyzed, analyze one blank sample and one standard. Analyze under the same conditions used for the initial calibration standards.

(c) HPLC Identity Confirmation: For confirmation of the identity of pyridostigmine bromide by HPLC, a second set of HPLC conditions is

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employed. HPLC confirmation of the identity of pyridostigmine bromide is performed by analysis under a second set of HPLC conditions. Compare the retention times and relative responses of the pyridostigmine bromide reference standard and sample peak for structural confirmation in conjunction with the first set of HPLC results and NMR conclusions.

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

Mobile Phase: 60 percent buffer (see Section G.5.c), 40 percent acetonitrile.
Detector: UV @ 254 nm.

Flow Rate: 1.0 mL/min.

Injection Volume: 20 µL.

For confirmation purposes, analyze a pyridostigmine bromide standard and a sample from the formulation under these HPLC conditions.

7. Data Reduction: The NMR spectra obtained in Section G.6.a are compared with the reference NMR spectra for pyridostigmine bromide to verify structural identity. The HPLC samples analyzed in Section G.6 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 pyridostigmine bromide 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 area ratios for each standard and sample as described in the instruction manual. Prepare a standard curve from the peak area ratios versus concentration of the standards.

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

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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 need are reanalyzed.

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

(d) HPLC Dose Verification: The identity of pyridostigmine bromide used during dose administration is verified by analyzing the administered dosage formulation by the HPLC method described in Section G.6.b. The response is compared to that obtained from a series of standards prepared from the analytical reference material to verify identity.

H. Emergency Procedures: All personnel involved in the MREF's laboratory operations must be familiar with FSSP SOP MREF-18.I.

First Aid Procedures: First aid and self aid at the MREF are to be conducted as specified in FSSP SOP MREF-19.

J. References:

1. "Assay of Syrup Preparation of Pyridostigmine Bromide, Syrup Mestinon, WR-250710AJ, BL08189," Draft Report No. 509, Contract No. DAMD17-85-C-5141, SRI International Project No. 8504, July 25, 1985.

TLH:CAH

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STANDARD OPERATING PROCEDURE
MREF SOP-89-60

TITLE: Analysis of Serum or Plasma Samples for Diazepam and Metabolite, Desmethyldiazepam by Gas Chromatography

LABORATORY: MREF, HML, or King Ave. SOP APPROVAL DATE: 9/12/90

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 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) or Hazardous Materials Laboratory (HML) job site at all times.

Submitted By: Timothy K Hayes 8/31/90
Signature/Date

Timothy L. Hayes, Principal Research Scientist
Printed Name/Title

Approved By: David W. Holmes 8/31/90
Signature/Date

Garrett S. Dill, D.V.M., Manager
Printed Name/Title

Approved By: David Stitcher 9-12-90
Signature/Date

David L. Stitcher, CIH, Safety/Surety Officer
Printed Name/Title

Revised August 31, 1990

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Approved By: Richard A. Swanda 9-18-90
Signature/Date

Quality Assurance Unit
Health and Environment Group
Printed Name/Title

Approved By: Chuck K. Burdick 9/18/90
Signature/Date

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

Revised August 31, 1990

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SIGNATURES

I have read and understand the contents of MREF SOP-89-60.

Signature	Date	Signature	Date

Revised August 31, 1990

STANDARD OPERATING PROCEDURE 89-60

Analysis of Serum or Plasma Samples for Diazepam and Metabolite, Desmethyldiazepam, by Gas Chromatography

I. Scope: This SOP describes a procedure for the analysis of serum or plasma samples for Diazepam (DZ) and a pharmacologically active metabolite, Desmethyldiazepam (DMDZ), in either serum or plasma samples. The analytical methods discussed are based upon a compilation of previously published methodology. Our approach differs from the other methods in that minor modifications were made to simplify sample preparation and improve method sensitivity, precision, and accuracy.

To perform this assay, blood samples are drawn from the test subject and either clotted for serum samples using non-treated collection tubes, or not clotted for plasma samples using anticoagulant treated collection tubes. Commonly used anticoagulants that have been successfully used with this method are heparin and ethylenediaminetetraacetic acid (EDTA). Once the samples have been properly collected they are centrifuged to separate the two phases and the liquid top layers removed for extraction. A liquid-liquid extraction is then performed on a known volume of serum or plasma using benzene as the extraction media. The sample extract is analyzed directly by gas chromatography (GC) using an Electron Capture Detector (ECD) with the ^{63}Ni element installed or a Nitrogen Phosphorous Detector (NPD). Sample pre-concentration was found not to be necessary or practical since a background interference observed in "DZ-free" samples typically ranges from 20 to 30 percent of the reported lower calibration limit.

The analytical method has been validated over an expected working range of 1 to 500 ng/mL in the plasma or serum samples. The findings of the certification phase indicate that, following the procedures described in this SOP, concentrations of DZ and DMDZ in this operational range can be quantified with less than 10 percent relative error. The observed therapeutic dose range in monkeys is typically 2 to 200 ng.

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

A. Responsibility:

All technical staff will be familiar with the safe handling practices of chemical surety 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.

B. Materials To Be Used:

1. Solvents and Chemicals: DZ, DMDZ, midazolam (MID), medazepam (MED), high purity compressed gases (He, N_2, and air), benzene, methanol (MeOH), acetone, water, or other appropriate solvent specified by test protocol.

C. Equipment:

1. Freezer, refrigerator, labels, first-aid kit, plastic-backed, absorbent paper, brown paper, squirt bottles, wiping tissues, beakers, bottles, maxi-vials, volumetric pipettes, pipette bulbs, tissue paper, laboratory coat, safety shoes, safety glasses, spatula, precision syringes, volumetric glassware, 13 mL x 100 mm screw-cap glass culture tubes, analytical balance, rotating extraction apparatus, centrifuge, crimp-cap GC vials, vortex mixer, Hewlett-Packard 5890 GC equipped with a ^{63}Ni ECD, NPD or equivalent, Neslab Cool-Flow recirculating refrigerator, Re-pipettor, scrub suit, and latex gloves.

D. Hazards Involved:

1. Solvents: The solvents used this SOP material may have hazards associated with their use. A copy of the Material Safety Data Sheets (MSDS) are available in the MREF office or through Battelle's Safety Services Department at 505 King Avenue.

E. 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 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 magnehelic 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.

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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 8-inch line in the hood.

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

3. Equipment Preparation:

   a. Glassware: All glassware shall be cleaned before use. If desired, the glassware may be silanized with hexamethyl disilizane (HMDS) prior to use. This will minimize adsorption of traces of the compounds of interest on otherwise active glass surfaces.

      All glassware is washed three times each with 5 percent detergent solution, such as alconox, followed by three MeOH rinses, and finally three acetone rinses, the glassware is then heated in a drying oven until dry.

      To silyate the clean glassware, place it in a vacuum oven which is sealed, and evacuated with an aspirator or vacuum pump to 20- to 25-mm Hg. The oven is heated to 180 ± 10 C and injected with 1-mL of a HMDS solution. Following the addition of the HMDS solution the oven is held at temperature for 2-3 hr. While the oven is still under vacuum, turn off the heater and allow the oven to cool to room temperature (overnight). Once the oven has thoroughly cooled vent the oven and remove the glassware. Glassware treated in this manner is now ready for use.

   b. Instrument Preparation: Install the proper column into the ECD detector and injector ports and leak test the joints. Connections must be made with high temperature vespel ferrules to maintain a leak free system. If the column has not been in use, following proper installation and parameter settings, condition at 320 C by programming from 50 C to 320 C at 5 C/min and holding at 320 C for 3 hr prior to installation into the detector. The GC (Hewlett-Packard 5890 or equivalent) is prepared for use with the following settings:

      (1) Column - 25 m x 0.32-mm inside diameter (I.D.) RSL-300 (Bonded OV-17, Alltech Associates) with 0.33-μm film thickness or equivalent.

Note: Set the gas flow rates with the column oven at operating temperature (260 C) as prescribed. Using a stopwatch or flow calibrator, set the carrier linear velocity. A typical column head pressure will be 11 psi with the following settings.

To make the flow measurements attach a soap bubble flow meter to the gas outlet from the detector with only the carrier and make-up gases on. Record the flow time and corresponding flow volume. Calculate the flow rate.

$$\text{Flow rate (mL/min)} = \frac{\text{Volume (mL)}}{\text{Time (min)}}$$

Adjust and repeat measurements until the prescribed value is reached.

(2) Carrier linear velocity = 35 cm/sec ± 3 cm/sec

(3) Carrier (He) + make-up flow rate = 34 mL/min ± 2 mL/min

(4) Septum Purge flow rate = 5 mL/min ± 2 mL/min

(5) Split/Splitless Purge flow rate = 30 mL/min ± 5 mL/min
(purge on at 0.75 min)
(purge off before end of program)

(6) Injection mode: 5 µL splitless

(7) Injector Temperature: 310 C

(8) Detector (ECD) Temperature: 340 C
(NPD) Temperature: 300 C

(9) Autosampler Tray Temperature: Cooled to 8-10 C via Cool-Flow

(10) Oven Program:

Initial temperature = 60 C
Initial time = 0.75 min

Level 1: Program rate = 35 C/min
Final temperature = 235 C
Final time = 0.0 min

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Level 2: Program rate = 2.5 C/min
Final temperature = 270 C
Final time = 0.0 min

Level 3: Program rate = 35 C/min
Final temperature = 330 C
Final time = 2.0 min

(11) Enter integration parameters into the data system or integrator which give the most reliable and reproducible peak integration. This may require some experimentation and adjustment.

4. Solution Preparation:

a. Preparation of Stock Solutions: Prepare individual stock solutions of MED, DZ, DMDZ, and MID at a concentration of approximately 1.0 mg/mL in MeOH.

(1) Using an analytical balance, weighing paper and spatula, accurately weigh 10.0 ± 0.1 mg of each compound and quantitatively transfer to a corresponding pre-labeled 10-mL volumetric flask. Dissolve each weighed quantity of material in approximately 5 mL MeOH with the aid of vortex mixing. Dilute each volumetric flask to volume using MeOH. Transfer each solution to a properly labeled teflon cap lined glass vial and store frozen at -70 C until use.

b. Preparation of Working Solutions:

(1) 200 µg/mL MED Surrogate Spiking Solution in MeOH: Dispense 2.00-mL (using 2500-µL gas-tight syringe) of the 1.0 mg/mL MED stock prepared in Section G.4.a. into a 10 mL volumetric flask and q.s. to the mark with MeOH. Mix 60 sec on a vortex mixer. Aliquot 0.5 mL volumes into crimp-cap GC autosampler vials and store at -70 C until use.

(2) 25 µg/mL DZ/DMDZ Spiking Solution: Dispense into a 50 mL volumetric flask containing approximately 20 mL MeOH, 1.25 mL (using 1,250-µL gas-tight syringe) each of the DZ and DMDZ stocks prepared in Section G.4.a. Mix the solution for 60 sec on a vortex mixer. The volume of the volumetric is brought to the mark with additional MeOH and the solution re-mixed on a vortex mixer for

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60 sec. Aliquot 1.0 mL volumes into crimp-cap GC autosampler vials and store at-70 C in GC vials until use.

(3) 2.5 µg/mL DZ/DMDZ Spiking Solution: Dispense into a 25 mL volumetric flask containing approximately 10 mL MeOH, 2.5 mL (using 2,500-µL gas-tight syringe) of the DZ/DMDZ spiking solution prepared in Section G.4.b.(2). Mix the solution for 60 sec on a vortex mixer. The volume of the volumetric is brought to the mark with additional MeOH and the solution re-mixed on a vortex mixer for 60 seconds. Aliquot 1.0 mL volumes into crimp-cap GC autosampler vials and store at -70 C in GC vials until use.

(4) 0.25 µg/mL DZ/DMDZ Spiking Solution: Dispense into a 25 mL volumetric flask containing approximately 10 mL MeOH, 2.5 mL (using 2,500-µL gas-tight syringe) of the DZ/DMDZ spiking solution prepared in Section G.4.b.(3). Mix the solution for 60 sec on a vortex mixer. The volume of the volumetric is brought to the mark with additional MeOH and the solution re-mixed on a vortex mixer for 60 seconds. Aliquot 1.0 mL volumes into crimp-cap GC autosampler vials and store at -70 C in GC vials until use.

(5) 20 µg/mL MID Internal Standard Spiking Solution in benzene: Dispense 200 µL (using a 250 µL gas-tight syringe) of the MID stock prepared in Section G.4.a. into a 10-mL volumetric flask which contains approximately 5 mL benzene. The solution contained in the volumetric flask is mixed for 60 sec on a vortex mixer and then the volume of the volumetric is brought to the mark with additional benzene. The solution is then re-mixed for 60 sec on a vortex mixer. Aliquot 0.5 mL volumes into crimp-cap GC autosampler vials and store at -70 C until use.

c. Preparation and Extraction of Calibration Standards:

Instrument calibration is performed using calibration standards that have been extracted from control plasma or serum. The method was validated using extracted calibration standards prepared at 1.0 ng/mL, 2.0 ng/mL, 5.0 ng/mL, 10.0 ng/mL, 20.0 ng/mL, 50.0 ng/mL, 100.0 ng/mL, 200.0 ng/mL, and 500.0 ng/mL for the entire range. If an abbreviated range is to be

used select the appropriate standards and only prepare those necessary. If a reduction in calibration standards is desired a confirmation of method precision and accuracy must be conducted prior to modification in SOP. The calibration standards are prepared in duplicate by spiking 1.00 mL volumes of drug-free control plasma or serum with appropriate volumes of the OZ/DMOZ spiking solutions prepared in Section G.4.b.(2)-(4) followed by extraction. The amounts of spiking solution needed and extraction process is detailed below.

(1) 500 ng/mL OZ,DMOZ Standard: Dispense into a pre-labeled 13-mL screw-cap culture tube, 1.00 mL (using 1,000-µL gas-tight syringe) of drug-free serum or plasma. Spike the serum or plasma with 5.0 µL (using a 10-µL gas-tight syringe) of the 200 µg/mL MED surrogate prepared in Section G.4.b.(1) and 20 µL (using a 25 µL gas-tight syringe) of the 25 µg/mL OZ,DMOZ spike solution prepared in Section G.4.b.(2). Cap and vortex this solution for 10 sec. Using a calibrated pipettor dispense 1 ± 0.05 mL benzene to the contents of the culture tube.

Note: The calibration of the Re-pipettor must be performed daily to ensure accurate delivery of the extraction solvent. Calibration is performed by simply delivering ten stroke volumes to a 10-mL volumetric flask. Adjust stroke volume and repeat as necessary until the bottom of the meniscus is at the calibration mark.

Vortex the mixture 30 sec and set aside until the entire set of standards have been prepared for extraction. The entire calibration standard set and actual samples are extracted for 30 min on a rotating extraction apparatus at about one revolution per second. Following the extraction phase the tubes are removed from the rotating extractor and centrifuged for 30 min at approximately 1,500 x G. Using a 500 µL gas-tight syringe transfer 500 µL of the clear (top) benzene layer to corresponding crimp-cap GC vials and crimp immediately forming a gas-tight seal. Spike each extract with 5.0 µL (using a 10-µL gas-tight syringe) of the 20 µg/mL MID internal standard spiking solution in Section G.4.b.(5). Mix the spiked extracts for 10 sec using a vortex mixer and analyze by GC using the equipment and parameters shown in Section G.3.b. above.

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(2) 200 ng/mL DZ,DMDZ Standard: Dispense into a pre-labeled 13-mL screw-cap culture tube, 1.00 mL (using 1,000-µL gas-tight syringe) of drug-free serum or plasma. Spike the serum or plasma with 5.0 µL (using a 10-µL gas-tight syringe) of the 200 µg/mL MED surrogate prepared in Section G.4.b.(1) and 8.0 µL (using a 25-µL gas-tight syringe) of the 25 µg/mL DZ,DMDZ spike solution prepared in Section G.4.b.(2). Cap and vortex this solution for 10 sec. Using a calibrated pipettor dispense 1.0 ± 0.05 mL of benzene to the contents of the culture tube.

Vortex the mixture 30 sec and set aside until the entire set of standards have been prepared for extraction. The entire calibration standard set and actual samples are extracted for 30 min on a rotating extraction apparatus at about one revolution per second. Following the extraction phase the tubes are removed from the rotating extractor and centrifuged for 30 min at approximately 1,500 x G. Using a 500-µL gas-tight syringe transfer 500 µL of the clear (top) benzene layer to corresponding crimp-cap GC vials and crimp immediately forming a gas-tight seal. Spike each extract with 5.0 µL (using a 10-µL gas-tight syringe) of the 20 µg/mL MID internal standard spiking solution G.4.b.(5). Mix the spiked extracts for 10 sec using a vortex mixer and analyze by GC using the equipment and parameters shown in Section G.3.b. above.

(3) 100 ng/mL DZ,DMDZ Standard: Dispense into a pre-labeled 13-mL screw-cap culture tube, 1.00 mL (using 1,000-µL gas-tight syringe) of drug-free serum or plasma. Spike the serum or plasma with 5.0 µL (using a 10-µL gas-tight syringe) of the 200 µg/mL MED surrogate prepared in Section G.4.b.(1) and 4.0 µL (using a 10-µL gas-tight syringe) of the 25 µg/mL DZ,DMDZ spike solution prepared in Section G.4.b.(2). Cap and vortex this solution for 10 sec. Using a calibrated pipettor dispense 1.0 ± 0.05 mL of benzene to the contents of the culture tube.

Vortex the mixture 30 sec and set aside until the entire set of stanuards have been prepared for extraction. The entire calibration standard set and actual samples are extracted for 30 min on a rotating extraction apparatus at about one revolution per second. Following the extraction phase the tubes are removed from the rotating

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extractor and centrifuged for 30 min at approximately 1,500 x G. Using a 500-µL gas-tight syringe transfer 500 µL of the clear (top) benzene layer to corresponding crimp-cap GC vials and crimp immediately forming a gas-tight seal. Spike each extract with 5.0 µL (using a 10 µL gas-tight syringe) of the 20 µg/mL MID internal standard spiking solution in Section G.4.b.(5). Mix the spiked extracts for 10 sec using a vortex mixer and analyze by GC using the equipment and parameters shown in Section G.3.b. above.

(4) 50 ng/mL DZ,DMDZ Standard: Dispense into a pre-labeled 13-mL screw-cap culture tube, 1.00 mL (using 1,000-µL gas-tight syringe) of drug-free serum or plasma. Spike the serum or plasma with 5.0 µL (using a 10-µL gas-tight syringe) of the 200 µg/mL MED surrogate prepared in Section G.4.b.(1) and 20 µL (using a 25-µL gas-tight syringe) of the 2.5 µg/mL DZ,DMDZ spike solution prepared in Section G.4.b.(3). Cap and vortex this solution for 10 sec. Using a calibrated pipettor dispense 1.0 ± 0.05 mL of benzene to the contents of the culture tube.

Vortex the mixture 30 sec and set aside until the entire set of standards have been prepared for extraction. The entire calibration standard set and actual samples are extracted for 30 min on a rotating extraction apparatus at about one revolution per second. Following the extraction phase the tubes are removed from the rotating extractor and centrifuged for 30 min at approximately 1,500 x G. Using a 500-µL gas-tight syringe transfer 500 µL of the clear (top) benzene layer to corresponding crimp-cap GC vials and crimp immediately forming a gas-tight seal. Spike each extract with 5.0 µL (using a 10-µL gas-tight syringe) of the 20 µg/mL MID internal standard spiking solution in Section G.4.b.(5). Mix the spiked extracts for 10 sec using a vortex mixer and analyze by GC using the equipment and parameters shown in Section G.3.b. above.

(5) 20 ng/mL DZ,DMDZ Standard: Dispense into a pre-labeled 13-mL screw-cap culture tube, 1.00 mL (using 1,000-µL gas-tight syringe) of drug-free serum or plasma. Spike the serum or plasma with 5.0 µL (using a 10 µL gas-tight syringe) of the 200 µg/mL MED surrogate prepared in Section G.4.b.(1) and 8.0 µL (using a 10 µL gas-tight syringe) of the 2.5 µg/mL DZ,DMDZ spike solution prepared

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in Section G.4.b.(3). Cap and vortex this solution for 10 sec. Using a calibrated pipettor dispense 1.0 ± 0.05 mL of benzene to the contents of the culture tube.

Vortex the mixture 30 sec and set aside until the entire set of standards have been prepared for extraction. The entire calibration standard set and actual samples are extracted for 30 min on a rotating extraction apparatus at about one revolution per second. Following the extraction phase the tubes are removed from the rotating extractor and centrifuged for 30 min at approximately 1,500 x G. Using a 500-µL gas-tight syringe transfer 500 µL of the clear (top) benzene layer to corresponding crimp-cap GC vials and crimp immediately forming a gas-tight seal. Spike each extract with 5.0 µL (using a 10-µL gas-tight syringe) of the 20 µg/mL MID internal standard spiking solution in Section G.4.b.(5). Mix the spiked extracts for 10 sec using a vortex mixer and analyze by GC using the equipment and parameters shown in Section G.3.b. above.

(6) 10 ng/mL OZ,DMOZ Standard: Dispense into a pre-labeled 13-mL screw-cap culture tube, 1.00 mL (using 1,000-µL gas-tight syringe) of drug-free serum or plasma. Spike the serum or plasma with 5.0 µL (using a 10-µL gas-tight syringe) of the 200 µg/mL MED surrogate prepared in Section G.4.b.(1) and 4.0 µL (using a 10-µL gas-tight syringe) of the 2.5 µg/mL OZ,DMOZ spike solution prepared in Section G.4.b.(3). Cap and vortex this solution for 10 sec. Using a calibrated pipettor dispense 1.0 ± 0.05 mL of benzene to the contents of the culture tube.

Vortex the mixture 30 sec and set aside until the entire set of standards have been prepared for extraction. The entire calibration standard set and actual samples are extracted for 30 min on a rotating extraction apparatus at about one revolution per second. Following the extraction phase the tubes are removed from the rotating extractor and centrifuged for 30 min at approximately 1,500 x G. Using a 500-µL gas-tight syringe transfer 500 µL of the clear (top) benzene layer to corresponding crimp-cap GC vials and crimp immediately forming a gas-tight seal. Spike each extract with 5.0 µL (using a 10-µL gas-tight syringe) of the 20 µg/mL MID internal standard spiking solution in Section G.4.b.(5). Mix the spiked extracts for 10 sec using a vortex mixer and

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analyze by GC using the equipment and parameters shown in Section G.3.b. above.

(7) 5 ng/mL DZ,DMDZ Standard: Dispense into a pre-labeled 13-mL screw-cap culture tube, 1.00 mL (using 1,000-µL gas-tight syringe) of drug-free serum or plasma. Spike the serum or plasma with 5.0 µL (using a 10-µL gas-tight syringe) of the 200 µg/mL MED surrogate prepared in Section G.4.b.(1) and 20 µL (using a 25-µL gas-tight syringe) of the 0.25 µg/mL DZ,DMDZ spike solution prepared in Section G.4.b.(4). Cap and vortex this solution for 10 sec. Using a calibrated pipettor dispense 1.0 ± 0.05 mL of benzene to the contents of the culture tube.

Vortex the mixture 30 sec and set aside until the entire set of standards have been prepared for extraction. The entire calibration standard set and actual samples are extracted for 30 min on a rotating extraction apparatus at about one revolution per second. Following the extraction phase the tubes are removed from the rotating extractor and centrifuged for 30 min at approximately 1,500 x G. Using a 500-µL gas-tight syringe transfer 500 µL of the clear (top) benzene layer to corresponding crimp-cap GC vials and crimp immediately forming a gas-tight seal. Spike each extract with 5.0 µL (using a 10-µL gas-tight syringe) of the 20 µg/mL MID internal standard spiking solution in Section G.4.b.(5). Mix the spiked extracts for 10 sec using a vortex mixer and analyze by GC using the equipment and parameters shown in Section G.3.b. above.

(8) 2 ng/mL DZ,DMDZ Standard: Dispense into a pre-labeled 13-mL screw-cap culture tube, 1.00 mL (using 1,000-µL gas-tight syringe) of drug-free serum or plasma. Spike the serum or plasma with 5.0 µL (using a 10-µL gas-tight syringe) of the 200 µg/mL MED surrogate prepared in Section G.4.b.(1) and 8.0 µL (using a 10-µL gas-tight syringe) of the 0.25 µg/mL DZ,DMDZ spike solution prepared in Section G.4.b.(4). Cap and vortex this solution for 10 sec. Using a calibrated pipettor dispense 1.0 ± 0.05 mL of benzene to the contents of the culture tube.

Vortex the mixture 30 sec and set aside until the entire set of standards have been prepared for extraction. The

entire calibration standard set and actual samples are extracted for 30 min on a rotating extraction apparatus at about one revolution per second. Following the extraction phase the tubes are removed from the rotating extractor and centrifuged for 30 min at approximately 1,500 x G. Using a 500-µL gas-tight syringe transfer 500 µL of the clear (top) benzene layer to corresponding crimp-cap GC vials and crimp immediately forming a gas-tight seal. Spike each extract with 5.0 µL (using a 10-µL gas-tight syringe) of the 20 µg/mL MID internal standard spiking solution in Section G.4.b.(5). Mix the spiked extracts for 10 sec using a vortex mixer and analyze by GC using the equipment and parameters shown in Section G.3.b. above.

(9) 1 ng/mL DZ, DMDZ Standard: Dispense into a pre-labeled 13-mL screw-cap culture tube, 1.00 mL (using 1000-µL) gas-tight syringe) of drug-free serum or plasma. Spike the serum or plasma with 5.0 µL (using a 10-µL gas-tight syringe) of the 200 µg/mL MED surrogate prepared in Section G.4.b.(1) and 4.0 µL (using a 10 µL gas-tight syringe) of the 0.25 µg/mL DZ,DMDZ spike solution prepared in Section G.4.b.(4). Cap and vortex this solution for 10 sec. Using a calibrated pipettor, dispense 1.0 ± 0.05 mL of benzene to the contents of the culture tube.

Vortex the mixture 30 sec and set aside until the entire set of standards have been prepared for extraction. The entire calibration standard set and actual samples are extracted for 30 min on a rotating extraction apparatus at about one revolution per second. Following the extraction phase, the tubes are removed from the rotating extractor and centrifuged for 30 min at approximately 1,500 x G. Using a 500-µL gas-tight syringe transfer 500-µL gas-tight syringe, transfer 500 µL of the clear (top) benzene layer to corresponding crimp-cap GC vials and crimp immediately forming a gas-tight seal. Spike each extract with 5.0 µL (using a 10-µL gas-tight syringe) of the 20 µL/mL MID internal standard spiking solution in Section G.4.b.(5). Mix the spiked extracts for 10 sec using a vortex mixer and analyze by GC using the equipment and parameters shown in Section g.3.b. above.

(10) Blank Standard Extract: Dispense into a pre-labeled 13-mL screw-cap culture tube, 1.00 mL (using 1,000-µL gas-tight syringe) of drug-free serum or plasma. Spike the serum or plasma with 5.0 µL (using a 10-µL gas-tight syringe) of the 200 µg/mL MED surrogate prepared in Section G.4.b.(1). Cap and vortex this solution for 10 sec. Using a calibrated pipettor dispense 1.0 ± 0.05 mL of benzene to the contents of the culture tube.

Vortex the mixture 30 sec and set aside until the entire set of standards have been prepared for extraction. The entire calibration standard set and actual samples are extracted for 30 min on a rotating extraction apparatus at about one revolution per second. Following the extraction phase the tubes are removed from the rotating extractor and centrifuged for 30 min at approximately 1,500 x G. Using a 500-µL gas-tight syringe transfer 500 µL of the clear (top) benzene layer to corresponding crimp-cap GC vials and crimp immediately forming a gas-tight seal. Spike each extract with 5.0 µL (using a 10-µL gas-tight syringe) of the 20 µg/mL MID internal standard spiking solution in Section G.4.b.(5). Mix the spiked extracts for 10 sec using a vortex mixer and analyze by GC using the equipment and parameters shown in Section G.3.b. above. A blank must be included with each set of analyses.

d. Quality Control Samples:

(1) Control Sample A (200 ng/mL DZ,DMDZ): Into a 50-mL volumetric flask containing approximately 40 mL drug-free control serum or plasma, deliver 400 µL (using a 500-µL gas-tight syringe) of the 25 µg/mL DZ/DMDZ spiking solution prepared in Section G.4.b.(2).

Dilute to volume with drug-free serum or plasma and vortex 60 sec. Using appropriate precision syringes or volumetric pipettes, transfer as many 1.00 mL aliquots to pre-labeled 13-mL screw-cap culture tubes as can be recovered. Cap and store frozen at -70 C until use.

(2) Control Sample B (50 ng/mL DZ,DMDZ): Into a 50-mL volumetric flask containing approximately 40 mL drug-free control serum or plasma, deliver 100 µL (using a 100-µL gas-tight syringe) of the 25 µg/mL DZ/DMDZ spiking solution prepared in Section G.4.b.(2).

Dilute to volume with drug-free serum or plasma and vortex 60 sec. Using appropriate precision syringes or volumetric pipettes, transfer as many 1.00 mL aliquots to pre-labeled 13-mL screw-cap culture tubes as can be recovered. Cap and store frozen at -70 C until use.

5. Storage of Samples and Reagent Solutions: Serum or plasma samples may be stored for up to 60 days prior to extraction provided they are kept frozen at -70 C. Following extraction, the extract may be stored for up to 30 days at -70 C in a freezer.

All stock and standard solutions must be kept at -70 C in a locked freezer when not in use. Standard extracts must be prepared and processed fresh with each sample set. Stock solutions must be prepared fresh every 6 months.

Standard spiking solutions made from the stock solutions must be discarded and remade every 60 days. These solutions must be stored at -70 C in a freezer.

6. Sample Preparation Procedure:

a. The blood samples are collected in the appropriate manner (see study specific SOP's for details) and then centrifuged to facilitate the separation of the aqueous material from the precipitous material in the blood. Using appropriate precision syringes or volumetric pipettes, measure and transfer a convenient quantity of serum or plasma to be analyzed (between 0.5 and 2.0 mL per sample) to a pre-labeled 13-mL glass culture tube. This volume must be accurately measured and recorded for entry into the final calculations. This is necessary to arrive at the final concentration of DZ and DMDZ in the serum or plasma. Total method detection limit may be reduced by extracting larger volumes due to the excellent extraction efficiency of the analytes using this method (typically 100 percent). Smaller volumes may be extracted when sample availability is limited, however, the certification process has only been tested down to 0.5 mL of sample. Once the samples have been measured and placed into the appropriate culture tube it is recommended that they be immediately spiked with 5 µL of the 200 µg/mL MED/MeOH surrogate prepared in Section G.4.b.(1). The surrogate standard is used in this method to track the stability and recovery of the analytes and is best when the standard is used to certify storage as well as extraction and analysis conditions. Cap the culture tube and vortex 10 sec to ensure

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homogenous dissolution of the surrogate. This will result in a 1,000 ng/mL concentration of MED in the final extracts at a theoretical recovery of 100 percent. This concentration of MED is equivalent to that of the analytical standards previously prepared.

The samples are extracted with identical rotating extractor and centrifuge conditions as used for calibration standards. When sample size and equipment permits it is recommended that unknown samples and calibration samples be extracted and handled as a single set.

7. Sample Extraction Procedure:

a. If the samples have been stored frozen they will need to be brought to room temperature and thoroughly mixed prior to extraction. If the samples have not been spiked previously with 5 µL of the 200 µg/mL MED/MeOH surrogate prepared in Section G.4.b.(1) this will need to be performed at this time.

b. Using a Re-pipettor calibrated to deliver 1.00 ± 0.05 mL deliver 1.0 mL of benzene into each sample tube. Cap the culture tube and vortex the mixture for 30 sec. Samples prepared to this step may be set aside until a reasonable number are ready to complete the extraction process.

c. Once a group of samples have been appropriately spiked with surrogate and benzene they can be placed in an appropriate test tube rack and extracted for 30 min on rotating extraction apparatus set at approximately one revolution per second.

d. After the extraction period remove the tubes from the extraction apparatus and centrifuge the mixtures for 30 min at approximately 1,500 x G.

e. Once the extracts have been separated from the samples by centrifugation transfer 500 ± 1 µL (using 500-µL gas-tight syringe) of each sample extract (top layer) to corresponding pre-labeled GC autosampler vials and cap immediately forming a gas-tight seal. Spike each of the extracts with 5 µL of the 20 µg/mL MID internal standard spike solution prepared in Section G.4.b.(5) and vortex each vial 10 sec. This spike level will result in a concentration of 200 ng/mL MID (provided volume of extract is 500 µL) which is equivalent to the concentration in the analytical standards previously

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prepared. Analyze the extracts by GC using the instrument parameters detailed in Section G.3.b.

8. Calibration:

Instrument calibration is performed by making splitless injections of all calibration standards prepared in Section G.4.c, plus the reagent blank. A complete set of calibration standards must be analyzed and detector linearity confirmed prior to analysis of any unknown sample extracts. Once the calibration of the instrument has been checked, the sample extracts are analyzed with at least every sixth sample being a calibration standard or a control sample to check the stability of the instrument. All calibration standards analyzed during the unknown sample analyses are used to develop a complete calibration curve for quantitation of the sample extracts. The only exception to this would be if a calibration sample analysis was flawed due to an instrumental malfunction which would need to be noted and recorded with the analysis form. Unknown sample analyses which result in peak areas outside of the calibrated range must be reported as greater than or less than the closest calibration standard concentration. This method does not support quantitative measurements outside the calibration range.

Any sample response that exceeds the largest calibration standard will be reported as greater than the highest calibration standard, and must be either diluted to within range or the calibration range can be extended for quantification of the sample if it is within the certified calibration range.

9. Analysis of Samples: Samples and calibration standards are analyzed in using the same procedures. At least every sixth analysis must be a calibration standard extract or a quality control samples prepared in Section G.4.d.

10. Calculations:

a. The samples are analyzed using a multiplicative ($y = ax^b$) regression analysis with internal and surrogate standard corrections. The values obtained for a and b parameters, slope and intercept respectively, from the regression of the calibration standards are used to calculate analyte concentrations in the samples.

b. For each calibration standard and sample injection, calculate the corrected peak area ratio (CPAR) by dividing the Analyte

(either OZ or OMOZ) response by the internal standard MID response and then dividing the resulting quotient by the result of the ratio of the peak area response of the surrogate standard MED to the peak area response of internal standard MID divided by the average MED to MID ratio for all the calibration standards.

$CPAR_{OZ}$ = [(OZ/MID)/((MED/MID)/(average MED/MID ratio calculated from calibration standards)]

c. Using a multiplicative regression program, generate the slope, intercept, and correlation coefficient for the OZ and OMOZ CPAR's in the calibration data. The resulting calibration curve will be used to calculate the observed concentrations of OZ and OMOZ in the samples.

d. Enter the corrected peak area as the ordinate (x-value) and the corresponding standard concentration in ng/mL as the abscissa (y-value).

e. Enter each data point obtained from the calibration standards and calculate percent relative standard deviation (%RSD) between replicate standards. Do not include the blank in the calibration calculations as this will weight the regression toward zero.

f. Identify the analyte and internal standard peaks in the sample chromatograms; record the peak area.

g. Perform a regression on the data points obtained, as shown above. Enter the values obtained for CPAR in the samples to obtain the observed concentration. The actual concentration will be equal to the observed concentration when the volume of serum or plasma extracted is 1.00 mL. If the volume extracted differs from 1.00 mL, the volume must be entered into the calculations to obtain the actual concentration by simply dividing the observed concentration by the volume (in mL) extracted.

11. Quality Control: Each step in the extraction and analysis must be done reproducibly to achieve good precision and accuracy. This includes standard spiking technique, preparation of samples, extraction and transfer of samples, and instrument operation. All sample chromatograms must be inspected for proper integration, peak retention time, column performance parameters, and possibility of interferences. If interferences pose a problem, chromatographic peaks must be confirmed by mass spectrometry or

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similar confirmation technique that can resolve the chromatographic interference.

Control charts shall be established for the analysis of the quality control samples A and B prepared in Section G.4.d. using the method certification data. The 95 percent confidence limits are defined as ± two standard deviations about the mean. All subsequent analyses of the controls should fall within these limits. The confidence limits must be re-established whenever fresh control samples are prepared.

12. Certification:

a. Range and Sensitivity: In the certification of this method, the upper limit was defined as 500 ng/mL for both DZ and DMDZ. The range from 1 ng/mL to 500 ng/mL in the sample has been tested for linearity. The working range has been defined for this method as between 1 and 500 ng/mL (or 1 to 500 parts per billion (ppb)) for DZ and between 2 and 500 ng/mL for DMDZ.

The instrumental detection limit, defined as that concentration that can be injected and still result in a signal-to-noise ratio of 5:1, is on the order of 1 ng/mL for each drug in the serum or plasma. Background interferences in drug-free serum or plasma are typically 20 to 30 percent of the response obtained for the 1 ng/mL calibration standard.

b. Precision and Accuracy: To document method precision and accuracy, the entire method, including sample preparation, extraction, concentration, and analysis was performed on 5 separate days with triplicate injections of each of the nine calibration standard solutions and duplicates of extracts from six "blind" samples using the chromatographic conditions and procedures described in this method. Percent RSD between replicate standards as well as blind sample extracts was found to be less than 10 percent at each concentration except at 1 ng/mL where the %RSD occasionally fell outside of 10 percent. Calculated concentrations of the blind samples using the multiplicative regression and calculations described herein resulted in percent relative error of less than 10 percent at each concentration level. A report on the method certification results has been prepared and details this process.

c. Recovery: Absolute recovery of the analytes was determined on two separate days by comparing standard extract responses to responses obtained for the analytes in prepared standards

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using the procedures detailed in this SOP. The recovery was estimated as 95 ± 5 percent over the certification range. There is a close correlation between DZ, DMDZ and the surrogate MED recoveries indicating the validity of using the MED as the surrogate in determining recovery of DZ and DMDZ in samples of unknown concentration. Relative recoveries are determined for each injection of unknowns based comparison of peak area ratios of the surrogate and internal standard in the unknowns to the standard extracts.

d. Interferences: The ECD and NPD are relatively specific detectors that respond to only electronegative, "electron capturing" or nitrogen containing compounds. Although these detectors are specific for a particular class of compounds their does exist a large number of compounds that detectors will respond to. Therefore, any compound which elutes in the chromatographic window of the compcunds of interest might pose interference problems. The chromatographic conditions described above have been found to successfully resolve the DZ and DMDZ from any extractable interferences present in serum or plasma which have been encountered to date. The only interference problem that has been observed throughout the method certification is the trace level of material found in human plasma that co-elutes with DZ. This material does appear to remain relatively small and constant which is readily corrected for by the extraction of the calibration standards using similar plasma. In addition, organic free glassware and caps lined with inert materials such as teflon are required.

13. 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 or weekend shut-down, set the oven temperature to the upper limit of the column in use for 1 to 3 hr (depending on extent of contamination of the column). Be sure that sufficient gases are supplied for continuous flow of carrier and make-up gases for the period of time that the system will be unattended. After the column conditioning period the analytical column can be set at a midrange temperature of the oven program, typically 150 to 200 C. This will help prevent the collection of possible contaminants in the carrier gas on the analytical column.

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c. If the carrier gases are going to be turned off for any period of time all GC temperature zones must be reduced to room temperature prior to the carrier gas being turned off. If the carrier gas has been turned off for an extended period of time the lines must be bleed to remove all air prior to heat up of the GC temperature zones.

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Revised August 31, 1990

STANDARD OPERATING PROCEDURE
MREF SOP-89-63

TITLE: High Performance Liquid Chromatographic Analysis For Diazepam

LABORATORY: MREF SOP APPROVAL DATE: November 3, 1989

PLACE OF OPERATION OR TEST: Throughout the MREF laboratory

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 11/2/89
Signature/Date

Timothy L. Hayes, Research Scientist
Printed Name/Title

Approved By: David W. Hobson 11/2/89
Signature/Date

Garrett S. Dill, D.V.M., Manager
Printed Name/Title

Approved By: Donald W Cagle 11/3/89
Signature/Date

Donald W. Cagle, CIH, Safety/Surety Officer
Printed Name/Title

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Approved By:

Signature/Date 11/3/89

Ramona A. Mayer, Manager, Regulatory Compliance
Printed Name/Title

Approved By:

Signature/Date 11/3/89

Anna D. Barker, Ph.D.
Group Vice President and General Manager
Health and Environment
Printed Name/Title

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SIGNATURES

I have read and understand the contents of MREF SOP-89-63.

Signature	Date	Signature	Date
Linda Adams	11-06-89		
James Blank	11/6/89		
James Clay	11/06/89		
Rebekah A. Harmon	11-10-89		
Carl T. Olson	12-11-89		
Malcolm L. Pearson	12-11-89		
Melissa Myers	12-11-89		
K. Braun	12/11/89		
Raymond L. Cunningham	12-11-89		
Robert C. King	12-11-89		
Pamela L. Cooley	2/12/90		
Susi J. Moore	3/30/90		

STANDARD OPERATING PROCEDURE 89-63

High Performance Liquid Chromatographic Analysis For Diazepam

A. Statement of Work: This SOP describes the method for the quantitative analysis of diazepam in an injectable multi-solvent solution. The prepared sample is analyzed by high performance liquid chromatography (HPLC). The sample preparation and analysis methods detailed here were developed in support of on-going tasks at the MREF.

B. Responsibility:

1. Personnel Qualifications: All technical staff will be familiar with working procedures within the MREF laboratory and the MREF Facility Safety and Surety Plan (FSSP). They must know the requirements of the Buddy System. 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 FSSP are followed.

d. Each 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.

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.

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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, OH 43201-2693.

C. Materials To Be Used:

1. Solvents and Chemicals: Potassium phosphate dibasic (K_2HPO_4), ammonium acetate, acetonitrile, propylene glycol, denatured ethyl alcohol, benzyl alcohol, isopropyl alcohol, methyl alcohol, millipore or distilled water, diazepam (pure crystalline form supplied by Hoffmann-La Roche), and phosphoric acid.

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

E. Hazards Involved:

1. Solvents and Chemicals: The solvents and chemicals used in preparing the dilute material may have hazards associated with their use. A copy of the Material Safety Data Sheets (MSDS) are available in the MREF office files or through Battelle's Safety Office at 505 King Ave.

2. 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 ± 10 lfpm. 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.

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2. Protective Equipment: 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
   safety glasses

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

3. First Aid: The location of the nearest eye-wash 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 magnehelic 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. Area Set Up: An area in Room 46 or another approved room will be used to prepare calibration standards and perform spiking and extraction procedures.

   The hood areas for solvent handling are covered with plastic-backed, absorbent paper. All materials for sample preparation are located in or near the hood area.

3. Equipment Preparation:

   a. Column Check: The integrity of the column needs to be checked before samples are analyzed. This is accomplished by analyzing a column test mix with appropriate conditions and comparing the resulting chromatogram with that of the sample chromatogram. The test mix and sample chromatogram are shipped with each column.

   b. Instrument Preparation: The HPLC is prepared for use with the following recommended initial settings:

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(1) Column - 8 cm x 4 mm inside diameter (I.D.) Zorbax ODS Cartridge Column with 5 μm partial size.

(2) Guard Column - 1.25 cm x 4 mm I.D. Zorbax ODS Cartridge Guard Column with 5 μm partial size.

(3) Mobile Phase: 50 percent buffer/50 percent acetonitrile.

(4) Mobile Phase Flow Rate: 1.0 mL/min.

(5) Injection Loop: 20 μL volume.

(6) Detector Wavelength: 300 nm.

(7) Absorbance Units Full Scale (A.U.F.S.) - 0.02.

c. Column Conditioning: The column needs approximately 30 min of conditioning before it can be used to analyze samples. This conditioning insures that all stationary phase has been "washed" with the mobile phase producing a homogeneous environment.

4. Solution Preparation:

a. Mobile Phase Buffer: Accurately weigh 2.44 ± 0.01 g potassium phosphate dibasic and 15.42 ± 0.01 g ammonium acetate onto weighing paper. Quantitatively transfer these chemicals into a 2-L volumetric flask containing approximately 500-mL millipore water. Dilute to volume with millipore water. Mix well and pH solution to pH 6.8 with a 0.1 M phosphoric acid solution. Filter the resulting solution through a 0.45 μm filter.

Prepare a solution which is approximately 0.1 M H_3PO_4 by dispensing approximately 0.5 mL of H_3PO_4 into a 50-mL beaker containing 10-mL millipore water. Mix well. CAUTION: Process is exothermic.

b. Multisol solvent: The multisol solvent is prepared by dispensing 200-mL propylene glycol, 50-mL denatured alcohol, and 7.5-mL benzyl alcohol into a 500-mL volumetric flask and diluting to volume with millipore water and vortexing to insure complete mixing.

c. Diazepam Stock Solution: The diazepam stock solution is prepared from pure crystalline diazepam supplied by Hoffman-La Roche.

(1) 1.0-mg/mL Diazepam Stock Solution: Accurately weigh 10 ± 0.1 mg of diazepam onto weighing paper. Quantitatively transfer the diazepam into a 10-mL volumetric flask

containing approximately 5-mL Multisol. Mix well using a vortex mixer. Multisol is a viscous liquid and requires a lot of mixing to get diazepam into solution until dissolved. Dilute to volume with millipore water and mix again. Specific density is 1.007 at 25 degrees C.

e. Preparation of Diazepam Analytical Standards:

(1) 0.777-mg/mL Analytical Standard: Aliquot 0.70-mL of the diazepam stock solution into each of two 1.8 mL auto-injection vials and dilute with 0.30-mL of mobile phase. Label the vials with the following information: (1) contents, (2) concentration of analyte (3) date of formulation. Store in the freezer at -20 C until use.

(2) 0.666-mg/mL Analytical Standard: Aliquot 0.60-mL of the diazepam stock solution into each of two 1.8 mL auto-injection vials and dilute with 0.40-mL of mobile phase. Label the vials with the following information: (1) contents, (2) concentration of analyte (3) date of formulation. Store in the freezer at -20 C until use.

(3) 0.555-mg/mL Analytical Standard: Aliquot 0.50-mL of the diazepam stock solution into each of two 1.8 mL auto-injection vials and dilute with 0.50-mL of mobile phase. Label the vials with the following information: (1) contents, (2) concentration of analyte (3) date of formulation. Store in the freezer at -20 C until use.

(4) 0.444-mg/mL Analytical Standard: Aliquot 0.40-mL of the diazepam stock solution into each of two 1.8 mL auto-injection vials and dilute with 0.60-mL of mobile phase. Label the vials with the following information: (1) contents, (2) concentration of analyte (3) date of formulation. Store in the freezer at -20 C until use.

(5) 0.333-mg/mL Analytical Standard: Aliquot 0.30-mL of the diazepam stock solution into each of two 1.8 mL auto-injection vials and dilute with 0.70-mL of mobile phase. Label the vials with the following information: (1) contents, (2) concentration of analyte (3) date of formulation. Store in the freezer at -20 C until use.

(6) 0.000-mg/mL Analytical Standard: Aliquot 0.10-mL of the multisol stock solution into each of two 1.8 mL auto-injection vials and dilute with 0.90-mL of mobile phase. Label the vials with the following information: (1)

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contents, (2) concentration of analyte (3) date of formulation. Store in the freezer at -20 C until use.

5. Collection and Storage of Samples:

   a. Samples are collected in 2-mL GC vials treated with hexamethyldisiloxane (HMDS) to prevent reaction with active sites in the glass. Diazepam samples generated this way can be stored in the Revco freezer at -70 C for up to 60 days until analyzed.

6. Sample Preparation: The samples are diluted to a concentration within the calibration range of the instrument before analysis. The same dilution procedures are used to dilute the samples as were used to prepare the calibration standards. Aliquot 0.10-mL of the diazepam sample into each of two 1.8 mL auto-injection vials and dilute with 0.90-mL of mobile phase. Label the vials with the following information: (1) contents, (2) concentration of analyte (3) date of formulation. Store in the freezer at -20 C until use.

7. Calibration: Instrument calibration is performed when quantitation of samples is required by injecting 20 µL each of analytical standard prepared in Section G.4 using an autosampler. A complete set of calibration standards is analyzed prior to analysis of any sample. Once the calibration of the instrument has been checked, the samples are analyzed with at least every sixth sample being a calibration standard to check the calibration of the instrument. A complete set of calibration standards is analyzed following the last sample. All calibration 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 calibration 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 calibration standard will be reported as greater than the highest calibration standard, and must be either diluted to within range or the calibration range extended for quantification of the sample.

8. Analysis of Samples: Samples and calibration standards are analyzed using the sample procedures. At least every sixth analysis should be a standard.

9. Calculations:

   a. The samples are analyzed using a regression analysis with internal standards.

   b. Using a linear regression program, generate the slope, intercept, and correlation coefficient for diazepam in the calibration data.

c. Enter the peak area of diazepam as the ordinate (x-value) and the corresponding standard concentration as the abscissa (y-value).

d. Enter each data point obtained from the calibration standards and calculate percent relative standard deviation (% RSD) between replicate standards. Do not include the blank in the calibration calculations as this will weigh the regression toward zero.

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

$$b = \frac{[(\Sigma y)(\Sigma x^2)-(\Sigma x)(\Sigma xy)]}{[n(\Sigma x^2)-(\Sigma x)^2]}$$

$$a = \frac{[n(\Sigma xy)-(\Sigma x)(\Sigma y)]}{[n(\Sigma x^2)-(\Sigma x)^2]}$$

$$r = \frac{[n(\Sigma xy)-(\Sigma x)(\Sigma y)]}{[(n(\Sigma x^2)-(\Sigma x)^2)^{1/2}(n\Sigma(y^2)-(\Sigma y)^2)^{1/2}]}$$

where: $y = ax + b$
a = slope
b = y-intercept
r = correlation coefficient
x = peak area (diazepam)
y = concentration of agent in mg/mL
n = number of replicates

f. Identify the analyte peak in the sample chromatograms; record the peak area. Using the regression values calculated from the calibration data, calculate the found concentration for each sample using the formula above.

10. Column Clean-up: After each analysis day, the column needs to be flushed with a mixture of acetonitrile, methanol and water. Flush the column with 33:33:34 mixture of ACN/MeOH/H_2O for approximately 30 min with a flow rate of 2-mL per min.

11. 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.

TLH/cah

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APPENDIX C
INDIVIDUAL ANIMAL PATHOLOGY DATA

Animal No. 79B Necropsy Date: 10/24/89 Group 1

Dose: 12.8 μg/Kg GD

Necropsy Results:
No gross lesions found.

Histopathology Results:
Not applicable.

APPENDIX C
INDIVIDUAL ANIMAL PATHOLOGY DATA

Animal No. 6EA Necropsy Date: 10/25/89 Group 1

Dose: 9.3 µg/Kg GD

Necropsy Results:
No gross lesions found.

Histopathology Results:
Not applicable.

APPENDIX C
INDIVIDUAL ANIMAL PATHOLOGY DATA

Animal No. 6PF Necropsy Date: 10/31/89 Group 1

Dose: 7.4 µg/Kg GD

Necropsy Results:
Lung, right apical - Bronchiectasia, focal, minimal.
Comment: Typical of lung mite-induced change.

Histopathology Results:
Not applicable.

APPENDIX C
INDIVIDUAL ANIMAL PATHOLOGY DATA

Animal No. 6EM Necropsy Date: 10/31/89 Group 1

Dose: 6.4 µg/Kg GD

Necropsy Results:
No gross lesions found.
Whipworms noted in cecum.

Histopathology Results:
Not applicable.

APPENDIX C
INDIVIDUAL ANIMAL PATHOLOGY DATA

Animal No. 6PC Necropsy Date: 2/22/90 Group 2

Dose: 74 μg/Kg GD plus pyridostigmine, atropine, 2-PAM, diazepam

Necropsy Results (perfusion-fixation performed):
- Ileum - hemorrhage
- Lungs - nodules, 2 x 2 x 2 mm, white/yellow
- Lungs - adhesions, rare (various lobes to diaphragm)
- Heart, right ventricle - focus, dark, 6 x 4 mm (found at trim)

Histopathology Results:
- Eye - no significant lesion (nsl)
- Brain - nsl
- Pituitary - no section present
- Spinal Cord - nsl
- Sciatic Nerve - nsl
- Brachial Plexus - nsl
- Ulnar Nerve - nsl
- Radial Nerve - nsl
- Phrenic Nerve - nsl
- Adrenal Glands - nsl
- Liver - nsl
- Kidneys - nsl
- Lung - Pneumonia, interstitial, lymphocytic, multifocal, minimal
  (corresponds to gross lung nodule; mite pigment noted)
  (significant pleural adhesions not present microscopically)
- Ileum - hemorrhage, acute, submucosal, moderate
  (corresponds to gross lesion)
- Stomach - nsl
- Diaphragm - nsl
- Biceps Muscle - nsl
- Common Digital Extensor Muscle - nsl
- Heart - hemorrhage, acute, subendocardial, mild (L. ventricular papillary muscle)
  - Degeneration, myocyte, subacute, multifocal, minimal

  Comment: Gross "dark focus" was congested coronary vein.

APPENDIX C
INDIVIDUAL ANIMAL PATHOLOGY DATA

Animal No. 6F8 Necropsy Date: 11/7/89 Group 2

Dose: 37 µg/Kg GD plus pyridostigmine, atropine, 2-PAM

Necropsy Results:
No gross lesions found.
Whipworm found in cecum.

Histopathology Results:
Not applicable.

APPENDIX C
INDIVIDUAL ANIMAL PATHOLOGY DATA

Animal No. 6MG Necropsy Date: 12/18/89 Group 2

Dose: 74 μg/Kg GD plus pyridostigmine, atropine, 2-PAM

Necropsy Results:
Kidneys, bilateral, cortex - pale

Histopathology Results:
Eye - no significant lesion (nsl)
Brain - neuronal necrosis, minimal to moderate
(cortex, amygdaloid, caudate, hippocampus, thalamus, midbrain, pons, medulla)
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, moderate, diffuse
Kidneys - nsl
Lung - nsl
Ileum - nsl
Stomach - nsl
Diaphragm - nsl
Biceps Muscle - nsl
Common Digital Extensor Muscle - nsl
Heart - nsl

APPENDIX C
INDIVIDUAL ANIMAL PATHOLOGY DATA

Animal No. 6R6 Necropsy Date: 1/10/90 Group 2

Dose: 74 µg/Kg GD plus pyridostigmine, atropine, 2-PAM, diazepam

Necropsy Results:
Ileum - distal intussusception (into cecum).
Comment: Interpreted to be terminal (agonal) event.
Whipworm noted in cecum.

Histopathology Results:
Not applicable.

APPENDIX C
INDIVIDUAL ANIMAL PATHOLOGY DATA

Animal No. 5VA Necropsy Date: 1/16/90 Group 2

Dose: 74 μg/Kg GD plus pyridostigmine, atropine, 2-PAM, diazepam

Necropsy Results:
Lungs - discoloration, red/purple, multifocal
- edema, diffuse, mild
Note: Trachea, bronchi plugged with white froth

Histopathology Results:
Not applicable.

APPENDIX C
INDIVIDUAL ANIMAL PATHOLOGY DATA

Animal No. GRX Necropsy Date: 2/6/90 Group 2

Dose: 74 µg/Kg GD plus pyridostigmine, atropine, 2-PAM, diazepam

Necropsy Results:
No gross lesions found.

Histopathology Results:
Not applicable.

APPENDIX C
INDIVIDUAL ANIMAL PATHOLOGY DATA

Animal No. 6EY Necropsy Date: 2/7/90 Group 2

Dose: 74 µg/Kg GD plus pyridostigmine, atropine, 2-PAM, diazepam

Necropsy Results:
No gross lesions found.

Histopathology Results:
Not applicable.

APPENDIX C
INDIVIDUAL ANIMAL PATHOLOGY DATA

Animal No. 6V5 Necropsy Date: 2/9/90 Group 2

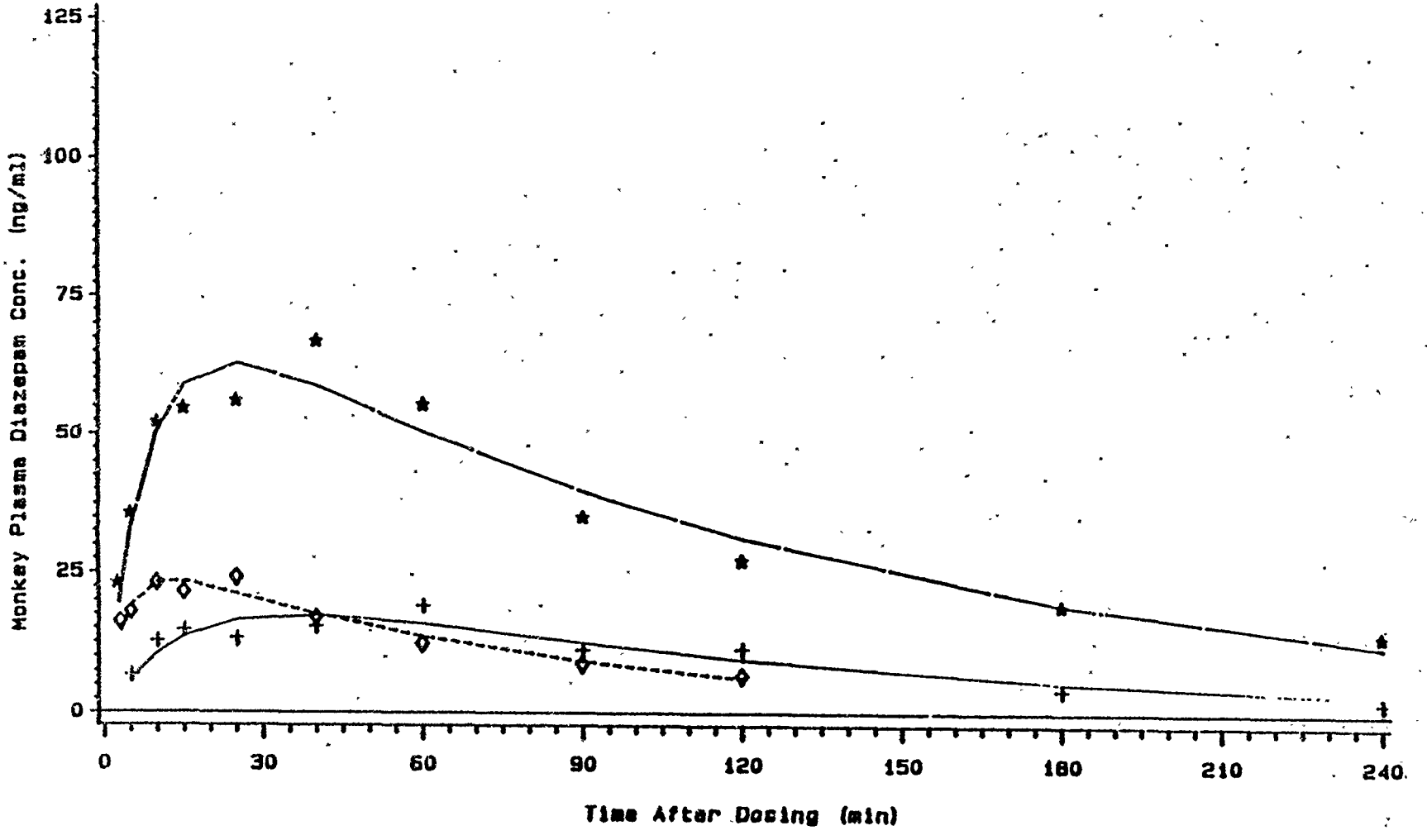
Dose: 74 µg/Kg GD plus pyridostigmine, atropine, 2-PAM, diazepam

Necropsy Results:
Ileum; serosa - hemorrhage, moderate

Histopathology Results:
Eye - no significant lesion (nsl)
Brain - nsl
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 - Fatty change, minimal
Kidneys - Pigment, intraepithelial, mild (origin undetermined)
Lung - Pneumonia, acute to subacute, mild, multifocal
Ileum - nsl (gross: hemorrhage - not confirmed)
Stomach - nsl
Diaphragm - nsl
Biceps Muscle - nsl
Common Digital Extensor Muscle - nsl
Heart - nsl (vacuolar change/artifact noted)

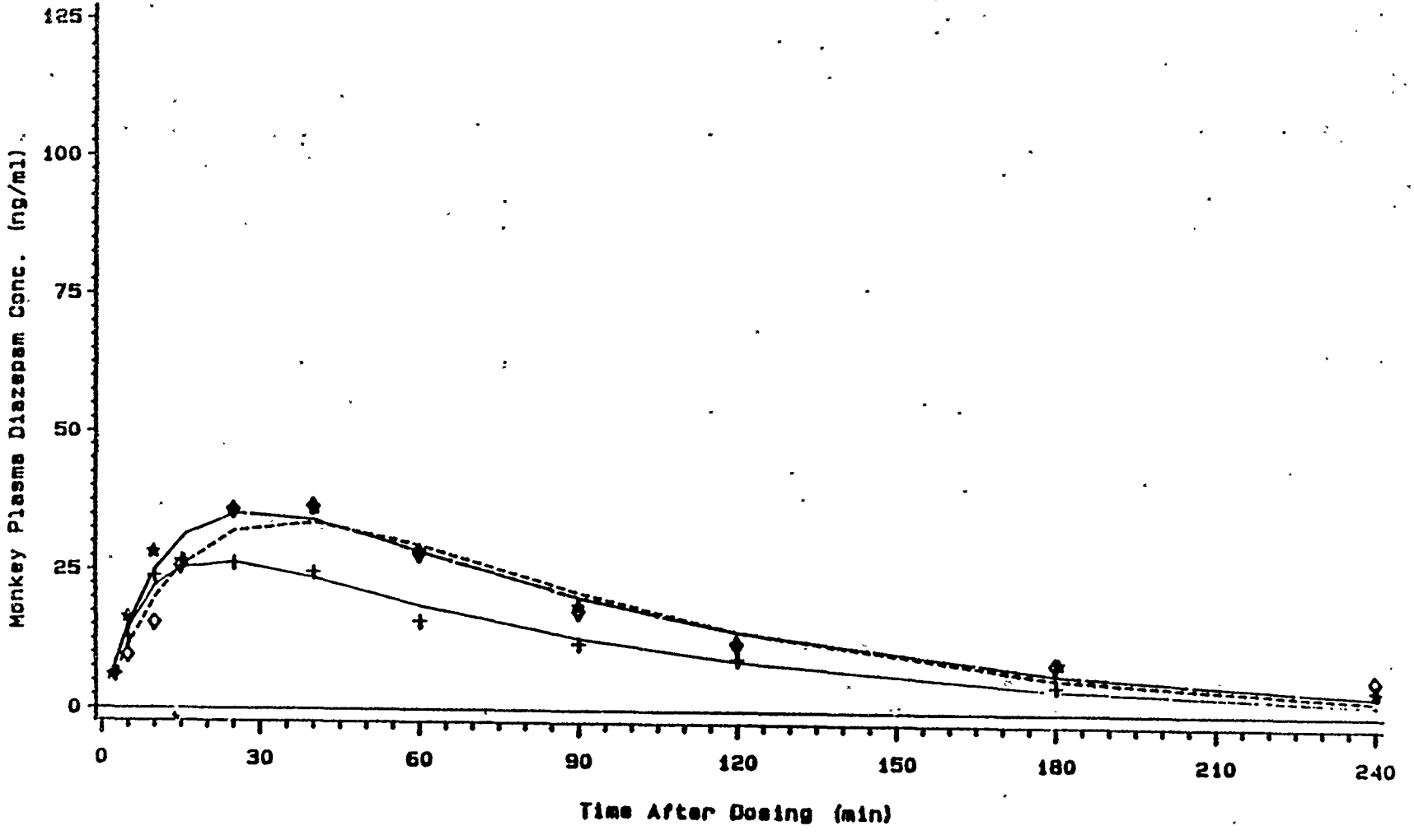
APPENDIX D

PHARMACOKINETICS OF DIAZEPAM ADMINISTERED AT APPROXIMATELY 70 ug/kg (+),
110 ug/kg (◊), and 220 ug/kg (★)
ANIMAL-71M

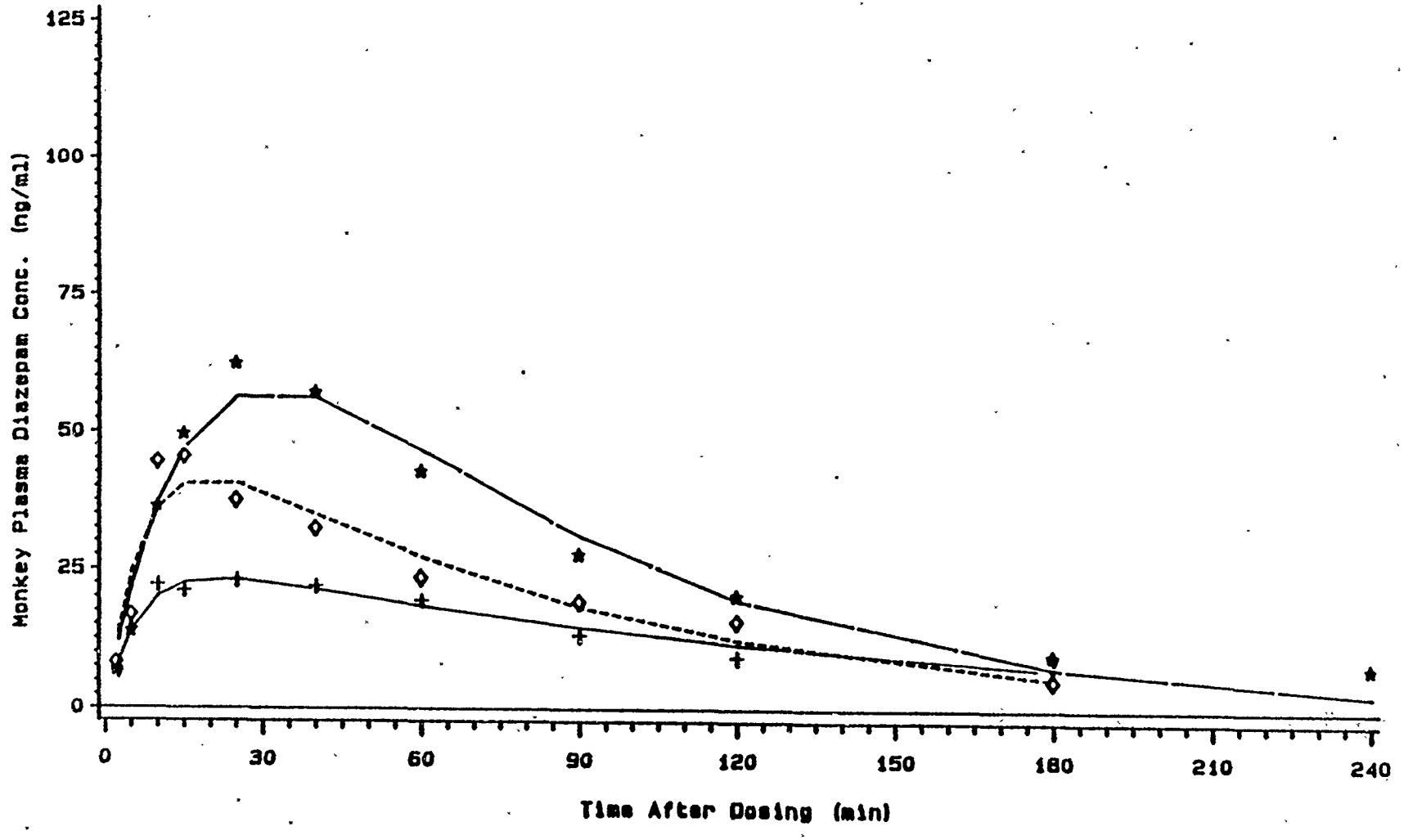


PHARMACOKINETICS OF DIAZEPAM ADMINISTERED AT APPROXIMATELY 70 ug/kg (+), 110 ug/kg (◇), and 220 ug/kg (★)

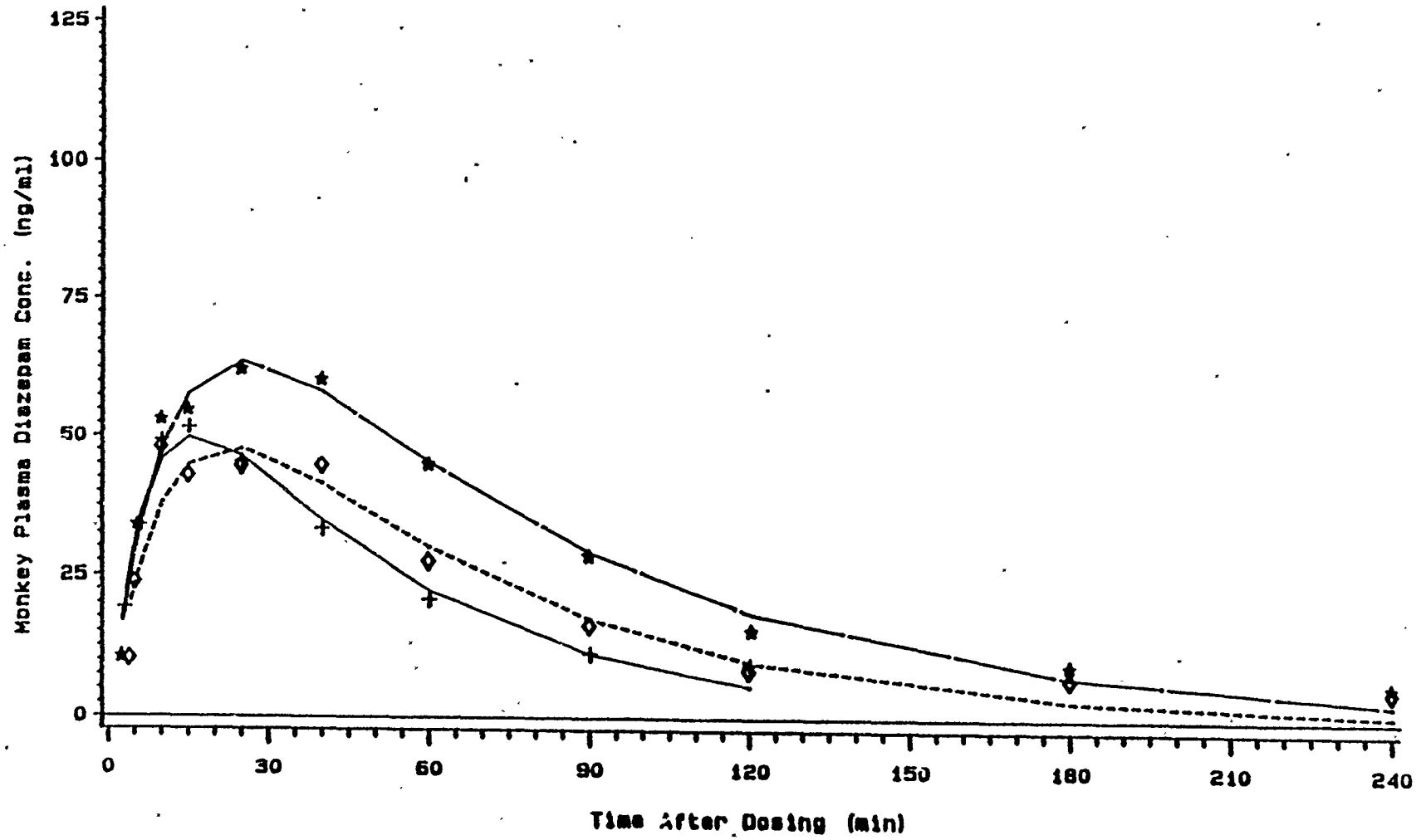
ANIMAL-6R1



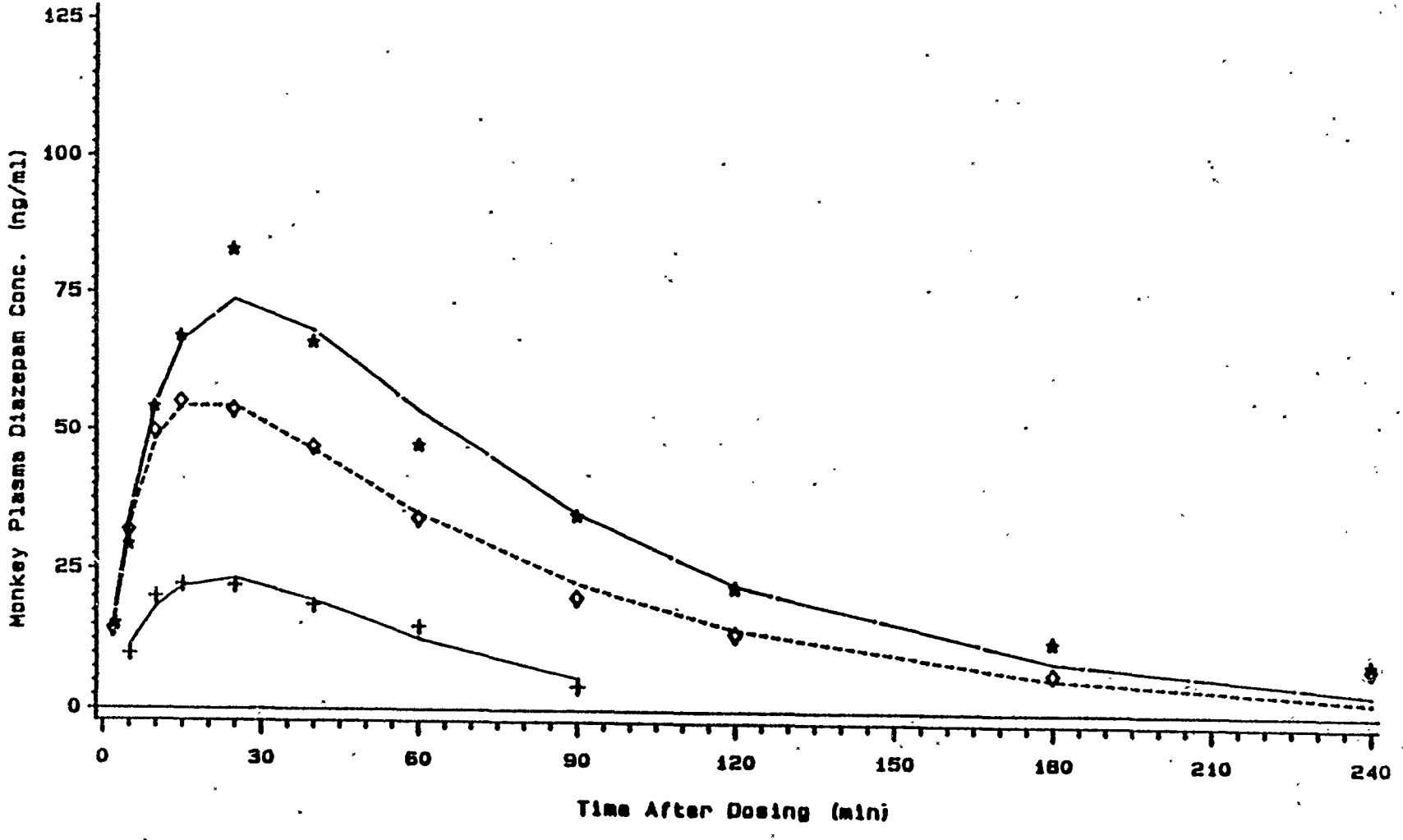
PHARMACOKINETICS OF DIAZEPAM ADMINISTERED AT APPROXIMATELY 70 ug/kg (+), 110 ug/kg (◊), and 220 ug/kg (★)
ANIMAL-6BJ



PHARMACOKINETICS OF DIAZEPAM ADMINISTERED AT APPROXIMATELY 70 ug/kg (+), 110 ug/kg (◇), and 220 ug/kg (★)
ANIMAL-6AS

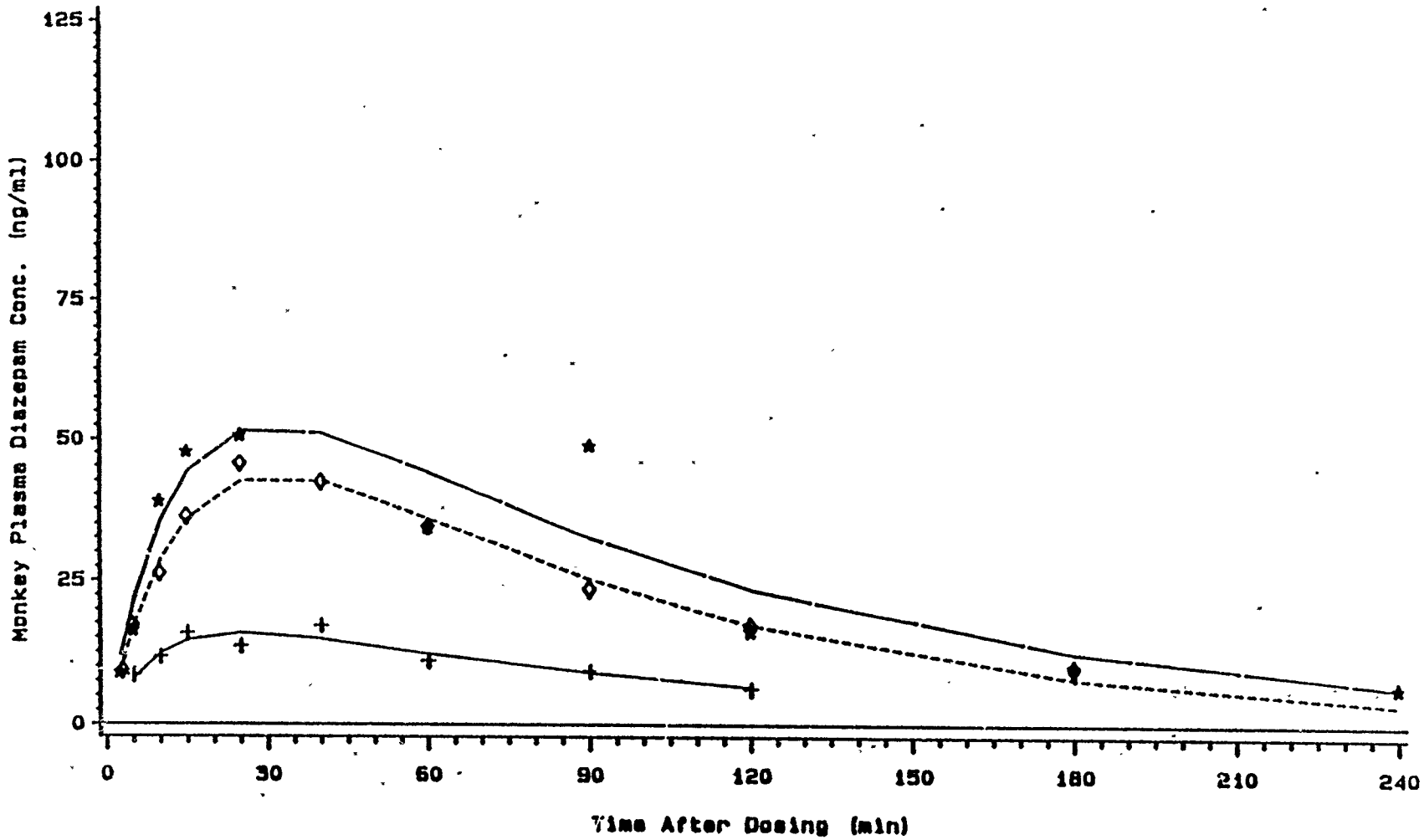


PHARMACOKINETICS OF DIAZEPAM ADMINISTERED AT APPROXIMATELY 70 ug/kg (+),
110 ug/kg (◊), and 220 ug/kg (★)
ANIMAL-6AR

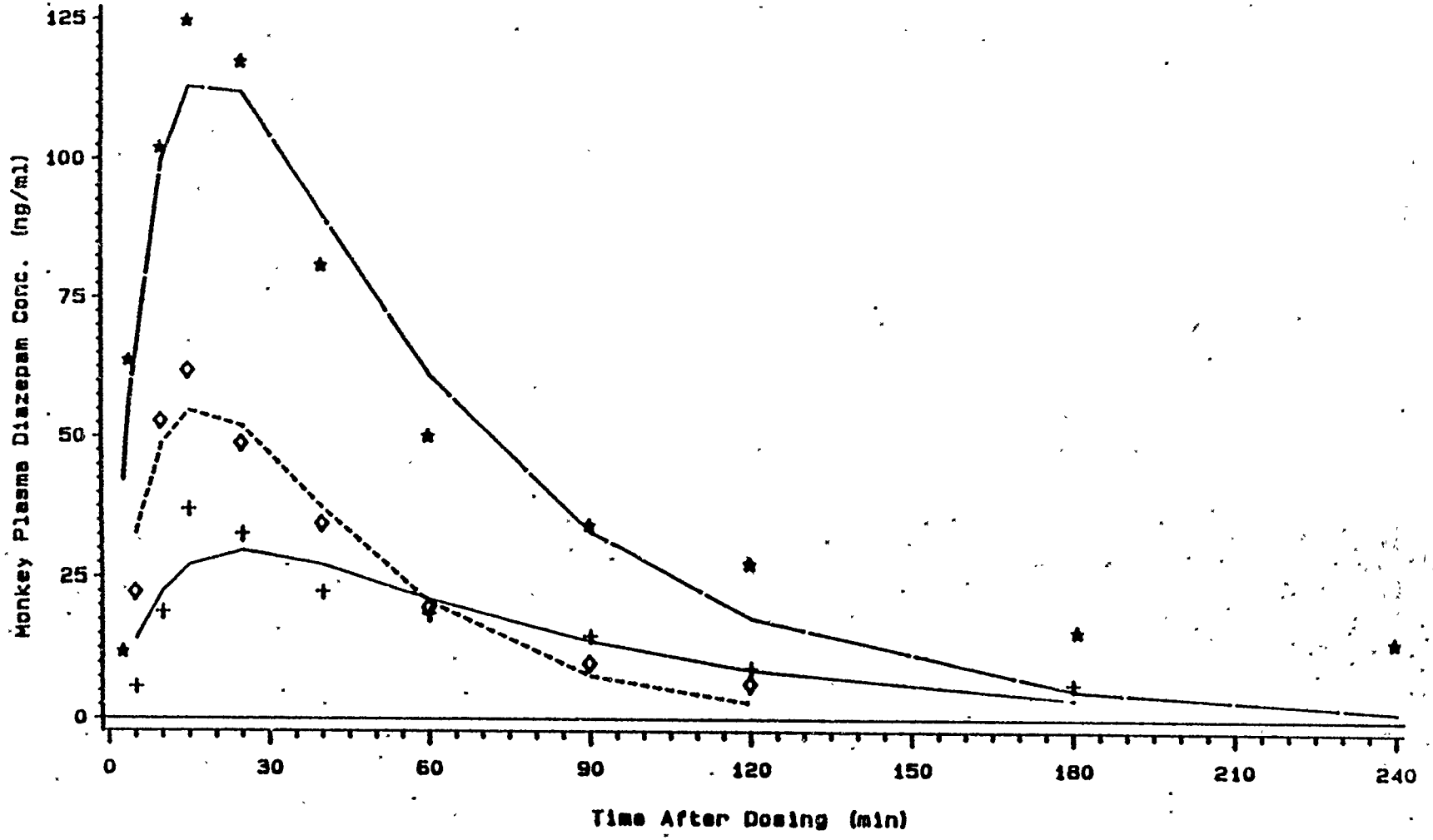


PHARMACOKINETICS OF DIAZEPAM ADMINISTERED AT APPROXIMATELY 70 ug/kg (+), 110 ug/kg (◊), and 220 ug/kg (★)

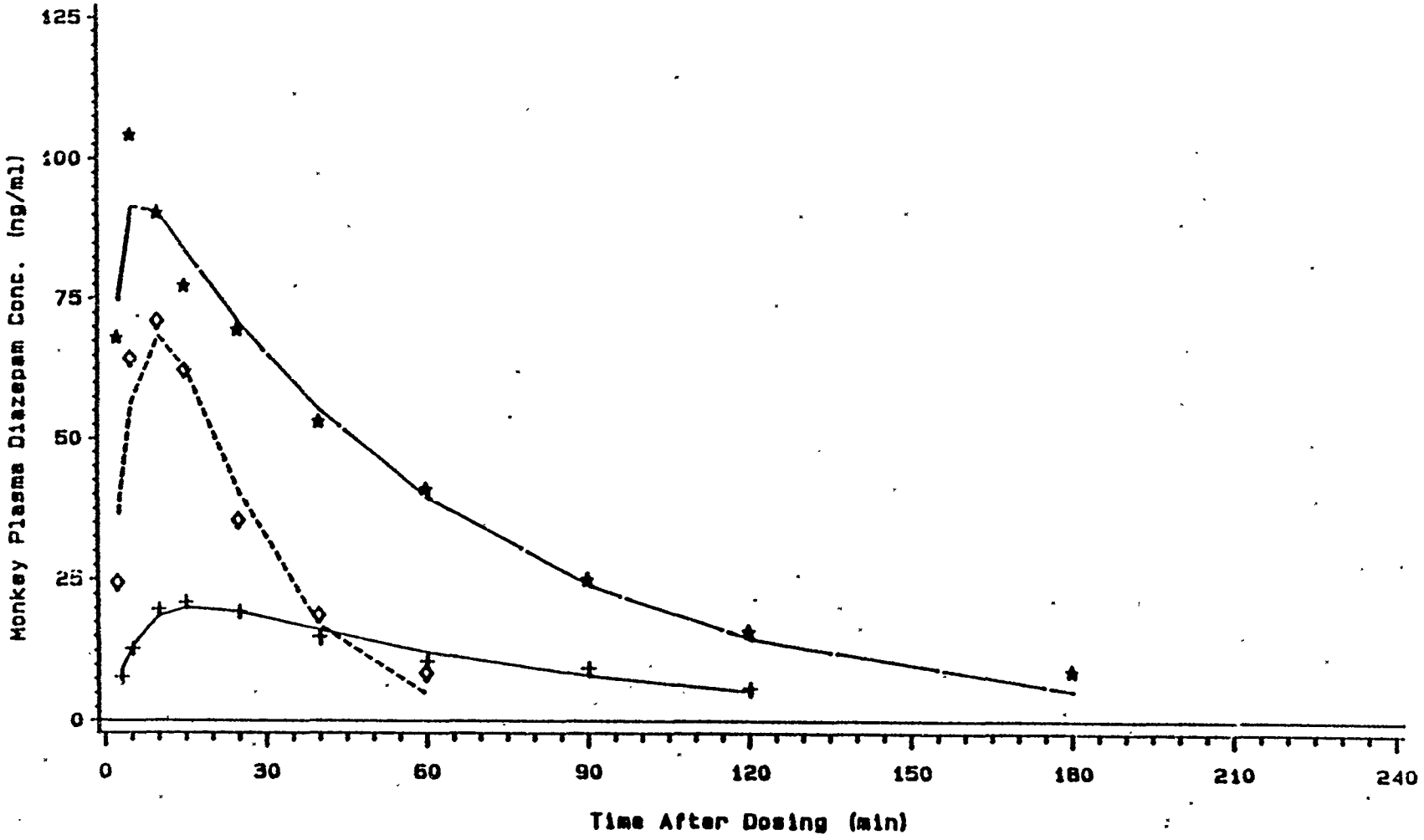
ANIMAL-68W



PHARMACOKINETICS OF DIAZEPAM ADMINISTERED AT APPROXIMATELY 70 ug/kg (+), 110 ug/kg (◊), and 220 ug/kg (★)
ANIMAL-61P

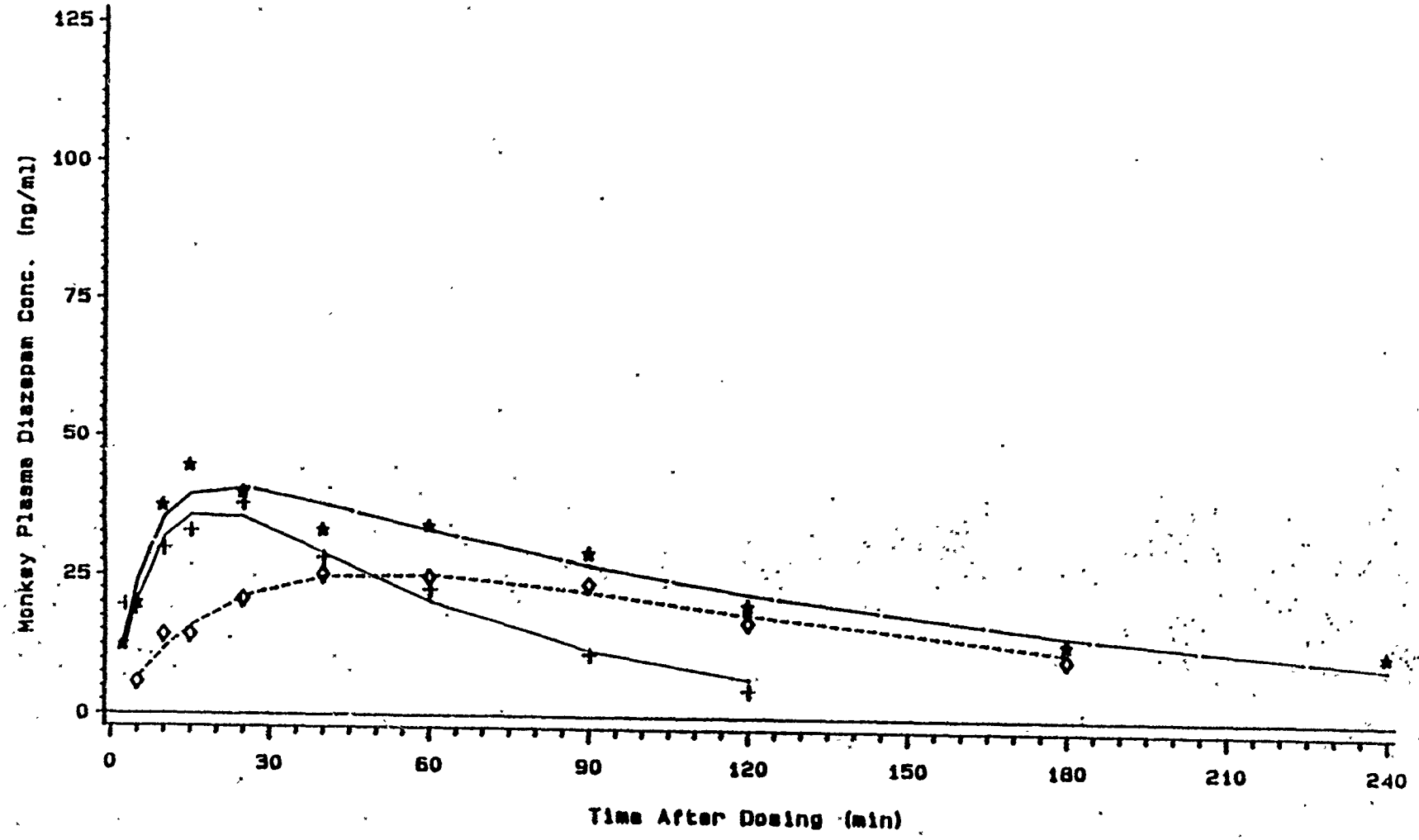


PHARMACOKINETICS OF DIAZEPAM ADMINISTERED AT APPROXIMATELY 70 ug/kg (+),
110 ug/kg (◊), and 220 ug/kg (★)
ANIMAL-5MF



PHARMACOKINETICS OF DIAZEPAM ADMINISTERED AT APPROXIMATELY 70 ug/kg (+), 110 ug/kg (◊), and 220 ug/kg (*)

ANIMAL-5V7



LISTING OF COMPUTER PROGRAM FOR PHARMACOKINETIC MODELING

```
LIBNAME LOW '[TSNIDER.08.LOW]';
OPTIONS LS=80;

*************************************************************;
* Program Name:  Singlecm.SAS    Written by:  Tom Snider    *;
*                                                           *;
* Program fits a single compartmental model to a single     *;
* animal, calculates several pharmacokinetic parameters,    *;
* and stores the results in a SAS database.                 *;
*************************************************************;

* Get the low diazepam dose results for Animal 5V7 ;
DATA TRUNC;
  SET LOW.ALLDATA;
  IF ANIMAL='5V7' AND CONC NE 0;

* Compute the maximum time for the diazepam concentration determinations;
PROC MEANS NOPRINT DATA=TRUNC;
  VAR TIME;
  ID ANIMAL;
  OUTPUT OUT=MAX MAX=MAXT;
DATA MAX2;
  SET MAX;
   TYPE_='FINAL';
PROC SORT;
  BY _TYPE_;

* Fit the Single Compartment Model;
PROC NLIN DATA=TRUNC CONVERGE=1E-6 METHOD=MARQUARDT OUTEST=ESTIM;
* Starting values for Ka, Ke, and Vd are calculated means from a
  Symphony program;
  PARMS KA=0.1488
        KE=0.0156
        V=1925;
  D=79000;
  T=TIME;
  EXPA=EXP(-KA*T);
  EXPE=EXP(-KE*T);
  KD=KA-KE;

* Single Compartment model being fitted to the data;
  MODEL CONC=(D/V)*(KA/KD)*(EXPE-EXPA);

* Compute the derivatives for Ka, Ke, and Vd;
  DER.KA=D/(V*KD)*((1-KA/KD)*(EXPE-EXPA)+KA*T*EXPA);
  DER.KE=D*KA/(V*KD)*((EXPE-EXPA)/KD-T*EXPE);
  DER.V=-(D*KA/KD)*(EXPE-EXPA)/(V*V);

  TITLE 'SINGLE-COMPARTMENT PHARMACOKINETIC MODEL WITHOUT IV DOSE';
  TITLE2 'TASK 89-08 PLASMA DIAZEPAM, LOW DOSE - ANIMAL 5V7';
  OUTPUT OUT=LOW.P5V7 P=CONCHAT L95M=LCL U95M=UCL;
```

```
PROC SORT DATA=ESTIM;
  BY _TYPE_;
PROC PRINT DATA=LOW.P5V7;

* Get the values of the estimated parameters, and merge the
  estimated parameter values with the time of the last measured diazepam
  concentration.  Several pharmacokinetic parameters are computed in
  this data step;
DATA EST;
  SET ESTIM;
  IF _TYPE_='FINAL';
DATA LOW.AN5V7;
  MERGE EST MAX2;
  BY _TYPE_;
  LABEL D_ug_kg = 'Dose in ug/kg'
        V_l_kg  = 'Apparent Volume in L/kg'
        KA      = 'Absorption rate constant'
        KE      = 'Elimination rate constant'
        CALCAUC = 'TOTAL AUC from MODEL'
        AUC_D   = 'Total AUC divided by Dose'
        TKA     = 'Absorption phase half life'
        TKE     = 'Elimination phase half life'
        TMAX    = 'Time to Cmax'
        CMAX    = 'Maximum Concentration'
        CMAX_D  = 'Cmax divided by dose'
        CL      = 'Clearance' ;
  KD=KA-KE;
  D=79000;
  V_l_kg=V/1000;
  D_ug_kg=D/1000;

* Calculate Areas under the Curve from the modeled data using the
  trapezoid method for interim times;
  DX=0.5;
  X=0;
  SUMY=0;
  DO UNTIL(X GE 480);
  * ALTERNATIVELY, THE ABOVE STATEMENT COULD READ DO UNTIL (X GE MAXT);
    Y=(D/V)*(KA/KD)*(EXP(-KE*X)-EXP(-KA*X))*DX;
    X=X+DX;
    SUMY=SUMY+Y;
    PART=Y/SUMY;
    IF X=2.5 THEN SUMY2_5=SUMY;
       ELSE IF X=5 THEN SUMY5=SUMY;
       ELSE IF X=10 THEN SUMY10=SUMY;
       ELSE IF X=15 THEN SUMY15=SUMY;
       ELSE IF X=25 THEN SUMY25=SUMY;
       ELSE IF X=40 THEN SUMY40=SUMY;
       ELSE IF X=60 THEN SUMY60=SUMY;
       ELSE IF X=90 THEN SUMY90=SUMY;
       ELSE IF X=120 THEN SUMY120=SUMY;
       ELSE IF X=180 THEN SUMY180=SUMY;
       ELSE IF X=240 THEN SUMY240=SUMY;
       ELSE IF X=480 THEN SUMY480=SUMY;
```

```
      END;* end of until statement;
      INTAUC=SUMY;

      * Calculate other pharmacokinetic parameters from Dose-D, Ka, Ke, and
Vd;
      CALCAUC=D/(V*KE);
      TKA=LOG(2)/KA;
      TKE=LOG(2)/KE;
      TMAX=LOG(KA/KE)/(KA-KE);
      CMAX=(D/V)*(KA/KD)*(EXP(-KE*TMAX)-EXP(-KA*TMAX));
      CMAX_D=CMAX/D_UG_KG;
      CL=D/CALCAUC;
      AUC_D=CALCAUC/D_UG_KG;
      DROP _TYPE_ _NAME_ _ITER_ SUMY D V KD X;

    PROC PRINT;
      TITLE1 'SINGLE-COMPARTMENT MODEL WITHOUT IV DOSE';
      TITLE2 'PARAMETERS FOR TASK 89-08 DIAZEPAM LOW DOSE: ANIMAL 5V7';
      VAR _SSE_--PART;
    PROC PRINT;
      VAR SUMY2_5--SUMY480;
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APPENDIX D. PLASMA DESMETHYLDIAZEPAM CONCENTRATION (ng/mL) IN INDIVIDUAL ANIMALS FOLLOWING THREE DOSE LEVELS OF DIAZEPAM

Time (min)	Animal No. / Dose (μg/kg) / Body Weight (kg)	6AS 67 2.8	6R1 71 3.4	5V7 79 4.3	61P 71 3.2	68W 74 3.9	5WF 71 4.1	6AR 72 3.4	71M 75 3.8	6BJ 71 4.8	Mean	STD
-10		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
2.5		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
5		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
10		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
15		0.0	0.0	0.0	0.0	0.0	6.7	0.0	0.0	0.0	0.74	2.23
25		10.8	0.0	0.0	0.0	0.0	6.3	0.0	3.4	0.0	2.29	3.90
40		20.8	8.0	0.0	6.9	0.0	12.5	9.5	6.4	11.8	8.42	6.42
60		25.0	11.8	11.9	9.5	0.0	15.2	13.6	17.4	12.0	12.94	6.66
90		27.0	16.7	17.5	10.9	10.0	25.2	22.2	4.2	15.7	16.60	7.47
120		23.9	12.4	20.1	11.5	11.7	21.7	16.0	6.0	26.5	16.65	6.80
180		25.5	14.4	12.3	9.2	11.6	14.9	12.5	8.0	24.8	14.78	6.26
240		20.7	17.2	9.3	12.5	8.4	15.8	15.3	7.1	0.0	11.79	6.28
480		15.5	10.2	0.0	0.0	0.0	11.7	13.0	0.0	0.0	5.60	6.78
1,440		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00

APPENDIX D.
(Continued)

Time (min)	Animal No. / Dose (μg/kg) / Body Weight (kg)	6AS* 112 3.0	6R1 111 3.4	5V7 112 4.3	61P 110 3.3	68W 110 3.4	5WF 114 4.0	6AR 109 3.3	71M 111 3.8	6BJ 111 4.9	Mean	STD
-10			0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
2.5			0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
5			0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
10			0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
15			0.0	0.0	0.0	0.0	11.2	0.0	0.0	0.0	1.41	3.98
25			0.0	0.0	0.0	0.0	22.6	11.5	0.0	0.0	4.27	8.45
40			0.0	0.0	4.1	10.8	27.7	24.6	0.0	18.4	10.69	11.51
60			14.3	8.9	11.3	15.9	26.9	35.8	10.5	25.8	18.67	9.67
90			13.6	18.6	14.9	21.9	25.7	37.3	14.0	28.2	21.89	8.41
120			13.5	18.6	14.4	26.6	25.4	37.9	17.2	34.9	23.57	9.24
180			13.9	20.7	13.8	27.8	17.7	39.3	14.6	29.7	22.18	9.24
240			13.1	28.3	12.8	25.8	16.3	36.1	0.0	33.2	20.71	12.22
480			0.0	22.5	8.2	24.1	0.0	24.7	0.0	21.3	12.60	11.63
1,440			0.0	6.1	0.0	0.0	0.0	0.0	0.0	0.0	0.77	2.16

APPENDIX D.
(Continued)

Time (min)	Animal No. / Dose (μg/kg) / Body Weight (kg)	6AS 224 3.0	6R1 221 3.3	5V7 224 4.3	61P 222 2.8	68W 225 3.7	5WF 229 3.8	6AR 220 3.3	71M 223 3.5	6BJ 221 4.6	Mean	STD
-10		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
2.5		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
5		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
10		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
15		0.0	0.0	0.0	0.0	0.0	16.9	0.0	0.0	0.0	1.88	5.65
25		7.8	0.0	5.4	14.6	10.2	28.1	12.3	11.7	13.6	11.52	7.70
40		20.5	8.7	20.4	29.8	**	45.0	25.4	20.1	19.8	23.69	10.48
60		29.2	14.1	20.7	38.7	27.8	64.4	36.8	33.3	30.0	32.78	14.11
90		41.8	17.4	28.7	49.5	20.1	76.7	50.3	34.1	40.4	39.89	18.08
120		46.1	30.5	30.8	51.3	41.8	73.0	50.9	42.6	40.5	45.27	12.78
180		53.2	32.5	27.3	48.9	33.2	74.3	49.6	57.2	36.7	45.89	14.92
240		44.8	30.1	26.3	42.6	31.3	64.1	45.8	53.7	33.7	41.36	12.32
480		30.9	24.3	17.3	17.5	21.8	33.5	33.1	42.3	23.8	27.16	8.35
1,440		0.0	29.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.25	9.75

*Unresolvable desmethyldiazepam peaks due to interference on gas chromatogram.
**Loss of plasma sample due to breakage of tube during centrifugation.