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Derivation of an Effective Concentration for 50% of the Population (ECt_{50}) for Whole Blood Acetylcholinesterase Inhibition by VX in the New Zealand White Rabbit

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This study established the effective concentration of *O*-ethyl-*S*-(2-diisopropylaminoethyl) methyl phosphonothiolate (VX) aerosol required to produce mild, moderate, and severe inhibition of whole blood acetylcholinesterase enzyme (WB-AChE) in the New Zealand white rabbit. A method was established by which an estimate for inhalation toxicity may be derived from known intravenous toxicity data for the rabbit. The calculated effective concentrations for 50% of the population for each severity level were as follows: mild (21–50% inhibition), 6.6 mg min/m^3 (95% confidence interval [CI], 4.3–10.1); moderate (51–80% inhibition), 12.2 mg min/m^3 (95% CI, 8.6–17.2); and severe (81–100% inhibition), 23.2 mg min/m^3 (95% CI, 12.7–42.3). We estimated that for the moderate severity level, the total VX dose administered was between 1.40 and 2.74 µg/kg. For comparison, a previous study found an effective dose for 50% of the population that resulted in greater than 50% WB-AChE inhibition after intravenous delivery of VX in rabbits to be 1.37 µg/kg. This represents a fairly accurate estimation of equipotent dosimetry between two differing routes of exposure.

15. SUBJECT TERMS

VX	Cholinesterase	Whole blood acetylcholinesterase enzyme (WB-AChE)
Blood	New Zealand white rabbit	Effective concentration for 50% (ECt_{50})

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PREFACE

The work described in this report was authorized under project no. CB3281. The work was started in November 2017 and completed in December 2017. At the time this work was performed, the U.S. Army Combat Capabilities Development Command Chemical Biological Center (CCDC CBC; Aberdeen Proving Ground, MD) was known as the U.S. Army Edgewood Chemical Biological Center (ECBC).

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This report has been approved for public release.

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EXECUTIVE SUMMARY

This study established the effective concentration of *O*-ethyl-*S*-(2-diisopropylaminoethyl) methyl phosphonothiolate (VX) aerosol required to produce mild, moderate, and severe inhibition of whole blood acetylcholinesterase (WB-AChE) in the New Zealand white rabbit. This work was performed to establish dosing levels for a follow-on study that is designed to identify novel physiological biomarkers of VX exposure at concentrations below those producing visible signs of intoxication. Finally, a method was established by which an estimate for inhalation toxicity may be derived from known intravenous (IV) toxicity data in the rabbit. A version of this method may be used in future studies to better estimate human toxicity from animal models.

The calculated effective concentrations for 50% of the population, after a 20 min exposure duration for each severity level, were as follows: mild (21–50% WB-AChE inhibition), 6.6 mg min/m^3 (95% confidence interval [CI], 4.3–10.1); moderate (51–80% WB-AChE inhibition), 12.2 mg min/m^3 (95% CI, 8.6–17.2); and severe (81–100% WB-AChE inhibition), 23.2 mg min/m^3 (95% CI, 12.7–42.3). We estimated that for the moderate severity level, the total VX dose administered was between 1.40 and 2.74 µg/kg. For comparison, Reutter-Christy et al. (2010) found an effective dose for 50% of the population that resulted in greater than 50% WB-AChE inhibition after IV delivery of VX in rabbits to be 1.37 µg/kg.* This represents a fairly accurate estimation of equipotent dosimetry between two differing routes of exposure. Further experimentation will be required to validate and verify this method for future use.

* Reutter-Christy, S.A.; Sommerville, D.R.; Hulet, S.W. *VX Studies in Support of the Contact Hazard Defense Technology Objective and Recommendations for Human Toxicity Estimates*; ECBC-TR-795; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2010; UNCLASSIFIED Report (ADB365653).

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1. INTRODUCTION

It is likely that the soldier of the future will have real-time monitoring of basic physiological processes available for the individual and commanders. One potential advantage of this information could be the early detection of exposure to chemical agent. The physiological effects of low-level (no-observed-adverse-effect level [NOAEL] or lower) chemical agent exposure is a topic that has received relatively little attention over the long history of chemical agent use and research. No direct link between sub-NOAEL levels of exposure and human pathology has been established. Despite this, it is likely that sub-NOAEL exposure may lead to subtle physiological changes that can serve as an early warning of chemical agent exposure. This report details the derivation of an effective concentration for 50% of the population (ECt_{50}) for whole blood acetylcholinesterase enzyme (WB-AChE) inhibition after a 20 min exposure to *O*-ethyl-*S*-(2-diisopropylaminoethyl) methyl phosphonothiolate (VX) aerosol in the New Zealand white rabbit. This dose–response curve will be used for future studies to investigate the effects of sub-NOAEL exposure to VX on cardiovascular homeostasis in the rabbit. Because a dose–response relationship between inhaled VX aerosol and WB-AChE activity has not been established for the rabbit, the work described herein is prerequisite to the effective execution of future NOAEL research.

2. BACKGROUND

2.1 Current Biomarkers for Anticholinesterase Exposure

The two most commonly used biomarkers for anticholinesterase exposure in mammalian species are whole blood cholinesterase inhibition (acetylcholinesterase [AChE] and butyrylcholinesterase [BChE]) with a focus on WB-AChE and the presence of pupillary miosis. Typically, pupillary miosis is the earliest indicator of anticholinesterase exposure when the exposure is airborne in nature and the eyes are left unprotected. In this scenario, miosis results from direct absorption of a cholinesterase-inhibiting agent into the eye, which exerts a local effect on cholinergic regulation of the pupillary sphincter muscle and results in constriction of the muscle that is observed as miosis. If an individual presents with pupillary miosis and has been in an environment where exposure to anticholinesterase compounds is possible, then miosis may be a reasonable diagnostic of exposure. It is important to note that miosis can occur with a non-airborne exposure; however, this will typically be associated with other mild signs of exposure because the anticholinesterase compound must enter the systemic circulation to reach the pupillary muscle and other tissues will be affected at the same time.

Generally speaking, whole blood cholinesterase inhibition is the easiest, definitive diagnostic for anticholinesterase exposure, particularly when the exposure is not airborne. In most cases, some degree of whole blood cholinesterase inhibition will occur before the appearance of overt signs of intoxication. Therefore, if samples can be obtained rapidly, blood cholinesterase inhibition may serve as a reasonable early warning biomarker for anticholinesterase exposure. However, it is not reasonable to regularly sample blood from a large population of individuals, and this method does not qualify as a passive monitoring technique. As a result, a need exists for the identification of novel biomarkers that can be easily applied to a large population and that are specific for anticholinesterase exposure.

2.2 Animal Model of Novel Biomarkers

Recent evidence from this laboratory indicates that changes in the duration of intervals of the electrocardiogram and/or changes to the pattern of heart rate variability may be specific for anticholinesterase exposure. We hypothesize that changes in these parameters occur at exposure concentrations that do not produce visible signs of intoxication. To test this hypothesis, an animal model of low-level anticholinesterase exposure will be utilized. That model will be nose-only inhalation of the prototypical chemical nerve agent, VX, by the rabbit. However, before this study can proceed, the determination of effective aerosol doses of VX that result in mild (21–50%), moderate (51–80%), and severe (81–100%) inhibition of whole blood cholinesterase activity must be determined. In this study, we defined an ECt_{50} for mild, moderate, and severe inhibition of WB-AChE after VX aerosol exposure in the rabbit.

3. MATERIALS AND METHODS

3.1 Animal Model

The male New Zealand white rabbit was chosen for this study based on the requirement of cardiomyocyte similarity for the follow-on study that this work supports. Rabbit cardiomyocyte sarcomere structure is more similar to that of humans than of other small animal models,[1] particularly with respect to the work-producing component, myofibril myosin. In human cardiomyocytes, the beta-myosin heavy chain (β-MyHC) protein is the predominant isoform, which represents 90% of total myosin, and the remainder is the alpha-myosin heavy chain (α-MyHC) protein.[2] In the rabbit, the β-MyHC protein represents 80% of the total myosin.[2] In the mouse, the opposite is true, and the α-MyHC protein represents about 95% of the total myosin.[2] The handling of adenosine 5'-triphosphate (ATP) and the rate of contraction between these two isoforms is different,[3,4] and this may result in differential handling of sodium, potassium, and calcium currents (rate and magnitude), which can be observed in the electrocardiogram.

Male New Zealand white rabbits were sourced from Covance, Inc. (Princeton, NJ); pathogen screened; and determined to weigh between 2.0 and 4.0 kg when used in this study. Rabbits were maintained on a 12/12 light/dark cycle, with a room temperature of 61–72 °F and 30–70% humidity. All work was performed in accordance with an animal use protocol approved by the U.S. Army Edgewood Chemical Biological Center (ECBC; now known as the

U.S. Army Combat Capabilities Development Command Chemical Biological Center; Aberdeen Proving Ground, MD) Institutional Animal Care and Use Committee.

3.2 Generation of VX Aerosol

This study used neat VX (lot number VX-U-1244-CTF-N, purity 90.2 ± 2.8%) that was acquired from the ECBC Agent Chemistry Branch (Appendix A).

(U) VX aerosol was generated using a coaxial needle atomization device (Figure 1). This device consists of a 32 ga stainless steel tube located within a blunt-ended, 22 ga stainless steel needle. Liquid VX is driven through the 32 ga tubing by a syringe drive, typically at a rate of 1.0 µL/min. Inert gas (nitrogen) is simultaneously supplied at 80 psi and 1.0 L/min through the 22 ga outer needle. The VX is atomized into a larger expansion chamber that is plumbed to a 12-port, nose-only exposure chamber (CH Technologies USA, Inc.; Westwood NJ). The nose-only exposure chamber is operated at 24.0 L/min total airflow.

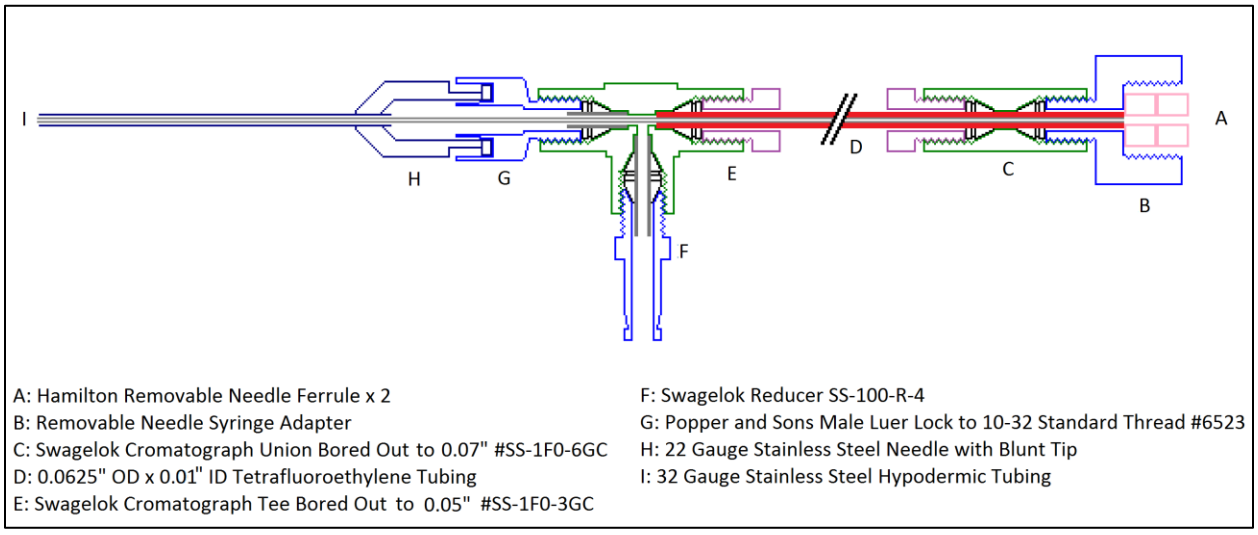

Figure 1. Schematic representation of the coaxial needle atomizer (patent no. 9016671 B1, McCaskey, David A.).

As shown in Figure 1, agent is delivered through the atomizer by a Hamilton Company (Reno, NV) gas tight syringe connected through two Teflon Hamilton removable needle ferrules (A). The syringe is connected through a custom-made, removable needle syringe adapter (B). Swagelok Company (Solon, OH) chromatograph fittings and a Popper and Sons, Inc. (New Hyde Park, NY) male luer lock fitting (C, E, F, and G) allow for a section of 32 ga hypodermic tubing (I, gray) to be sheathed by a section of Teflon tubing (D, red) and then pass through the middle of a 22 ga blunt-tipped needle (H, blue). Atomization gas flows through F and then the 22 ga needle, where it meets with agent that flows through I, and atomizes the agent for dispersion into the exposure chamber.

3.3 Analysis of Aerosol Samples

Primary analytical stocks were prepared by weighing known amounts of neat VX and then adding isopropyl alcohol to a known volume using Class A volumetric flasks. Individual stock solutions were prepared by certified laboratory personnel in accordance with standard operating procedure (SOP) RSC-366 and were prepared as needed by volumetric dilution of the stock solution. All solutions were stored at 4 °C while not in use. Reagent-grade solvents were obtained from Aldrich Chemical Company (Sigma-Aldrich Company; St. Louis, MO). Statistical requirements for each analytical method were met before they were used to produce data. Documentation for each analytical method is available upon request.

Calibration of analytical instrumentation was performed daily by certified personnel who evaluated the system performance or fit-for-use based on the results obtained from a series of challenges and review. Calibration and challenge for analytical methods included initial calibration and/or continuing calibration. A minimum of a five-point curve that covered the range to be analyzed was used to establish the calibration curve of the instrument. NMR purity analysis was performed on primary VX solutions before use.

To quantify exposure concentration and particle size, glass fiber filters and impactor stages were extracted with reagent-grade isopropyl alcohol (Sigma-Aldrich). Extraction volumes were 1.0 mL for glass fiber filters and 0.5 mL for stainless steel impactor stages. The concentration of VX in each extract was determined using gas chromatography (GC; model 6890, Agilent Technologies; Santa Clara, CA) with a flame photometric detector (FPD). After the extraction equilibrated for at least 1 h, it was removed and filtered through a 0.2 µm syringe filter to remove the particulates. Glass fiber filter samples were injected into a depot area air-monitoring system (DAAMS) tube with Tenax sorbent (Markes International; Sacramento, CA) for analysis on a GC/FPD system, and the impactor stage extracts were analyzed using an Agilent auto injector connected to a GC/FPD system. The Agilent GCs were mirrored to closely repeat the analysis.

3.4 Collection of VX Aerosol Samples and Quantification of Aerosol Concentration

VX aerosol was characterized by several methodologies during each animal exposure. Real-time monitoring of aerosol concentration was accomplished by laser diffraction with a pDR 1500 instrument (Thermo Fisher Scientific, Inc.; Waltham, MA) and was used to determine the stability of the aerosol concentration rather than the nominal concentration (Figure 2). Nominal aerosol concentrations were determined by analytical chemistry analysis of filter extracts. Particle sizing was achieved through analytical chemistry analysis of extracts from a CH Technologies, Mercer-style, seven-stage cascade impactor (Table 1).

Table 1. Aerosol Particle Size Data

Run No.	MMAD (µm)	GSD
1	1.36789	4.33789
2	0.94777	2.82639
3	1.56349	1.87330
4	1.58059	1.35994
5	1.13445	2.20333
6	1.63009	1.63097

MMAD, mass median aerodynamic diameter; GSD, geometric standard deviation.

During the 20 min exposure period, two aerosol samples were collected onto glass fiber filter pads through a 27 mm, in-line filter holder (In-Tox Products; Moriarty, NM). Each filter sample was collected from one of the 12 nose ports that were under constant vacuum with a flow rate of 500 mL/min for a total duration of 2 min. Aerosol samples were collected at 5 and 15 min during each exposure. To avoid vaporization of sample, 2 min sample times were used, and each filter was backed up using a DAAMS tube with Tenax sorbent to identify the presence of VX vapor. There were no instances of VX detection from the DAAMS tubes, which indicated that the VX remained a liquid aerosol and that the filter sample was representative of the true VX concentration.

Aerosol particle size was determined using a seven-stage cascade impactor (In-Tox Products). Cascade impactor samples were collected from one of the 12 nose ports in the chamber. The impactor was set to sample for the duration of the exposure at a rate of 1000 mL/min. The aerosol was separated into its component sizes based on increasingly smaller passages at each stage. A glass fiber filter after the last stage collected all residual aerosol. There was VX vapor formation from these samples; however, it was the distribution of the material, rather than the precise concentration, that was of interest.

3.5 Rationale for Aerosol Particle Size Selection

In this study, the aerosol particle size was selected to target maximal tracheobronchial and alveolar deposition. According to Asgharian et al., maximal lower airway deposition in rabbits occurs at approximately 2.0 µm.[5] In addition to having a maximal deposition in the lower airways (5% tracheobronchial and 5% alveolar) at this size, the deposition fraction in the upper airways of a rabbit is about 40%.[5] However, according to Raabe, et al., total airway deposition rates for rabbits are between 22 and 43% for particles ranging from 1.0 to 2.0 µm aerodynamic resistance diameter (D_{ar}).[6] (The mean was 31.8%, assuming that materials recovered from the rabbit gastrointestinal tract and skull were deposited in the upper airways.) Maximal lower airway deposition of aerosol particles in rabbits occurs at approximately 2.0 µm D_{ar}.[6] The effect of the location of aerosol deposition is primarily on the pharmacokinetic properties for VX because it is expected to absorb into systemic circulation from all mucous membranes. The information herein provides a frame of reference for the

amount of inhaled aerosol that may be deposited in the animal. This study was not designed to investigate aerosol distribution and absorption kinetics.

3.6 Acute Inhalation Protocol

Each aerosol exposure group consisted of four male rabbits placed into plastic nose-only exposure tubes (CH Technologies), which were connected to the stainless steel 12-port, nose-only exposure chamber in an ECBC glovebox facility. Each animal's exposure consisted of a 2 to 3 min baseline period (no aerosol generated), followed by the 20 min VX aerosol exposure period, and a brief (<1 min) chamber-clearing period. After the chamber was cleared of aerosol, which was determined through real-time aerosol monitoring, the animals were immediately removed from the chamber and transported to a chemical fume hood for observation and blood sampling.

3.7 Analysis of WB-AChE

Whole blood cholinesterase (AChE and BChE) activity was assayed by a modified Ellman method using the Cholinesterase Chemistry Set (model 700; EQM Research, Inc.; Cincinnati OH). Blood samples were collected by lancing the marginal ear vein and gathering the sample into 20 µL capillary tubes. Baseline blood samples were obtained from each animal immediately before VX aerosol exposure. Post-exposure blood samples were obtained 10 min after VX exposure. Previous studies in our laboratory have shown that 10 min is sufficient for blood cholinesterase inhibition to equilibrate after aerosol challenges of various anticholinesterase compounds. Blood samples were assayed for cholinesterase activity according to the manufacturer's instructions. The kinetic reaction was analyzed with a SpectraMax 5 photometer (Molecular Devices, LLC; San Jose, CA) using the SoftMax Pro version 5.0 software. Inhibition is expressed as percent inhibition of baseline values for each animal.

3.8 Statistical Analysis

The relationship between VX aerosol concentration and WB-AChE activity was described by a probit analysis of scored data to determine an effective concentration for three ranges of WB-AChE inhibition. Probit analysis of scored data was conducted as described by Reutter-Christy et al. in 2010.[7]

For probit analysis, a scoring system for several depression bins was devised, as shown in Table 2.

Table 2. Scoring System for Depression Bins

Score	AChE Depression Range (%)
0	0–20
1	21–50
2	51–80
3	81–100

Because normal variation in blood AChE and BChE activity by this assay can be as much as 20%, any inhibition value of 20% or less is considered to be baseline or uninhibited. Score 1 is considered to be mild depression, Score 2 is moderate depression, and Score 3 is severe depression.

4. RESULTS: PROBIT ANALYSIS OF WHOLE BLOOD CHOLINESTERASE INHIBITION

Six groups of four rabbits were exposed to VX aerosol in increasing concentrations across a complete dose–response range. Within any single group of four rabbits, a range of cholinesterase inhibition values were observed, as shown in Table 3. The calculated ECt_{50} values for each score (Table 4) were as follows: Score 1 (21–50% inhibition), 6.6 mg min/m^3 (95% confidence interval [CI], 4.3–10.1); Score 2 (51–80% inhibition), 12.2 mg min/m^3 (95% CI, 8.6–17.2); and Score 3 (81–100% inhibition), 23.2 mg min/m^3, (95% CI, 12.7–42.3). Binning in this way is an expansion upon the methods described by Hulet et al. in 2006[8] and Reutter-Christy et al. in 2010.[7] See Appendix B for a complete listing of individual animal responses.

Plasma BChE activity was also assessed for each exposure (Appendix C); however, minimal inhibition of this enzyme occurred at the VX doses administered in this study. The highest inhibition of plasma BChE (26%) was observed at a concentration–time (Ct) of 11.00 mg min/m^3, and 25% inhibition occurred at a Ct of 10.80 mg min/m^3. All other values fell at or below 20% inhibition, meaning they were within the normal variation in activity; therefore, no further analysis was conducted. See Appendix C for a complete listing of individual animal responses.

Table 3. Number of Rabbits, VX Aerosol Dose Concentrations, and Probit Scores

VX Dose (Ct) (mg min/m^3)	**Number of Rabbits Exhibiting Each Probit Score**			
	0	**1**	**2**	**3**
2.77	3	1	0	0
8.57	1	3	0	0
9.81	1	3	0	0
10.80	1	0	3	0
11.00	2	0	1	0
19.51	0	0	1	3
Total	8	7	5	3

Table 4. ECt_{50} and Probit Slopes for Each Severity Score

Score	**AChE Depression Range (%)**	**ECt_{50} (95% CI) (mg min/m^3)**	**Probit Slope (Standard Error)**
0	0–20	Not calculated	Not calculated
1	21–50	6.6 (4.3–10.1)	4.0 (1.33)
2	51–80	12.2 (8.6–17.2)	4.0 (1.33)
3	81–100	23.2 (12.7–42.3)	4.0 (1.33)

5. DISCUSSION

This study initially set out to determine a simple ECt_{50} for WB-AChE inhibition in rabbits exposed to VX aerosol. Given that aerosol exposures produce a range of effects across any single group of animals, analysis of the data in scored bins is a more realistic approach to the derivation of an ECt_{50}. ECt_{50} values that corresponded with the ranges of mild, moderate, and severe inhibition of WB-AChE resulted from this effort. In addition, more information was generated through this approach because estimates were provided for inhibition ranges above and below the ECt_{50} for moderate WB-AChE inhibition. The VX aerosol concentration required to produce 51–80% whole blood cholinesterase inhibition in the rabbit was found to be 12.2 mg min/m^3 (95% CI, 8.6–17.2) with a probit slope of 4.00 and a standard error of 1.33 (Table 4).

Although there is quite a bit of literature available in peer-reviewed and technical reports involving animal exposures to VX, it was difficult to find many studies involving aerosol or vapor exposures to VX. Among those studies, few actually provided data regarding WB-AChE inhibition for comparison with this study. The following referenced studies are intended to better frame the results of this study with the current body of literature on VX.

In 2007, Benton et al. performed a study to determine the effects of decontamination procedures on the toxicity of VX vapor in male and female rats.[9] Although generating an ECt_{50} for blood cholinesterase inhibition was not the primary objective of the study, the authors did report WB-AChE activity following VX vapor exposures. In male and female rats, the Ct values generally ranged between 30 and 35 mg min/m^3 and were able to produce 90% inhibition of WB-AChE activity.[9] This value is within the range found in this study; however, a lack of data at the higher range of the inhibition dose–response curve resulted in a wide range for the estimation of ECt_{50} for 80–100% WB-AChE inhibition.

In an earlier study by Benton et al. in 2004, the effects of VX vapor exposure on miosis in rats were investigated, and it was reported that at the highest concentrations for each exposure time, "significant" AChE depression occurred.[10] No actual values were given, and the highest doses were not reported. Rather, the ECt_{50} values for miosis were reported for 10, 60, and 240 min VX exposures in male rats as 0.102, 0.229, and 0.443 mg min/m^3, respectively. These values are at least 2 orders of magnitude lower than those of the present study. These results were not surprising because the whole-body exposure method used in the Benton 2004 study would have resulted in significant ocular absorption of VX. In this case, miosis was expected to occur before significant blood cholinesterase inhibition occurred.

Another point of comparison involved exposure of mice to VX aerosol. Carpin et al. (2005) described the derivation of a lethal concentration for 50% of the population (LCt_{50}) for mice exposed to VX aerosol for 10 min via the nose-only route.[11] The methods used in the study presented herein were very similar to those used by Carpin et al., who reported an LCt_{50} in mice of 16.1 mg min/m^3 (95% CI, 12.8–19.3) and a probit slope of 8.7.

Possibly, the best point of comparison comes from the investigation of VX vapor exposure in miniature swine. Hulet el al. (2006) conducted studies investigating lethal and sublethal effects of whole-body VX vapor exposure in swine.[8] As a part of that study, numerous blood samples were collected at specific time points during and after exposures to track the progression of WB-AChE depression over time. These data were then plotted, and the VX dosage was corrected for the time point at which it was collected. For example, if a blood sample was collected 5 min into a 20 min exposure, and the vapor concentration for the exposure was 1.0 mg/m^3, then the exposed dose for the 5 min blood sample would be 5 mg min/m^3. Using this method, a rough estimate of an ECt_{50} for WB-AChE inhibition in miniature swine was about 10 mg min/m^3. These data have not yet been published in a report and were the result of personal conversation and data review with Dr. Stanley Hulet.

Finally, understanding the factors that influence our ability to accurately estimate the effective aerosol dosage from a known effective intravenous (IV) dosage is a critical piece of information in generating toxicity estimates. Although the study presented herein was not designed to perform this task, we used the information presented by Raabe et al. (1988)[6] and the known respiratory mechanics that were measured under similar experimental conditions in rabbits by our laboratory to estimate the ECt_{50} for WB-AChE inhibition. Raabe et al. described total deposition rates between 22 and 43% (mean 31.8%) for particles ranging from 1.0 to 2.0 µm D_{ar}. The particle size in this study was 1.36 µm mass median aerodynamic diameter. The two most relevant particle sizes presented by Raabe et al. were 1.09 and 1.94 µm D_{ar},[6] and the total corresponding deposition percentages were 22.1 and 43.2%, respectively. These percentages were used to estimate the total dose delivered to each rabbit.

Using published average respiratory rates and tidal volumes from Suckow et al. (2010)[12] to calculate the deposited fraction of VX for each animal led to a low estimation of the dose delivered. Using the resting minute volume (described by Suckow) for this type of exposure scenario is inaccurate because it is known that rabbits restrained in exposure apparatus exhibit higher than normal respiratory rates and minute volumes. Data previously generated in our laboratory from rabbits with an average weight of 2.95 kg that were connected to a face-mask pneumotachometer and restrained in similar apparatus present an average minute volume of 1707 mL/min (578 mL/min/kg; n = 7). This was compared with 1062 mL/min for a theoretical 2.95 kg rabbit, in accordance with the data available in Suckow et al. (2010).[12]

Using the minute volume (578 mL/min/kg), the ECt_{50} for 51–80% WB-AChE inhibition of 12.2 mg min/m^3, a mean animal weight of 3.26 kg (from the present study), and total deposition rates of 22.1 and 43.2%, we estimated the total VX dose administered to be between 1.40 and 2.74 µg/kg (eq 1a,b). For comparison, Reutter-Christy et al. (2010) presented data in which the effective dose for 50% of the population (ED_{50}) for AChE inhibition after IV delivery of VX in rabbits was found to be 1.37 µg/kg.[7] This relationship suggested that it may be possible to accurately derive effective aerosol doses in rabbits based on data generated from IV studies. This finding also encourages the continued development of models for estimating human deposition based on known translational elements between animal models and humans.

Equation 1a,b is an estimation of the deposited dose of VX in rabbits (the units of milligrams per cubic meter [mg/m^3] equate to micrograms per liter [µg/L]).

$$12.2 \text{ mg min/m}^3 \times 1.884 \text{ L/min inhaled} = 22.98 \text{ µg total inhaled VX dose} \quad (1a)$$

$$22.98 \text{ µg VX dose} \times 0.902 \times 0.221 = 4.58 \text{ µg VX deposited / 3.26 kg rabbit} = 1.40 \text{ µg/kg deposited VX dose} \quad (1b)$$

where 0.902 = VX purity (%); and 0.221 = total deposition of 1.09 µm D_{ar} particle (%).

Figure 2 shows the stability of the aerosol concentration that was typical of each exposure in this study. Data are recorded every second before the beginning of VX aerosol exposure and after the end of exposure. Outlier data points at 5, 15, and 20 min after the start of exposure correlate with when sampling devices were removed from the chamber.

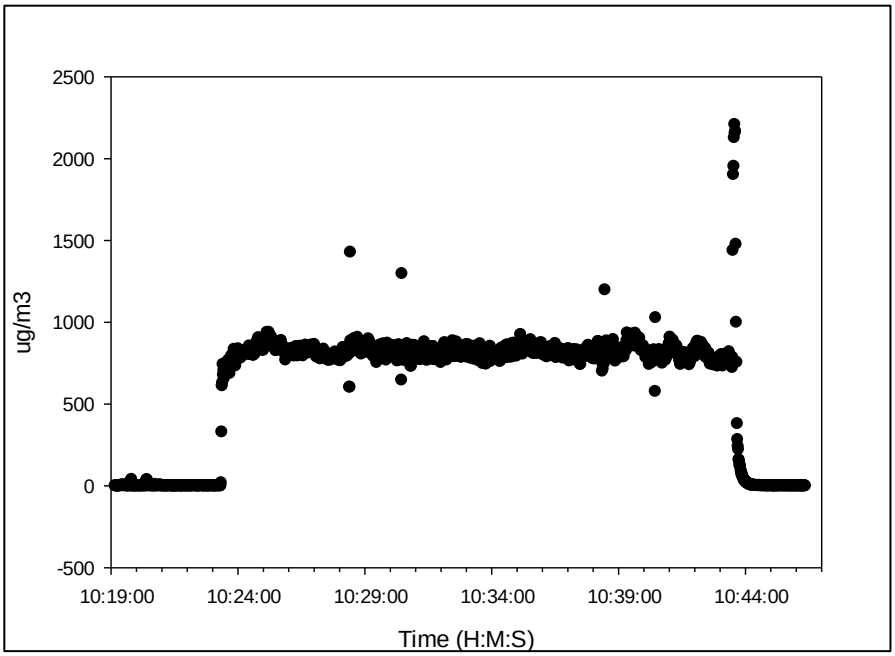

Figure 2. Representative real-time aerosol data.

In summary, this study establishes an ECt_{50} for three severity levels of WB-AChE inhibition after exposure to VX aerosol in male New Zealand white rabbits. These data are a required prerequisite for future studies that are designed to identify physiological biomarkers of VX exposure before signs of toxicity occur. In addition, we have identified a reasonable method by which an estimate of effective aerosol VX doses from known effective IV doses may be derived in the rabbit. The application of this method to future studies will be required to validate the process.

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ACRONYMS AND ABBREVIATIONS

α-MyHC	alpha-myosin heavy chain
β-MyHC	beta-myosin heavy chain
AChE	acetylcholinesterase enzyme
ATP	adenosine 5'-triphosphate
BChE	butyrylcholinesterase
CI	confidence interval
Ct	concentration–time (of aerosol)
DAAMS	depot area air-monitoring system
D_{ar}	aerodynamic resistance diameter
ECBC	U.S. Army Edgewood Chemical Biological Center
ECt_{50}	effective concentration for 50% of population (inhalation route)
ED_{50}	effective dose for 50% of the population (parenteral route)
FPD	flame photometric detector
GC	gas chromatography
GSD	geometric standard deviation
IV	intravenous
LCt_{50}	lethal concentration for 50% of population (inhalation route)
MMAD	mass median aerodynamic diameter
NOAEL	no-observed-adverse-effect level
VX	*O*-ethyl-*S*-(2-diisopropylaminoethyl) methyl phosphonothiolate
WB-AChE	whole blood acetylcholinesterase enzyme

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APPENDIX A
VX PURITY REPORT

REPORT OF ANALYSIS

To: **Michael Horsmon** **Toxicology Division** **ECBC R&T**	**CUSTOMER SAMPLE NO.:** VX-U-1244-CTF-N
	STORAGE NO.: VIAL #174-2 SRC-1246
	LAB I.D. NO: NB097P121A
	DATE RECEIVED: 6 DEC 2017
	DATE TESTED: 6 DEC 2017
	CONDITION OF SAMPLE: neat
From: **CDR US ARMY SBCCOM** **RDCB-DRC-T** **ANALYTICAL TOXICOLOGY TEAM** **APG, MD 21010-5424**	**ANALYST:** /D. McGarvey
	METHOD NO.: RSM-408, ECBC-TR-253
	INSTRUMENT: JEOL ECS-400 NMR (400 MHz)
	UNCERTAINTY: 2.0%
	INSTRU. DET. LIMIT: 1%
	METHOD DET. LIMIT: 1%

RESULTS:

GB Purity

Average wt%	90.2%
Std. Dev.	0.8%

Sample Preparation:

Neat agent sample was pipeted and weighed. Internal standard TEP was added and weighed. The solvent $CDCl_3$ (400 µl) was added. The solution was transferred to a 4 mm PTFE insert inside a 5 mm tube and both tubes were sealed. The tube was analyzed seven times by ^{31}P NMR for quantitation of phosphorus compounds relative to the internal standard.

Signature of Analyst: **Date**	**Checked by:** **Date**
Printed Name: William R. Creasy, Ph.D. 6 DEC 2017	**Printed Name: David McGarvey, Ph.D.**

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APPENDIX B
WHOLE BLOOD ACETYLCHOLINESTERASE (WB-AChE) DATA

Individual Animal Data

Data are expressed in units of activity and are corrected for hemoglobin (HgB) content of the sample.

Table B-1. Individual Animal WB-AChE Inhibition

6-December-2017								
Group	**Animal No.**	**Weight (kg)**	**Dose (Ct)**	**Pre (U/mL)**	**HgB mg/dL**	**20 min Post (U/mL)**	**HgB mg/dL**	**% Inhibition**
1	50	3.45	8.57	1195	10.7	623	10.6	48
1	51	3.27	8.57	1033	11.2	507	10.5	51
1	52	3.12	8.57	1011	10.6	577	9.5	43
1	53	3.14	8.57	1071	11.5	785	10.4	27
			Mean	**1077.5**		**623**		**42**
			SEM	**41.1**		**59**		**5**
					Pre/HgB	**20 min/ HgB**		**Corrected % Inhibition**
					111.7	58.8		47
					92.2	48.4		48
					95.4	60.7		36
					93.1	75.5		19
				Mean	**98.1**	**60.8**		**38**
				SEM	**4.6**	**5.6**		**7**
7-December-2017								
Group	**Animal No.**	**Weight (kg)**	**Dose (Ct)**	**Pre (U/mL)**	**HgB mg/dL**	**20 min Post (U/mL)**	**HgB mg/dL**	**% Inhibition**
2	54	3.30	10.80	1227	12.1	379	10.7	69
2	55	3.09	10.80	1213	10.7	280	10.5	77
2	56	3.13	10.80	1391	12.7	947	10.1	32
2	57	3.38	10.80	1112	10.3	239	10.0	79
			Mean	**1235.8**		**461**		**64**
			SEM	**57.7**		**165**		**11**
					Pre/HgB	**20 min/ HgB**		**Corrected % Inhibition**
					101.4	35.4		65
					113.4	26.7		76
					109.5	93.8		14
					108.0	23.9		78
				Mean	**108.1**	**44.9**		**58**
				SEM	**2.5**	**16.5**		**15**

(table continued)

Table B-1. Individual Animal WB-AChE Inhibition (continued)

8-December-2017								
Group	**Animal No.**	**Weight (kg)**	**Dose (Ct)**	**Pre (U/mL)**	**HgB mg/dL**	**20 min Post (U/mL)**	**HgB mg/dL**	**% Inhibition**
3	58	3.31	19.51	983	10.0	82	9.6	92
3	59	3.19	19.51	1107	9.2	107	10.0	90
3	60	3.15	19.51	1191	9.9	420	9.4	65
3	61	3.16	19.51	1036	9.7	102	9.4	90
			Mean	**1079.3**		**178**		**84**
			SEM	**45.1**		**81**		**7**
					Pre/HgB	**20 min/ HgB**		**Corrected % Inhibition**
					98.3	8.5		91
					120.3	10.7		91
					120.3	44.7		63
					106.8	10.9		90
				Mean	**111.4**	**18.7**		**84**
				SEM	**5.4**	**8.7**		**7**

11-December-2017								
Group	**Animal No.**	**Weight (kg)**	**Dose (Ct)**	**Pre (U/mL)**	**HgB mg/dL**	**20 min Post (U/mL)**	**HgB mg/dL**	**% Inhibition**
4	62	3.21	2.77	860	10.0	705	9.7	18
4	63	3.14	2.77	1130	9.7	821	9.2	27
4	64	3.52	2.77	947	10.0	990	10.4	–5
4	65	3.30	2.77	842	10.4	734	9.8	13
			Mean	**944.8**		**813**		**13**
			SEM	**65.9**		**64**		**7**
					Pre/HgB	**20 min/ HgB**		**Corrected % Inhibition**
					86.0	73		15
					116.5	89		23
					94.7	95		–1
					81.0	75		7
				Mean	**94.5**	**83**		**11**
				SEM	**7.8**	**5**		**5**

(table continued)

Table B-1. Individual Animal WB-AChE Inhibition (continued)

12-December-2017								
Group	**Animal No.**	**Weight (kg)**	**Dose (Ct)**	**Pre (U/mL)**	**HgB mg/dL**	**20 min Post (U/mL)**	**HgB mg/dL**	**% Inhibition**
5	66	3.31	11.00	953	10.3	902	11.5	5
5	67	3.27	11.00	1182	10.7	1021	10.3	14
5	68	3.21	11.00	1135	10.2	456	9.0	60
5	69	3.18	11.00	1196	10.0	Died in Tube		
			Mean	**1116.5**		**793**		**26**
			SEM	**56.0**		**172**		**17**
					Pre/HgB	**20 min/ HgB**		**Corrected % Inhibition**
					92.5	78		15
					110.5	99		10
					111.3	51		54
					119.6			
				Mean	**108.5**	**76**		**27**
				SEM	**5.7**	**14**		**14**
13-December-2017								
Group	**Animal No.**	**Weight (kg)**	**Dose (Ct)**	**Pre (U/mL)**	**HgB mg/dL**	**20 min Post (U/mL)**	**HgB mg/dL**	**% Inhibition**
6	70	3.44	9.81	989	9.5	718	9.1	27
6	71	3.28	9.81	946	9.9	498	9.4	47
6	72	3.34	9.81	973	11.2	828	11.0	15
6	73	3.26	9.81	1187	9.9	840	9.8	29
			Mean	**1023.8**		**721**		**30**
			SEM	**55.1**		**79**		**7**
					Pre/HgB	**20 min/ HgB**		**Corrected % Inhibition**
					104.1	79		24
					95.6	53		45
					86.9	75		13
					119.9	86		29
				Mean	**101.6**	**73**		**28**
				SEM	**7.0**	**7**		**6**

Pre, values before *O*-ethyl-*S*-(2-diisopropylaminoethyl) methyl phosphonothiolate (VX) exposure; Post, values after VX exposure; U, unit of an enzyme's catalytic activity; and SEM, standard error of the mean. Blank cells do not apply.

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APPENDIX C
PLASMA BUTYRYLCHOLINESTERASE (BChE) DATA

Individual Animal Data

Data are expressed in units of activity and percent inhibition.

Table C-1. Individual Animal Plasma BChE Inhibition

6-December-2017						
Group	**Animal No.**	**Weight (kg)**	**Dose(Ct)**	**Pre (U/mL)**	**20 min Post (U/mL)**	**% Inhibition**
1	50	3.45	8.57	169	135	20
1	51	3.27	8.57	356	336	6
1	52	3.12	8.57	156	135	13
1	53	3.14	8.57	198	168	15
			Mean	**219.8**	**194**	**14**
			SEM	**46.3**	**48**	**3**
7-December-2017						
Group	**Animal No.**	**Weight (kg)**	**Dose(Ct)**	**Pre (U/mL)**	**20 min Post (U/mL)**	**% Inhibition**
2	54	3.30	10.80	451	364	19
2	55	3.09	10.80	198	111	44
2	56	3.13	10.80	201	156	22
2	57	3.38	10.80	427	365	15
			Mean	**319.3**	**249**	**25**
			SEM	**69.3**	**67**	**7**
8-December-2017						
Group	**Animal No.**	**Weight (kg)**	**Dose(Ct)**	**Pre (U/mL)**	**20 min Post (U/mL)**	**% Inhibition**
3	58	3.31	19.51	200	113	44
3	59	3.19	19.51	391	359	8
3	60	3.15	19.51	195	144	26
3	61	3.16	19.51	380	369	3
			Mean	**291.5**	**246**	**20**
			SEM	**54.3**	**68**	**9**
11-December-2017						
Group	**Animal No.**	**Weight (kg)**	**Dose(Ct)**	**Pre (U/mL)**	**20 min Post (U/mL)**	**% Inhibition**
4	62	3.21	2.77	168	157	7
4	63	3.14	2.77	478	457	4
4	64	3.52	2.77	176	186	–6
4	65	3.30	2.77	610	689	–13
			Mean	**358.0**	**372**	**–2**
			SEM	**110.7**	**125**	**5**

(table continued)

Table C-1. Individual Animal Plasma BChE Inhibition (continued)

12-December-2017						
Group	**Animal No.**	**Weight (kg)**	**Dose (Ct)**	**Pre (U/mL)**	**20 min Post (U/mL)**	**% Inhibition**
5	66	3.31	11.00	427	403	6
5	67	3.27	11.00	455	454	0
5	68	3.21	11.00	411	425	–3
5	69	3.18	11.00	209		100
			Mean	**375.5**	**427**	**26**
			SEM	**56.2**	**15**	**25**

13-December-2017						
Group	**Animal No.**	**Weight (kg)**	**Dose (Ct)**	**Pre (U/mL)**	**20 min Post (U/mL)**	**% Inhibition**
6	70	3.44	9.81	190	164	14
6	71	3.28	9.81	321	329	–2
6	72	3.34	9.81	170	172	–1
6	73	3.26	9.81	411	428	–4
			Mean	**273.0**	**273**	**1**
			SEM	**56.9**	**64**	**4**

Pre, values before *O*-ethyl-*S*-(2-diisopropylaminoethyl) methyl phosphonothiolate (VX) exposure; Post, values after VX exposure; U, unit of an enzyme's catalytic activity; and SEM, standard error of the mean. Blank cells do not apply.

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