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CHEMICAL BIOLOGICAL CENTER

U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND

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CHEMICAL WARFARE AGENT OPERATIONAL EXPOSURE HAZARD ASSESSMENT RESEARCH: FY07 REPORT AND ANALYSIS

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Contact hazard, defined as the toxicological hazard arising from any potentially contaminated surface that is capable of transferring toxic quantities of chemical agent to the skin, is studied in a series of animal models. Physiological parameters were developed in the guinea pig model to compare toxicity and kinetic data for the nerve agents, VX, GB, or GF, and to determine lethal percutaneous (PC) levels of VX. Calibration of Physiologically-based pharmacokinetic biomarkers (dose-metrics) were determined in a miniature swine model, achieving very sensitive detection levels for GB/GF products in plasma and tissue partition coefficients for VX. Rabbit studies extended the dose-response data for PC exposure to VX. The comparison of neat and dilute VX doses indicates that for a PC exposure, the VX/IPA (VX in isopropyl alcohol) solution was two times more potent than the neat agent for the $ED_{50(mild)}$ and $ED_{50(moderate)}$.

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Contact hazard	Chemical warfare agent	Percutaneous
VX	PBPK	Dilute VX

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PREFACE

The work described in this report was performed at the request of the Program Manager, Operational Toxicology, Joint Science and Technology Office, in support of the Operational Toxicology Research Program. This work was started in October 2006 and completed in September 2007. This report presents results of a review of the contact hazard data and human toxicity estimates for selected persistent agents with recommendations for further studies.

In conducting these studies, investigators adhered to the "Guide for the Care and Use of Laboratory Animals," National Institutes of Health (NIH) Publication No. 86-23, 1985, as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council, Washington, DC. These investigations were also performed in accordance with the requirements of AR 70-18, "Laboratory Animals, Procurement, Transportation, Use, Care, and Public Affairs." All animals were cared for as stated in this research protocol and as specified in NIH Publication No 85-23, 1985, (or updates).

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EXECUTIVE SUMMARY

This report is the first in a series that will capture the scientific studies, data analysis, and operational applications resulting from a five-year program to refine and deliver human hazard exposure estimates relevant to military operations. The primary focus will be on the contact hazard, *i.e.*, any potential hazard from the transfer of agent to skin whether or not it is in direct contact with a contaminated surface. For practical purposes on today's battlefield, "Contact Hazard" must be defined as the toxicological hazard arising from any potentially contaminated surface that is capable of transferring toxic quantities of chemical agent to the skin.

Extrapolation of data to human exposures is always challenging. Physiologically-based Pharmacokinetic/Pharmacodynamic (PBPK/PD) mathematical models of the absorption, distribution and elimination of chemical warfare nerve agents can be used to improve toxicity estimates, extrapolate to humans, and predict response to a range of nerve agent exposures. PBPK parameters were developed in a series of studies in the guinea pig model to compare toxicity and kinetic data for VX, GB or GF and to determine lethal percutaneous (PC) levels of VX. Calibration PBPK biomarkers (dose-metrics) were determined in a minipig model, achieving highly sensitive detection levels for GB/GF products in plasma and tissue partition coefficients for VX. This method was used to estimate VX-G exposure levels in a human following inadvertent laboratory exposure, demonstrating its utility as a biomarker for predictive and retrospective exposure assessments.

Rabbit studies extended the dose-response data for PC exposure to VX. Intravenous dosing of VX was used as a comparison to determine the influence of dermal exposure and penetration for this agent. The appearance and sequence of clinical signs differed by dose-route. ED_{50}s for effects ranging from lethality to threshold acetylcholinesterase depression were estimated. Comparison of neat and dilute VX doses indicates that the VX/IPA (VX in isopropyl alcohol) solution was two times more potent than the neat agent for the $ED_{50(mild)}$ and the $ED_{50(moderate)}$, for a PC exposure.

Planned studies will investigate the toxicokinetics and general cognitive performance in a non-human primate model following PC VX exposure.

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GLOSSARY/ACRONYM

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ACh	Acetylcholine
AChE	Acetylcholinesterase
AFRL/HEPC	Air Force Research Laboratory, Counterproliferation Branch
ANOVA	Analysis of Variance
AUC	Area under the Curve
BChE	Butyrylcholinesterase
BDL	Below Detection Limit
C	Concentration
CASARM	Chemical Agent Standard Analytical Reagent Material
CDC	Centers for Disease Control
ChE	Cholinesterase
CI	Chemical Ionization
CID	Collision-Induced Dissociation
CL	Clearance
C_{max}	Maximum Concentration
CMPA	Cyclohexyl Methylphosphonic Acid
CW	Chemical Warfare
CWA	Chemical Warfare Agent
CWNA	Chemical Warfare Nerve Agents
DAEMS	2-(Diisopropylaminoethyl) Methyl Sulfide
DHHS	Department of Health and Human Services
DPBS	Dulbecco's Phosphate Buffered Saline
DTNB	5,5-Dithiobis-2-Nitrobenzoic Acid
ECBC	Edgewood Chemical Biological Center
EDTA	Ethylenediaminetetraacetic Acid
ESI	Electrospray Ionization
FY	Fiscal Year
GA	Tabun
GB	Sarin
GC	Gas Chromatography
GC-FID	Gas Chromatography-Flame Ionization Detection
GC-FPD	Gas Chromatograph-Flame Photometric Detection
GC-MS	Gas Chromatography-Mass Spectrometry
GC-MS/MS	Gas Chromatography-Tandem Mass Spectrometry
GD	Soman
GF	Cyclosarin
HPLC	High Performance Liquid Chromatography
HQC	High Quantitation Concentration
IACUC	Institutional Animal Care and Use Committee
IMPA	Isopropyl Methylphosphonic Acid
IPA	Isopropyl Alcohol
IS	Internal Standard
IV/i.v.	Intravenous
k	Toxic Load Constant
LC	Liquid Chromatography
LC-ESI-MS/MS	Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LLOQ	Lower Limit of Quantitation
LOD	Limits of Detection
LOQ	Limit of Quantitation
MeOH	Methanol
MQC	Medium Quantitation Concentration

MRICD	U.S. Army Medical Research Institute of Chemical Defense
MRM	Multiple Reaction Monitoring
MRT	Mean Residence Time
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MSD	Mass Spectrometric Detector
NMR	Nuclear Magnetic Resonance
NZW	New Zealand White Rabbits
OP	Organophosphorous
PBPK	Physiologically based pharmacokinetic
PBPK/PD	Pharmacokinetic/Pharmacodynamic
PC	Percutaneous
pKa	Ionization Constant
p-p S/N	Peak-to-Peak Signal-to-Noise Ratio
QC	Quantitation Concentration
RBC	Red Blood Cells
rGB	Regenerated Sarin
rGF	Regenerated Cyclosarin
RSD	Relative Standard Deviation
SC	Subcutaneous
SE	Standard Errors
SIM	Selected Ion Monitoring
S/N	Signal-to-Noise Ratio
SPE	Solid Phase Microextraction
SPR	Serial Probe Recognition
t	Exposure Duration
$t_{½}$	Half-life
TEP	Triethyl Phosphate
t_{max}	Time to Maximum Concentration
TOF	Time of Flight
VX	S-[2-(diisopropylamino)ethyl]-O-ethyl Methylphosphonothioate
VX-G	Regenerated VX, Ethyl Methylphosphonofluoridate
WCX	Weak Cation Exchange
WPL	Worker Population Limit

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CHEMICAL WARFARE AGENT OPERATIONAL EXPOSURE HAZARD ASSESSMENT RESEARCH: FY07 REPORT AND ANALYSIS

1 INTRODUCTION AND BACKGROUND

This report is the first in a series that will capture the scientific studies, the data analysis, and the operational applications resulting from a five-year program to refine and deliver human hazard exposure estimates relevant to military operations. The primary focus will be on the contact hazard, *i.e.*, any potential hazard from the transfer of agent to skin whether or not it is in direct contact with a contaminated surface. Some selected compounds with the inhalation route of exposure will also be addressed in the out years.

The Operational Hazard Assessment Research Program is managed through the Joint Science and Technology Office of the Defense Threat Reduction Agency. Building on the successful completion of the Low Level Chemical Warfare Agent (CWA) Research Program, which addressed the inhalation exposure hazard for traditional CWAs, this program extends that legacy to address the contact hazard from selected CWAs as well as relevant nontraditional compounds. The current research program began in FY07.

1.1 Identifying the Need

As a consequence of the first Gulf War (2 August 1990-28 February 1991), military and scientific communities questioned the accuracy of the (then) current understanding of the residual hazard posed by the intentional use of CWAs against U.S. military personnel. Clearly, the very public discussion surrounding Gulf War Veterans' Illness (GWVI) and the care of individuals with legitimate health issues drove extensive activity to determine, if possible, any linkage to chemical exposures within the battle theater to subsequent multi-symptom clinical manifestations. Numerous possible etiologies have been investigated including, but not limited to, chemical weapon agent release, intentional use of drug prophylaxis, vaccinations and various insecticidal and repellant compounds. In addition, multiple environmental exposure scenarios have been studied, as well as epidemiological analysis addressing stress, concomitant endemic disease exposures and even nutritional factors. Federal funding for research on GWVI totaled $274.0 million for the period from FY97 through FY06 (1). It is fair to say that there has been considerable controversy over whether or not Gulf War syndrome is a physical medical condition related to sufferers' Gulf War service (or relation to a Gulf War veteran). Finally, the entire focus of these efforts has been on hazards posed by the inhalation of selected CWAs. Contact transfer of traditional agents was not investigated.

The Operational Hazard Assessment Research Program is *strictly focused on the immediate effects on exposed military personnel that may impact their ability to execute mission tasks*. A pivotal report (2) questioned the "common wisdom" toxicity values used for years in chemical warfare doctrine, training, and materiel development. Numerous reviews resulted, culminating in a report by the National Research Council, Committee on Toxicology (COT) (3). Those reviews concluded that the Reutter and Wade report had indeed captured all relevant information and that the analysis and conclusions reached were, with slight modifications, valid. A multiservice workgroup was convened at the Institute for Defense Analysis (IDA) in May 1998 that included representation from the operational and medical communities of each service. The report from that workshop (4) noted the "...inherent tension between the stringency of protective measures and the operational burden of implementing them." The workshop was charged to reach a consensus position on those exposure standards as first articulated by Reutter and Wade and reviewed by the COT. Consensus on the exposure standards was reached, and in December 2001 the Assistant to the Secretary of Defense for Nuclear, Chemical and Biological Defense (ATSD/NCBD) granted interim approval (5).

1.2 Addressing the Need with a Dedicated Research Program

Interim certification was granted with the direction that a research program be established to address the data gaps identified within Reutter and Wade and the subsequent reviews. Accordingly, the ATSD/NCBD chartered a Low Level Toxicology Working Group to develop a research plan. The Department of Defense Low Level CWA Exposure Research Master Plan was delivered to ATSD/NCBD in June 2003. It described a broad scope of exposure scenarios, with priority placed on acute, single exposure events deemed most probable by the military operational reviewers, with the first priority to refine the CWA inhalation hazard standards. A research program was created under a Defense Technology Directive (DTO) CB.51:Low-Level CWA Exposure. Progress within the DTO program and the research master plan was reviewed over a two year period by the National Research Council, COT (6). The COT concluded that the master research plan was in general, "...well planned and many of the proposed research tasks are likely to provide valuable information in protecting military personnel from low-level exposure to CWAs and avoiding performance decrements." That program was completed in 2007 and the results published, completing the revision of the inhalation operational exposure values.

1.3 Operational Exposure Standards

Implicit in the research program is the requirement to express CWA exposure risks in terms that are immediately useful for command decisions during military operations. Operational exposure standards must have key characteristics for that purpose. First and foremost, they must provide sufficient information to predict expected casualties and their probability. To accomplish that end, they must provide an ability to extrapolate in time across various mission profiles. In addition, science should never be designed only to dictate to a commander what an "acceptable" level of risk should be. Rather, useful operational exposure standards must allow the commander the ability to anticipate consequences at various levels of risk.

Contact hazard evaluation is substantially more difficult than is the case with inhalation exposures. The exposure component, *i.e.,* how much agent is available for potential transfer to the skin, is complicated and driven by the physical chemistry of the compound, the nature of the material it impacts, reactivity of the agent with the material, as well as the usual time and temperature considerations. A separate program, the Agent Fate Research Program, is designed to characterize those parameters for the compounds of interest on materials of operational significance such as concrete and painted surfaces. The amount of agent that is transferred to the skin – and thus potentially available for absorption and systemic distribution – is poorly characterized for any number of agent/substrate combinations. Determination of this "bioavailability" for a specified agent/substrate will be a critical component in an overall operational hazard assessment. Finally, the nature of skin itself brings complications that are not present with an inhalation exposure. Penetration of a compound through the skin and its absorption into the system varies by compound, moisture content of the skin and by the body region where the contact and deposition occur. Layered upon all of these is the fact that this program will work with compounds for which there are only minimal data to inform a robust understanding of the chemistry and toxic potencies. Progress will be measured with the parallel advances in the Agent Fate Program combined with novel approaches to modeling the bioavailability and physiological absorption, distribution, metabolism and excretion of each compound.

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2 CONTACT HAZARD STUDIES

2.1 Hazard Assessment Studies—Perspectives on the "Contact Hazard"

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2.1.1 Background

2.1.1.1 Historical Definitions of Contact Hazard

The most commonly used definition of "Contact Hazard" was given by Klein (1983).

> Given a surface that has been contaminated with a liquid chemical agent and that surface undergoes a process after which the agent no longer can be detected as a liquid, contact hazard is that situation in which a toxicological hazard can result if an individual then touches that surface with the bare skin.

Schwope et al. (1985) amplified the definition to include soldier contact, not just bare skin, and pointed out that agent contamination could not be detected by liquid chemical detectors. Hence, personnel would not be able to assess the degree of hazard associated with decontaminated equipment.

"The Residual Agent and Contact Hazard Workshop" (Stuempfle and Klein, 1988) limited the definition of contact hazard to the hazard presented by residual chemical agent in surfaces following removal or decontamination of the BULK surface contamination. (The subsequent phenomenon is that chemical agent in the sub-surface can diffuse to the surface and become a hazard.) Later, Klein (1987) described contact hazard as a process dependent upon the diffusion of agent "sorbed" into a surface—meaning either adsorbed or absorbed.

"All contact hazard scenarios have in common the starting point that a toxic material must be delivered on surfaces by an agent delivery system." Although surfaces have been decontaminated, agent can reappear as it desorbs from substrates, and the persistence and magnitude of any potential residual hazard must be ascertained (Carlon, 1990). As re-stated by Carlon (1990), the ability to detect vapor will not suffice to warn troops of a contact hazard. Both authors stressed that the degree of physiological response from a contact hazard is often greater than that predicted from detectable vapor coming off the surface or the PC toxicity vapor data.

In 1990, Carlon stated that Klein's (1983) definition of contact hazard was too narrow for several reasons:

(1) "...what solid particles such as agents of biological origin (ABO) are contacted on surfaces?"

(2) "What if toxic vapors are contacted percutaneously, rather than by the respiratory route?"

(3) "What if the skin is clothed?"

(4) "What if contamination is first picked up on the fingers and then transferred to the skin or to the mouth where it is ingested?"

(5) "What about the inhalation threat from particulates that are re-aerosolized from surfaces?"

(6) "What about secondary vapor threat from contaminated surfaces as they weather, especially in enclosed areas?"

(7) "What about the threats from liquid/solid mixes such as "dusty" agents that use solid carriers in which liquids are absorbed from improved dissemination?"

2.1.1.2 The Issues at Hand and Theories of Contact Hazard

Implicit in the notion of contact hazard is the fact that the agent is somewhat persistent. [The Department of the Army (1963) defined persistency as "an expression of the duration of effectiveness of a war gas, which is dependent on physical and chemical properties of the gas, weather, methods of dissemination, and condition of terrain".] Rubber, plastic, paint, crevices, and porous surfaces absorb chemical agent and then release it after the surface of the equipment has been decontaminated. When the object is held in contact with the skin, some of the agent can migrate (diffuse) to the surface of the object and transfer to the skin. The hazard is a function of contact time, amount of agent, and agent migration rates. As stated by Armour and Sturgeon (1992), the extent of the contact hazard depends on the initial degree of contamination, the meteorological conditions, the surface type, and the time between the attack and the resumption of operations.

There are several different theories and models for what produces a contact hazard. It is not clear that any one theory or model is applicable to all situations. The two primary models of contact hazard were described by Manthei *et al.* (1988). In the first (the diffusion/vapor transfer model), agent is assumed to have been sorbed into a material, then it diffuses back to the surface from which it evaporates and is absorbed as vapor by a contacting surface (*e.g.*, skin). In the second (diffusion/surface distribution transfer model), it is postulated that the surface of the contaminated material can be compared to a pseudo-liquid, and agent transfers across the interface to the contacting surface. In the latter case, the rate of transfer is faster than that for vapor.

The diffusion/vapor transfer model was proposed first and was the driver for determining acceptable levels of decontamination [or an acceptable level of contamination—how "dirty" is clean enough?]. The levels of acceptable decontamination were actually defined by head-space analysis of decontaminated materials and dominated the experimental data for a number of years (Hall *et al.*, 1989). However, it was subsequently realized that a flowing airstream may not be as efficient a sink for desorbing agent as a contacting surface and that the vapor hazard did not relate to the amount of agent remaining in painted surfaces. The latter mechanism has since been substantiated, and analysis of the data indicates that both mechanisms may play roles in contact hazard—depending upon the volatility of the agent and the surface.

In fact, Klein (1987) stated that the agent flux from a surface is no measure of the contamination in the surface or of the hazard to the soldier. He further stated that meaningful interpretation of the degree of hazard associated with a surface requires that the contamination history and physical properties of the surface are well known, and this information is more important than the data collected from the surface itself. Carlon (1990) amplified this by saying the data suggest that the hazard from a liquid-free surface can exceed predicted hazard based on directly measured vapor evolution rates.

It is intuitive that if a persistent agent produces sufficient agent vapor to trigger a vapor detector a contact hazard is likely to exist. However, surfaces that have been decontaminated and/or otherwise appear to be clean may still present significant contact hazards *via* transfer of pseudo-liquid when skin is directly contacting a surface or—depending upon the agent and surface, *via* transfer of minute amounts of vapor when the skin is apposed to the surface but does not directly touch it. For practical purposes on today's battlefield, "Contact Hazard" must be defined as the toxicological hazard arising from any potentially contaminated surface that is capable of transferring toxic quantities of chemical agent to the skin.

2.1.2 Data

There are two recent reviews of the existing data on contact hazard (Reutter *et al.*, 2006; Reutter and Sommerville, *September 2009*). There are two primary problems in assessing contact hazard: 1) The existing data are limited and were designed to answer questions different from those

being posed today. 2) The percutaneous (PC) toxicity of the nerve agents and non-traditional agents has not been adequately modeled, and the confidence in the human toxicity estimates is extremely low. In short, the hazards of PC exposure to residual agent cannot be adequately characterized until there is better comprehension of their intrinsic toxicity.

2.1.2.1 PC Toxicity

There is low confidence in the human nerve agent toxicity estimates for PC exposure (Reutter and Wade, 1994; COT, 1997; Reutter et al., 2003), and the potency of the liquid agents may be underestimated.[a] Sommerville (2004)[b] performed a statistical review of the existing PC vapor lethality data for mammalian whole-body exposures. Using linear regression, three statistical trends were identified:

1) On an absolute dosage basis, larger species are less sensitive to PC vapor exposures than smaller species. This is probably due to differing surface area to volume ratios of large animals *versus* small animals.
2) PC vapor potency increases as volatility decreases.
3) The intrinsic toxicity of an agent—as represented by the intravenous (IV) toxicity, is the most important factor for scaling PC vapor lethality, followed by equal contributions from species body mass and agent volatility.

Unfortunately, the quality of the above datasets is such that rather large error bars are associated with the human toxicity estimates. Additional mammalian data are required to improve the precision of the estimates. More precise human toxicity estimates are required before "contact hazard" can begin to be understood. Indeed, Elskamp *et al.* (1973) stated that to evaluate contact hazard, it is necessary to know the minimum amount of agent producing an effect on human skin.

2.1.2.2 Existing PC Data

Human testing is no longer done, and the existing human data for nerve agents are sparse and limited to rather mild effects[c] in relatively healthy, young male volunteers. Animal studies have been primarily designed to determine severe and lethal effects, and these studies were not adequately designed to provide data for extrapolation to humans.

The situation is further complicated by the fact that modeling human toxicity estimates is especially difficult for PC exposures. There are huge species differences in skin, and there are body region differences within species (Wester and Noonan, 1980). The effects of these differences on PC absorption span orders of magnitude. Hence, effective toxic doses can vary markedly from one body region to the next and can be a function of anything affecting skin permeability—the physical properties of the agent, heat, humidity, perspiration, *etc.* (Effective dosages in humans are also a function of the presence or absence of clothing [or hair]).[d] Toxic effects in animals can be a function of the presence or absence of fur, as well as the manner in which the fur was removed [Muir and Callaway, 1951]. [Unlike humans, most laboratory species have fur and do not perspire.])

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[a] A meta-analysis was performed on the intravenous (IV) and PC data for nerve agents in multiple animal species to develop a model for estimating human toxicity. The effort provided a good statistical basis to indicate that many of the human toxicity estimates are too high—they underestimate the potency to humans.

[b] Sommerville, DR. *Review and Statistical Analysis of Mammalian Nerve Agent Percutaneous Vapor Lethality Data*, Internal ECBC Technical Note. US Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 1 March 2004.

[c] In Cullumbine *et al.* (1954) one individual died and another had very severe effects; many other subjects were exposed to these and higher doses and suffered only mild effects. The cause of these apparently untoward responses can only be speculated.

[d] Depending upon the agent and exposure scenario hair and/or clothing can be protective; however, they can also trap the agent and in the case of volatile agents, effectively increase the delivered dose.

The sensitivity of animals—relative to humans, has not been systematically researched for any of the chemical agents. Because animal data are required for modeling human toxicity, understanding the relative sensitivity of humans and various test species is critical to establishing robust human toxicity estimates. A metric must be established for extrapolating animal data to humans.

2.1.3 Contact Hazard Dogma

Two fallacious lines of reasoning have heretofore plagued the science of understanding contact hazard. The first is the notion that if agent cannot be detected, the surface is "safe". The second is that less absorptive surfaces are better. Both perceptions were invalidated by the work of Manthei *et al.* (1983, 1985, 1986, 1988)[6], but these have yet to fully register with the collective consciousness regarding contact hazard.

The first notion largely stemmed from the fact that chemical agent detectors were designed to detect vapor. Unfortunately, a significant amount of research into contact hazard was predicated on detecting agent vapor—rather than directly assessing the toxic hazard of a formerly contaminated surface, and it was erroneously concluded that if an agent were not detected, there was none present, and the surface was safe.

The second notion stemmed from the work to develop agent-repellant surfaces (*e.g.*, paints, coatings, *etc.*) and "success" was measured in terms of how little agent was absorbed—rather than from the perspective of determining the bioavailability and resulting hazard from the sorbed agent. A material to which agent is tightly bound (sorbed) may be less of a hazard than a material to which a significantly lesser quantity of agent is less tightly bound. A corollary to this latter concept is the recognition (and prevention) of conditions causing a material to release its sorbed agent.

2.1.4 The Way Forward

In order to set levels for detection, decontamination, and protection, it is important to better understand the PC toxicity of the agents in question and have defensible human toxicity estimates for the quantities of agent that are likely to be toxic. The latter can be accomplished only with appropriate toxicological studies.

Further, the data indicate that one cannot divorce chemical and toxicological assays—both are important. Finally, "Contact Hazard" must be broadly viewed as any potential hazard from the transfer of agent to skin whether or not it is in direct contact with a contaminated surface.

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2.2 Evaluation of the Toxicokinetics of Subcutaneous Exposure to Sub-Lethal Doses of VX, GF and GB in the Guinea Pig

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Mathematical models of the absorption, distribution and elimination of chemical warfare nerve agents (CWNAs) can be used to improve toxicity estimates, extrapolate to humans and predict response to a range of nerve agent exposures. These studies were performed to compare toxicity and kinetic data following subcutaneous (SC) exposure to different nerve agents in male guinea pigs. Animals were given a single sub-lethal SC dose of VX (S-[2-(diisopropylamino)ethyl]-O-ethyl methylphosphonothioate) sarin (GB), or cyclosarin ([GF] [either 0.1 or 0.4 x LD_{50}]). Blood levels of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), as well as of regenerated VX, ethyl methylphosphonofluoridate (as the G-analog or VX-G), GB (rGB) or GF (rGF), were evaluated at multiple time points following exposure. In plasma, for each exposure of VX, GF or GB, toxicokinetic parameters such as the area under the curve (AUC), maximum concentration (Cmax), time to maximum concentration (tmax) and clearance (CL) were similar for each respective dose for rGB and rGF; however, the values for these parameters except CL were different for VX-G (much lower for AUC and Cmax and higher for tmax). Furthermore, the half-life (t½) for VX-G in plasma was four or five times as long, respectively, compared to rGF and rGB. In red blood cells (RBCs), similar kinetic results occurred for the G-agents except that CL from RBC was higher for rGF than rGB and the t½ for the 0.1 x LD_{50} GB dose was higher than the other RBC t½ values. The RBC AUC, Cmax and t½ for VX-G were much less compared to the G-agents; however, RBC CL of VX-G was similar to rGF and VX-G tmax was slightly higher than the G-agents. For all agents, the respective tmax was higher for RBC than plasma, while the reverse was true for Cmax. It appears that VX and GF have a greater inhibitory action upon guinea pig AChE activity. It occurs earlier and persists longer than GB; however, both GF and GB had a much greater inhibitory effect upon plasma BChE than VX. Although some toxicokinetic parameters were similar for VX-G, rGB and rGF, differences that were likely due to the different physicochemical properties of the agents did occur. These data will be utilized in a physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) model developed to simulate low-level nerve agent exposures in various species, and the results will be applied to risk assessment determinations.

2.2.1 Introduction

Current nerve agent risk assessments depend on animal data, which is mainly based on the parenteral route of administration. A sizeable SC database has been established using the guinea pig model for studies of lethal and sub-lethal effects of CWNAs. SC injections have proven to be a convenient method for exposing guinea pigs to nerve agents in order to study mechanisms of action and related prophylactic and therapeutic treatment approaches. Nevertheless, in order to better utilize such data for assessing risks involving the more probable route of battlefield exposure for most nerve agents, *i.e.*, the inhalation route, a PBPK/PD method for relating and integrating nerve agent toxicity data across different routes of exposure would be helpful in risk extrapolation. It is known that the route of exposure is an important determinant of the toxicity observed (1). Also PBPK/PD models are useful to simulate the time course concentration of chemicals in experimental animals and humans, to better determine estimates of actual chemical doses delivered to target tissue, and to thereby provide a better prediction of response. These models require that a common dose metric be used to relate uptake and distribution/elimination of nerve agent in the blood and organs between the different routes of exposure for doses of agent with similar levels of toxic effect (2; 3; 4).

2.2.2 Objectives

The goal of the current study was to compare uptake and CL kinetics of sub-lethal doses of GB, GF, and VX in the blood of unanesthetized male guinea pigs exposed to these agents by the SC route of administration. Another study, designed similarly to this one, examined the uptake, distribution and kinetics of GB in blood and tissue following sub-lethal whole body inhalation exposure in guinea pigs (5). The results from these types of studies will later be used to integrate the toxicity data across different routes of exposure in guinea pigs. The animals were given a single sub-lethal SC dose of either GB or GF. Two sub-lethal doses of each agent were evaluated (0.1 and 0.4 x LD_{50}). Regenerated sarin, regenerated cyclosarin and VX (6) were measured from serial blood samples and used as the dose metric to compare the kinetics of each agent administered SC to guinea pigs. The distribution of rGB, rGF and VX-G in various tissues at different time points was also measured. Cholinesterase (ChE) inhibition and recovery was also evaluated in blood samples. These data will be used in a PBPK/PD model, developed to simulate low-level exposures. Earlier a physiologically based model (7) was developed that described the in vivo toxicokinetics of an intravenous (IV) bolus of C(±)P(±)-soman (GD) at 0.8 to 6 LD_{50} doses in the male guinea pig; however, in this study the animals were anesthetized, atropinized and artificially ventilated.

2.2.3 Materials and Methods

2.2.3.1 Chemicals and Reagents

Isopropyl methylphosphonofluoridate (sarin or GB; CAS No. 107-44-8; also known as EA 1208) was obtained from ECBC chemical agent standard analytical reagent material (CASARM) stock (certified purity > 97%). Prior to use CASARM-grade GB (lot # GB-U-6814-CTF-N [GB2035]) was verified by quantitative ^{31}P-NMR and stored in sealed ampoules containing nitrogen. Cyclohexyl methylphosphonofluoridate (GF or cyclosarin; CAS No. 329-99-7; also known as EA 1212) was also used for SC exposures in this study. Cyclosarin (lot # GF-93-0034-109 (GF-S-6092-CTF-N-1)) was also distilled at ECBC and verified as 98.87 ± 0.50% pure (as determined by quantitative ^{31}P-NMR) and stored in sealed ampoules containing nitrogen (note: this agent was not CASARM-grade). S-[2-(diisopropylamino)ethyl]-O-ethyl methylphosphonothioate (VX; CAS No. 50782-69-0; also known as EA 1701) was also used in this study. Before use CASARM-grade VX (lot # VX-U-5055-CTF-N) was verified by quantitative ^{31}P-NMR to be 95.7% pure and was stored in sealed ampoules containing nitrogen. Sealed GB, GF and VX ampoules of <2 mg agent/mL in saline were provided to the MRICD where the agent was diluted for injection. Triethyl phosphate ([TEP] 99.9% purity; Aldrich Chemicals, Milwaukee, WI) was used as the internal standard (IS) for GB, GF and VX purity assays. Chemical analysis and agent transfer records are kept on file with all chemical analysis records associated with animal exposure records for GB, GF and VX. Also $^{2}H_{6}$-sarin ($^{2}H_{6}$-isopropyl methylphosphonofluoridate) and $^{2}H_{11}$–cyclosarin ($^{2}H_{11}$-cyclohexyl methylphosphonofluoridate) of purity greater than 97% and $^{2}H_{5}$-VX-G with 93% deuterium purity were procured from ECBC APG and used as gas chromatography-mass spectrometry (GC-MS) ISs.

Analytical grade solvents used in this study were obtained from Sigma-Aldrich (St. Louis, MO). Gases for the analytical instruments were obtained from Messer, Inc. (Chattanooga, TN) and had a minimum purity of 99.999%. All chemicals, solvents, and gases were used as obtained with no further purification.

2.2.3.2 Animals (Guinea Pigs)

The animal model of choice for this study was the male Duncan Hartley guinea pig (*Cavia porcellus*) (Crl: (HA) BR COBS; Charles River Labs, Kingston, NY). Upon arrival, the animals were quarantined for a week and tested for evidence of disease. They were individually housed in polycarbonate cages in temperature (21 ± 2°C) and humidity (50 ± 10%) controlled animal quarters maintained on a 12 hr light-dark full spectrum lighting cycle with lights on at 0600. Guinea pig laboratory chow and water were available *ad libitum* whenever the animals were in home cages. The research environment and protocols for animal experimentation were approved by the institutional animal care and

use committee. The animal facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

The guinea pig has a number of advantages over other rodent species, such as the mouse and rat, for such studies. The lower circulating levels of carboxylesterases in the guinea pig, compared to the rat or the mouse, minimize the influences of these enzymes (non-specific binding sites) on the distribution/elimination calculations for organophosphate nerve agents (8; 9). Compared to other rodents, guinea pigs are more similar to non-human primates in their response to pyridostigmine pretreatment for protection against oxime-resistant nerve agents. In addition, AChE data for this species has been extensively examined in-house (10; 11). Further, the guinea pig is the best approximation to non-human primate sensitivity to CWNA among rodent species (12). This study complements other in-house and extramural nerve agent study protocols in which data were generated with the use of guinea pigs. Male animals were used to avoid possible variation due to changes in steroid hormone levels. At the time of the single SC agent exposure the animals weighed between 300-375 g.

2.2.3.3 Surgery

Male guinea pigs were identified by number on an ear, using a permanent marker to identify individual animals prior to surgery and exposure. On the day before agent exposure for the kinetic studies, a surgery was performed on the animals to place a cannula in the right carotid artery for repeated blood draws. The guinea pigs were anesthetized using isoflurane (3% induction, 1.5-2% maintenance; with oxygen). All procedures were performed using aseptic technique as described in SOP-VMSB-306 (dated Jan 2004), entitled "Surgery". Once a surgical plane of anesthesia was reached, the animal was placed on its back, the neck area was clipped and prepped with betadine, and the animal covered with a sterile surgical drape. The planned incision areas in the ventral and dorsal neck were infused with SC injections of 0.1% lidocaine. For carotid cannula placement, a midline incision was made in the neck just over the trachea extending toward the heart. The carotid, which runs parallel to the trachea, was identified and isolated by blunt dissection; two lengths of 4-0 silk suture were passed under the carotid ~0.5 cm apart and loosely tied; the carotid was gently lifted and a small incision made in the wall of the artery through which a piece of polyethylene tubing, filled with a saline/anticoagulant solution (sodium Heparin®, 100 Units/mL), was inserted ~1.0 cm toward the heart and tied in place with the sutures. A small trochar was then run under the skin from the ventral neck incision site to the dorsal neck area behind the head and pushed through a small snip in the skin; the piece of tubing was threaded through the trochar leaving 2-3 cm exposed, and then anchored in place in the skin using a small suture. The neck incision was closed with wound clips or sutures. The cannula was checked for patency, flushed again with the saline/anticoagulant solution and then capped with a stainless steel plug. The animal was then placed in a surgical recovery chamber equipped with a covered warming pad until normal righting reflex returns. Pain medication was given SC after surgery (10.5 µg/animal of buprenorphine hydrochloride, trade name Buprenex®). The animals were returned to the home cage when complete recovery was observed. On the following day, they were exposed to SC GB, GF, or VX.

2.2.3.4 SC GB, GF and VX Exposure

The SC exposure study was conducted at MRICD. These studies employed an SC dosing model previously established for studies of repetitive sub-acute, sub-chronic, and chronic exposures in the guinea pig (11). The GB, GF and VX SC LD_{50}s for male guinea pigs are, respectively, 42, 57, and 8 µg/kg (11). The animals were injected SC in the right flank area with either GB, GF, or VX (0.1 or 0.4 x LD_{50}) dissolved in sterile saline. Doses were calculated for delivery in 0.5 mL/kg of saline. During the study, the guinea pigs were observed for clinical manifestations of agent toxicity such as localized muscle fasciculations, tremors, and salivation; however, at the sub-lethal concentrations used in this study, overt clinical signs of agent toxicity were not observed.

2.2.3.5 Blood and Tissue Samples

Approximately 0.5 to 1.0 mL of whole blood was removed from each animal at each time point (collected before exposure to determine baseline ChE activity and at various time intervals from 1 min to 48 hr for post-exposure ChE activity and for regenerated agent; however, the total volume

removed from each animal was no more than 1% of its body weight and a volume of physiological saline equal to the amount removed at each interval was administered via the carotid cannula) and collected in BD Microtainer® plastic tubes with dipotassium EDTA additive to prevent clotting (BD, Franklin Lakes, NJ). The blood was separated into RBCs and plasma fractions by centrifugation (15,000 x g for 5 min) and used for the regeneration assays. Whole blood used for the ChE assays (either 40 or 80 µL whole blood) was diluted in distilled water and snap frozen on dry ice before analysis. Heparinized arterial blood was used to examine various physiological parameters. Blood sodium, potassium, pH, pCO_2, pO_2, O_2 saturation, glucose, lactate, bicarbonate, hematocrit and hemoglobin were determined using an i-STAT analyzer equipped with EC4+ and CG4+ analysis cartridges or CG8+ analysis cartridges (13). In this study, the physiological values obtained using i-STAT analysis were within normal parameters for the guinea pig.

At the end of blood collection, guinea pigs were euthanized with Nembutal® (0.7 mL into the carotid artery; 50 mg/mL). Following euthanasia and saline perfusion, samples of heart, lung, brain, kidney, diaphragm, eyes and liver, as well as the GB, GF or VX injection skin site and skin from the contralateral non-injected side, were removed and snap frozen in liquid nitrogen. After freezing, the samples were sealed and stored at -70 °C for later analysis of tissue levels of ChE and fluoride-regenerated GB, GF and VX. These data will not be presented in this paper.

2.2.3.6 Fluoride Regeneration Product Determination Methods

For fluoride regenerated nerve agent assays, the erythrocyte (RBC) fraction and plasma were utilized. Regenerated GB (rGB), regenerated GF (rGF) or regenerated VX (VX-G) in these samples was analyzed by GC-MS after a C18 solid-phase extraction sample preparation that included fluoride ion addition and pH adjustment by the method of Jakubowski *et al.* (6). The measurements of rGB, rGF and VX-G (mw = 126) were conducted at ECBC. The fluoride regeneration assay is a powerful technique and has been utilized for other nerve agents as well (6, 14-16; 17; 18; 19).

2.2.3.7 ChE Determination Methods

The AChE and BChE activity in blood (using acetylthiocholine and butyrylthiocholine as the substrates) was determined by an automated method using a SPECTRAmax Plus Microplate Spectrophotometer (Molecular Devices Corp., Sunnyvale, CA). The analytical procedure used (20; 21) was based on the manual method of Ellman *et al.* (22) and modified for the microplate spectrophotometer. The AChE and BChE activity was analyzed at MRICD.

2.2.3.8 Data Analysis

Differences in pre-exposure *versus.* post-exposure ChE levels were expressed as a change in activity (Units/mL) resulting from GB, GF or VX exposure. This graded response was plotted against various combinations of exposure concentration and duration in order to determine if correlations exist as indicated by significant ($p<0.05$) correlation coefficients. Student's t-test was utilized to compare the peak rGB, rGF and VX-G concentrations in plasma and RBC between the different GB, GF or VX concentrations for significance at the $p<0.05$ level. The following pharmacokinetic parameters were calculated from the graph of plasma or RBC rGB, rGF, and VX-G concentration versus time: time to reach the maximal concentration (t_{max}), maximal concentration (C_{max}), CL, the K_{01}, K_{10}, K_{12} and K_{21} values, and the area under the plasma and RBC concentration time curves extrapolated to infinity (AUC) using WinNonlin® analysis program 5.0. The $t_{½}$ for rGB, rGF and VX-G in plasma and RBC was also calculated.

2.2.4 Results

2.2.4.1 Regenerated Agent Concentration and Kinetics in Guinea Pig Plasma and RBC Following Sub-Lethal SC Exposures to GB, GF or VX

At the 0.4 x LD_{50} SC exposure (Figure 1), much higher concentrations of rGB (~60 ng/g in plasma and ~9 ng/g in RBC) and rGF (~60 ng/g in plasma and ~5 ng/g in RBC) were found in both

plasma and RBC compared to VX-G (2 ng/g in plasma and ~0.5 ng/g in RBC). Similar results occurred at the 0.1 LD_{50} exposures as well (Figure 2). However, following the 0.1 LD_{50} VX exposure, it was not possible to measure RBC VX-G at any time point (the VX-G concentration was <0.005 ng/g) (Figure 2, bottom).

Although there are a number of differences in the toxicokinetic data at 0.4 x LD_{50} exposures (*i.e.*, AUC and C_{max} of G-agents *versus*. VX; RBC CL with GF and VX similar; plasma and RBC t_{max}; plasma and RBC $t_{½}$) (Tables 1 and 2) between the three agents, there are also a number of similarities for the agents (plasma CL rates for all agents; GF and VX RBC CL rates; G-agent plasma and possibly RBC AUC, plasma and RBC C_{max}, plasma and RBC t_{max}, plasma and RBC $t_{½}$ at 0.4 x LD_{50}) (Tables 1 and 2). In plasma, for each exposure of VX, GF, or GB, toxicokinetic parameters such as the AUC, C_{max}, t_{max} and CL, were similar for each respective dose for rGB and rGF. However, the values for these parameters except for CL, were different for VX-G (much lower for AUC and C_{max} and higher for t_{max}) (Tables 1 and 2). Furthermore, the $t_{½}$ for VX-G in plasma was four or five times as long, respectively, compared to rGF and rGB (Tables 1 and 2). In RBC, similar kinetic results occurred for the G-agents except that CL from RBC was higher for rGF than rGB (Tables 1 and 2) and the $t_{½}$ for the 0.1 x LD_{50} GB dose was higher than the other RBC $t_{½}$ values (Table 1). The RBC AUC, C_{max} and $t_{½}$ for VX-G were much less compared to the G-agents; however, RBC CL of VX-G was similar to rGF and VX-G t_{max} was slightly higher than the G-agents (Tables 1 and 2). For all agents the respective t_{max} was higher for RBC than plasma, while the reverse was true for C_{max} (Tables 1 and 2). Various toxicological and physical properties are given in Table 3. On a µmol/kg basis (*i.e.*, the guinea pig LD_{50}), GB and GF are equitoxic via SC exposure while VX is 10x more toxic (Table 3). Water and lipid solubility are similar for VX and GF (Table 3); GB is more soluble in water but less soluble in lipids (Table 3).

2.2.4.2 AChE and BChE Activity in Guinea Pig Plasma and RBC Following Sub-Lethal SC GB, GF, or VX Exposures

SC exposures of 0.1 x LD_{50} GB had minimal effects upon AChE activity (Figure 3, top), but at this concentration both GF and VX depressed AChE activity substantially (Figure 3, top). However, 0.4 x LD_{50} GB, GF and VX exposures depressed AChE activity markedly with slow recovery (Figure 3, bottom); however, GF and VX had a much greater effect upon AChE activity than GB at this concentration (Table 3). AChE recovery was not complete 24-48 hr after exposure (Figure 3, top and bottom). At 0.1 x LD_{50}, GB had minor effects upon BChE activity (Figure 4, top), but 0.4 x LD_{50} GB and both GF concentrations depressed BChE activity markedly (Figure 4, bottom) concomitant with recovery by 24 hr. In contrast, VX at both sub-lethal concentrations had no or minimal effects upon BChE activity (Figure 4, top and bottom; also see Table 3).

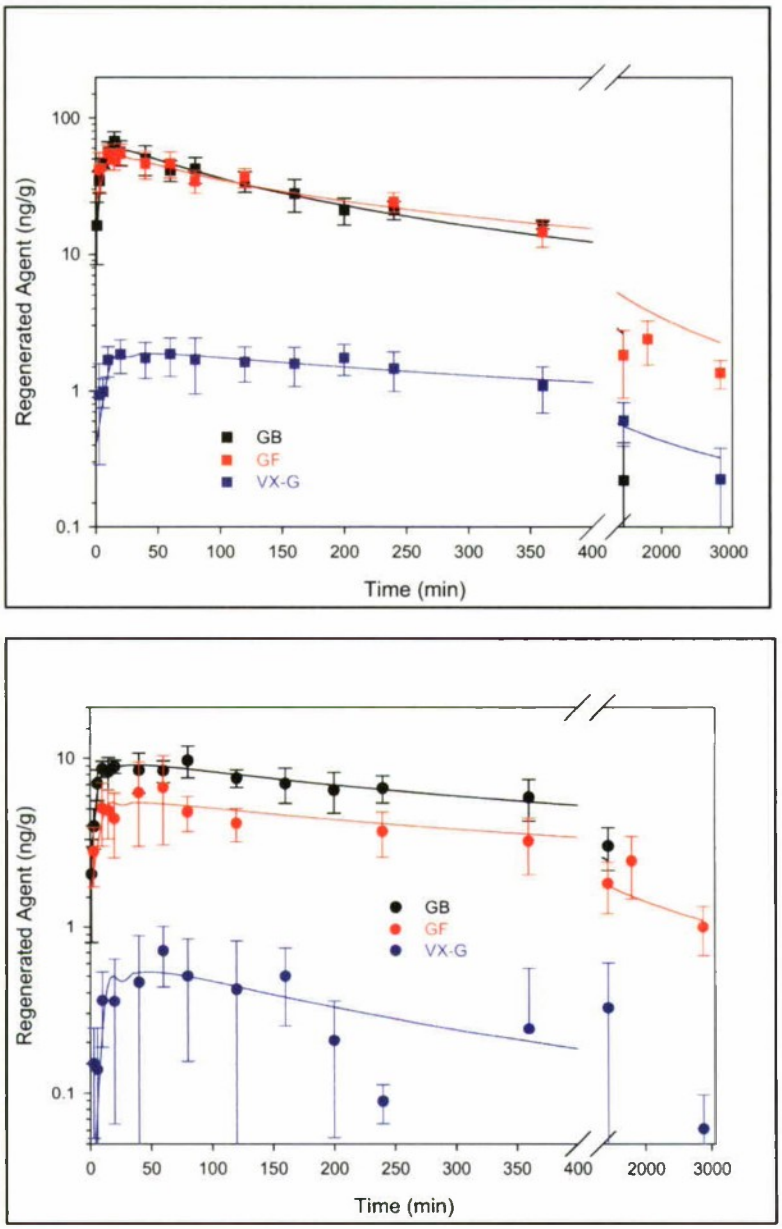


Figure 1. Regenerated Agent from Guinea Pig Plasma and RBC as a Function of Time Following SC CWNA Exposure at 0.4 x LD_{50}

Guinea Pig Plasma represented in top figure as colored squares. RBC represented in bottom figure as colored circles. The results were fitted according to y = ae(-0.5[ln(x/x0)/b]^2). Standard deviation bars are also shown.

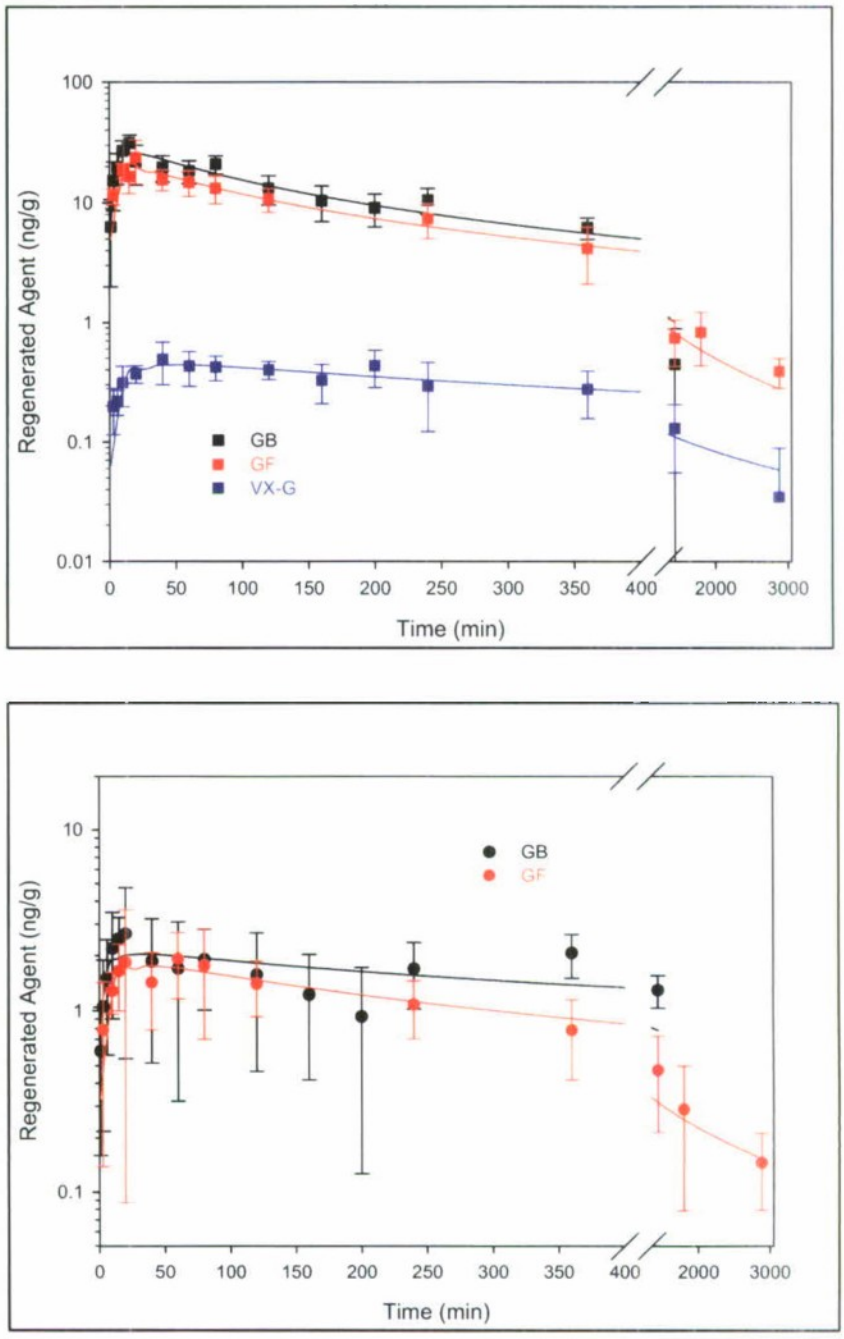


Figure 2. Regenerated Agent from Guinea Pig Plasma and RBC as a Function of Time Following SC CWNA Exposure at 0.1 x LD_{50}

Guinea Pig Plasma represented in top figure as colored squares. RBC represented in bottom figure as colored circles. (Note: RBC VX-G <<0.005 ng/g). The results were fitted according to y = ae(-0.5[ln(x/x0)/b]^2). Standard deviation bars are also shown.

Table 1. Toxicokinetic Parameters of rGB, rGF and VX-G in Guinea Pigs Following a Single SC Exposure of either 0.1 x LD_{50} or 0.4 x LD_{50} (GB, GF or VX)

Parameter	Agent	0.1 LD_{50}		0.4 LD_{50}	
		Plasma	RBC	Plasma	RBC
AUC (min-ng/g)	GB	6812	18183	14457	19935
	GF	6591	1848	20165	11099
	VX	504	NA	2473	1691
C_{max} (ng/g)	GB	26.5	2.50	60.9	9.03
	GF	19.7	1.81	53.7	5.79
	VX	0.45	NA	1.88	0.59
CL (g/min)	GB	0.18	0.07	0.37	0.27
	GF	0.25	0.90	0.33	0.60
	VX	0.45	NA	0.20	0.54
K_{01} (min^{-1})	GB	0.23	0.10	0.20	0.22
	GF	0.23	0.13	0.49	0.15
	VX	0.11	NA	0.17	0.02
K_{10} (min^{-1})	GB	0.004	0.0002	0.005	0.0005
	GF	0.003	0.001	0.003	0.006
	VX	0.001	NA	0.0008	0.0009
K_{12} (min^{-1})	GB	0.006	0.036	0.01	0.001
	GF	0.004	0.002	0.001	0.002
	VX	0.001	NA	0.0006	0.017
K_{21} (min^{-1})	GB	0.013	0.020	0.03	0.001
	GF	0.004	0.002	0.002	0.003
	VX	0.003	NA	0.002	0.003
t_{max} (min)	GB	14.3	18.5	14.6	22.6
	GF	15.6	29.5	9.89	27.6
	VX	40.0	NA	28.7	55.9
$t_{½}$ (min)	GB	165	3466	158	770
	GF	173	533	204	990
	VX	630	NA	866	161

NA = Data Not Available. The results were fitted with a two-compartment pharmacokinetic model with constant input and first-order output.

Table 2. Toxicokinetic Trends of Regenerated Nerve Agent in Guinea Pig Plasma and RBC Following a Single SC Exposure to either 0.4 x LD_{50} GB, GF or VX

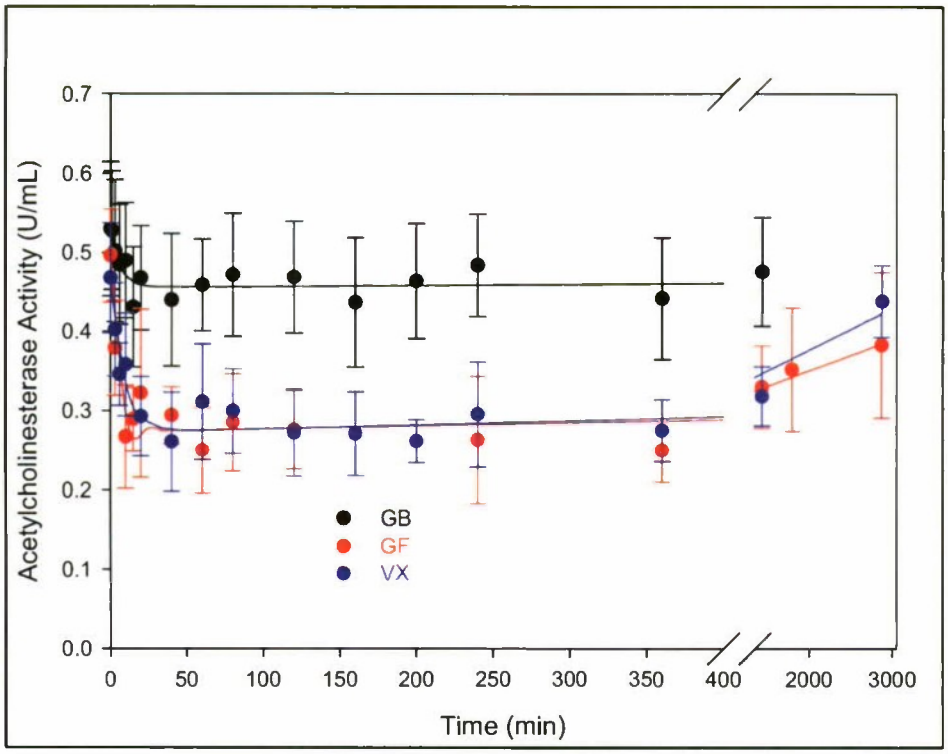
0.4 x LD_{50}				
Parameter	**Plasma**	**Plasma Trend**	**RBC**	**RBC Trend**
AUC GB	14457	=	19935	↑↑
AUC GF	20165	=	11099	↑
AUC VX-G	2473	↓↓↓	1691	↓↓↓
C_{max} GB	60.9	=	9.03	=
C_{max} GF	53.7	=	5.79	=
C_{max} VX-G	1.88	↓↓↓	0.59	↓↓↓
CL GB	0.37	=	0.27	↓
CL GF	0.33	=	0.60	=
CL VX-G	0.20	=/↓	0.54	=
t_{max} GB	14.6	=	22.6	=
t_{max} GF	9.89	=	27.6	=
t_{max} VX-G	28.7	↑	55.9	↑
$t_{½}$ GB	158	=	770	=
$t_{½}$ GF	204	=	990	=
$t_{½}$ VX-G	866	↑	161	↓↓↓

Table 3. Various Toxicological and Physical Properties of VX, GF and GB

Agent	**MW** (Daltons)	**LD_{50}** (µg/kg) [GP SC]	**Potency Ratio**	**LD_{50}** (µmol/kg) [GP SC]	**Potency Ratio**	**Water Solubility** (g/100 g @ room temp)	**Lipid Solubility*** (octanol:water partition coeff Log P)	**% AChE Inhib** (0.4xLD_{50})	**% BChE Inhib** (0.4xLD_{50})
VX	267.37	8	6	0.03	10	5	0.675 ± 0.070**	99	30
GF	180.14	57	1	0.32	1	3.7	1.038 ± 0.055	99	87
GB	140.10	42	1	0.30	1	100	0.299 ± 0.016	70	62

*(23)

**Other sources give a higher value for VX, *i.e.*, 2.09 (24)



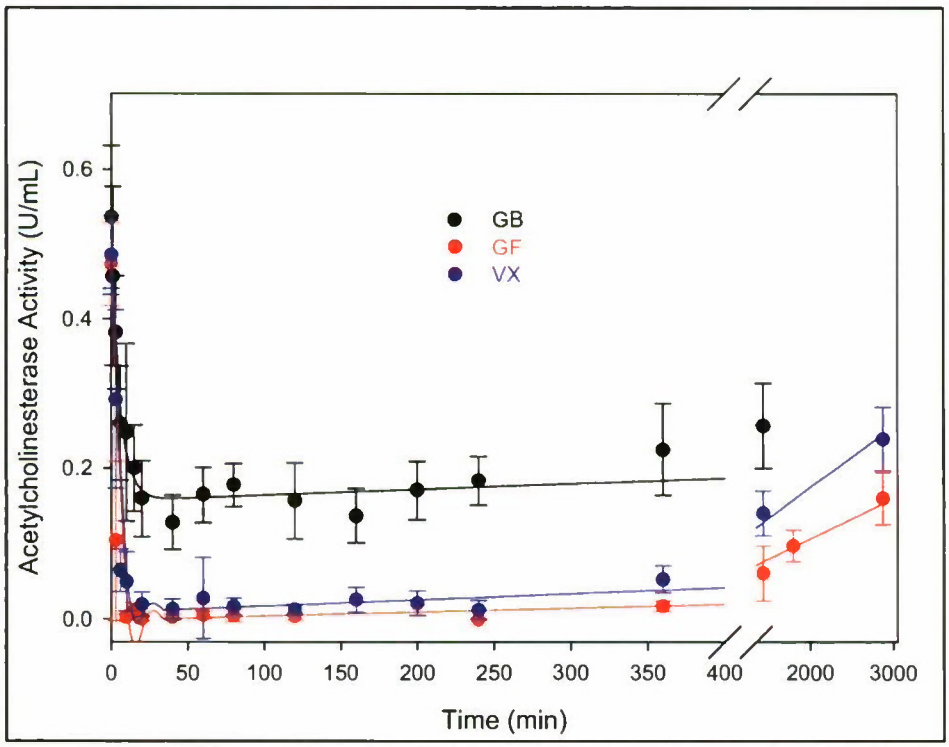
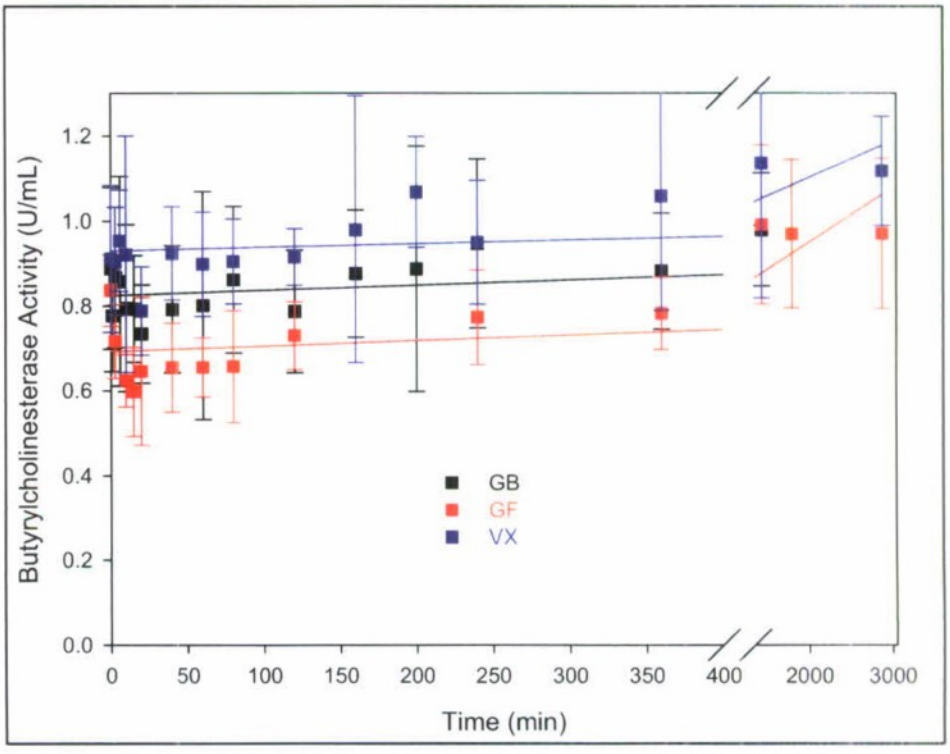


Figure 3. AChE Activities in Guinea Pigs as a Function of Time Following SC Exposure to CWNA

AChE Activities are represented by colored circles. 0.1 x LD_{50} (top); 0.4 x LD_{50} (bottom). The results were fitted according to y = y0 + ae-bx + cx. Standard deviation bars are also shown.



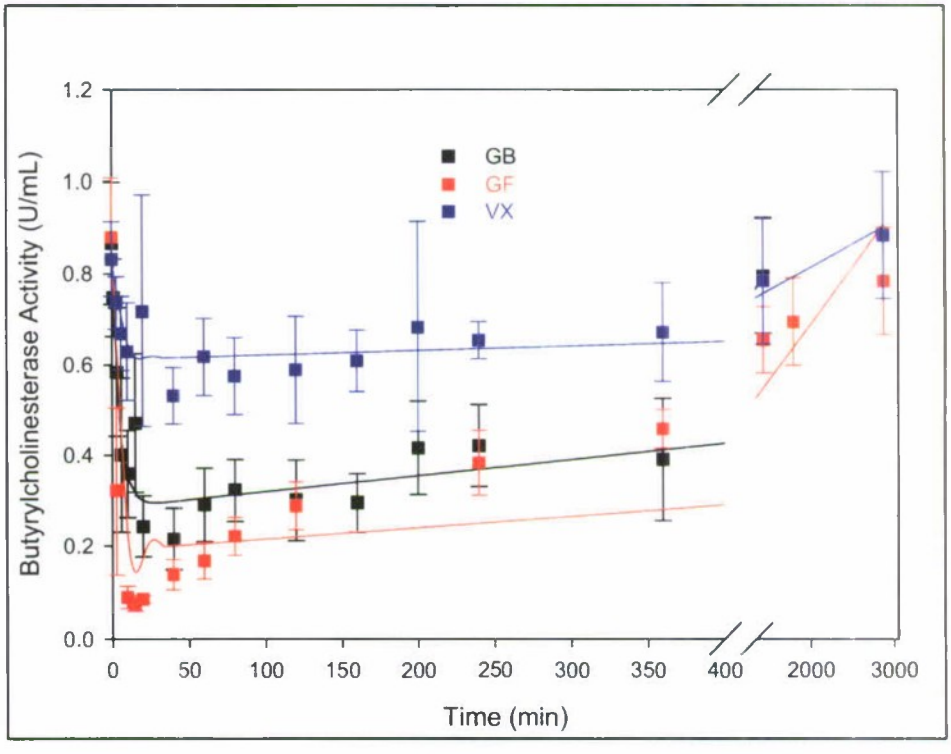


Figure 4. BChE Activities in Guinea Pigs as a Function of Time Following SC Exposure to CWNA

BChE Activities are represented by colored squares. 0.1 x LD_{50} (top); 0.4 x LD_{50} (bottom). The results were fitted according to y = y0 + ae-bx + cx. Standard deviation bars are also shown.

2.2.5 Conclusions

It appears that VX and GF have a greater inhibitory action upon guinea pig AChE activity, one that occurs earlier and persists longer, than GB; however, both GF and GB had a much greater inhibitory effect upon plasma BChE than VX. Although some toxicokinetic parameters were similar for VX-G, rGB and rGF, a number of differences did occur that were likely due to the different physicochemical properties of the agents.

It appears that (±)-VX is substantially more persistent *in vivo* than either GB or GD (25). In their study, toxicologically significant VX levels occurred in hairless guinea pig blood for 10-20 hr after IV administration (2 x LD_{50}) compared to less than 2 hr for either GB or GD (25).

It was surmised (26) that plasma BChE measurement was less relevant to CWNA toxicity than RBC AChE activity. For example, earlier studies (27; 28) showed that VX inhibited RBC AChE much more than plasma BChE. Although it has also been shown that GB preferentially inhibits RBC AChE, the extent was not the same as VX (29). Plasma BChE and RBC AChE can serve a protective function by binding with nerve agent to reduce the concentration of free agent available to bind with tissue AChE (26). It has also been demonstrated that plasma BChE was more labile than RBC AChE by being affected by gender, age, and oral contraceptive usage (30), and was affected by genetic determinants, disease states, nutritional status, hormonal changes, race and circadian rhythms (31; 32).

In humans, RBC AChE depressed 35-50% of normal after IV VX, returned to 80-90% normal within 14 days (28). After VX exposure, inhibited RBC AChE recovered 20-50% within 5 days (33). The rate of recovery was about 1% per day from the 12^{th} day to the 120^{th} day after VX intoxication (33). Like GB, the return of activity of VX-depressed plasma BChE approximated an exponential process (33).

When nerve agent enters the body, some combines rapidly with ChE, other esterases, and, possibly, other proteins. *In vitro* studies have demonstrated that the rate of combination with ChE is very rapid. This was demonstrated by the bimolecular rate constants (K_i). For example, the K_i obtained by the reaction of VX with human RBC AChE is 1.6×10^7 L/mol-min and for GB (human RBC) the K_i is 1.5×10^7 L/mol-min (33). Similar rate constants were obtained from kinetic studies using ChE from other animal species (33). However, the higher toxicity of VX in animals compared with GB is not accounted for by these rate constant comparisons. There is evidence for a greater specificity for AChE by VX versus other esterases, such as BChE as demonstrated in the present study and others (34), compared with GB or GF. There is a possibility that this specificity characteristic plays a role in the greater toxicity of VX compared to the G-agents, as postulated earlier by McNamara (33).

McNamara and coauthors (33) argued that recovery from CWNA intoxication (whole animal, brain AChE and plasma BChE) was satisfactorily described by a single or several exponential functions (*i.e.*, the recovery rates are proportional to the degree of depression). Recovery is rapid after an acute dose and becomes slower as time proceeds. The observed recovery could be a single or multiple processes (such as de novo enzyme synthesis, conversion of enzyme from inactive precursor to active form or spontaneous reactivation of agent-inhibited enzyme). Reactivation of inhibited enzyme is significant for VX intoxication but not with GB and accounts for the more rapid recovery from VX. This difference was reflected in the recovery of RBC AChE after intoxication with VX or GB (33). The recovery was a zero-order process with GB and coincided with RBC steady state turnover (33). This indicated no recovery of AChE in an individual agent exposed RBC but replacement by new RBC with a full amount of AChE. However, with VX, RBC AChE recovery was probably a combined zero-order process and pseudo first-order process occurring simultaneously. The initial rate of recovery of RBC AChE after VX intoxication (28; 33) likely reflected a dephosphorylation reaction at the esteratic site of the enzyme. A slow component of recovery was correlated with the formation of new RBC replacing those whose enzyme had been aged by VX. In the case of VX intoxication, de novo synthesis of RBC did not totally account for enzyme recovery in circulating RBC. Plasma BChE in humans returned to normal within 12 days (28; 33) after VX intoxication and some of the recovery was likely due to a spontaneous reversible process like RBC AChE and the rest by other recovery processes. In these studies, even the initial rate of plasma BChE recovery appeared faster than that for RBC AChE.

It has been demonstrated in male humans (young adults) that RBC AChE inhibited by IV VX spontaneously reactivates more rapidly than that inhibited by GB. VX-inhibited RBC AChE ages very little and is amenable to oxime reactivation for as long as 48 hr and the dose of oxime necessary to reactivate VX-inhibited RBC AChE is less than that needed to reactivate GB inhibited enzyme (35; 34). In this study, BChE was never depressed more than 20%. The inhibition of RBC AChE was dose-dependent and an IV dose of 1.5 µg/kg produced a 75% depression of enzyme activity. The inhibition was greatest about 1 hr after VX administration; however, in some individuals, maximal RBC AChE depression occurred as soon as 15 min and as late as 6 hr (35; 34). In the present study, depending upon concentration, maximal ChE inhibition occurred 20-40 min after exposure to CWNA (Figures 3 and 4). A lag time for maximal ChE inhibition has been reported for other anti-ChE compounds as well, such as echothiophate (36) and neostigmine (37). These authors demonstrated that spontaneous recovery of RBC AChE after VX intoxication occurred soon after maximal inhibition and proceeded at a rate of 1% per hr (34). VX has been reported to be a more potent anti-AChE compound compared to other nerve agents such as GB (38; 34). Grob and Harvey (38) reported that GB was much more potent than other anti-ChE compounds; however, in the present study, GF was a more potent inhibitor of guinea pig RBC AChE (Figure 3) and plasma BChE (Figure 4) than GB at both sub-lethal exposure concentrations.

The importance of a CWNA's structure has been demonstrated in therapeutic studies utilizing oximes to reactivate agent inhibited ChE enzymes. For example, the half-times quoted for aging of human RBC AChE *in vitro* are 2-6 min for GD, 3 or 5 hr for GB, 13 to >14 hr for tabun, 7.5 or 40 hr for GF and 48 hr for VX (39). Sidell and Groff (34) have argued that spontaneous reactivation and aging simultaneously affect phosphorylated (*i.e.*, inhibited) ChE enzyme. The rate of both reactions is dependent on the structure of the alkyl group (*i.e.*, alkyloxyl group) of VX and most G-agents attached to the oxygen molecule. The rate of spontaneous reactivation decreases rapidly when the alkyloxyl group increases in size. This phenomenon is due to steric factors as larger groups are more effective in preventing water from attacking the bond. The rate of spontaneous reactivation would be much slower if the alkyloxyl group is isopropyl (like GB) than when it is ethyl (like VX). Conversely, the aging rate is directly dependent upon the side groups attached to the first carbon of the alkyloxyl group which governs the ease by which the alkyloxyl group can stabilize a carbonium ion in the reaction transition state. This means that this reaction rate as well as aging is faster when the alkyloxyl group is isopropyl rather than ethyl. Due to these structural differences between VX and GB and the fact that oximes can only reactivate un-aged phosphorylated enzyme, Sidell and Groff (34) demonstrated that a lower dose of oxime was required to reactivate VX-inhibited ChE compared to GB-inhibited enzyme.

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2.3 Determination of LD_{50} of Percutaneous VX in Un-Anesthetized Guinea Pigs

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2.3.1 Introduction

Because of safety concerns, most studies examining the cutaneous application of VX have been done using anesthetized guinea pigs. In our laboratory, we previously determined the LD_{50} in anesthetized guinea pigs for the cutaneous application of 10% VX/90% methylene chloride to be 0.14 mg/kg (1). We also determined the LD_{50} in anesthetized guinea pigs for the cutaneous application of 10% VX/90% methylene chloride, covered with a plastic cap, to be 0.12 mg/kg (2).

2.3.2 Objectives

The objective of this project was to determine the LD_{50} in un-anesthetized guinea pigs for the cutaneous application of 10% VX/90% methylene chloride with the application site covered with a plastic cap.

2.3.3 Materials and Methods

Male guinea pigs (Crl HA [Br]), weighing 300-400 g, were used in all studies. Animals were quarantined and observed for evidence of disease for a minimum of five days prior to their use. Guinea pig ration and tap water were provided *ad libitum*. In conducting the research described in this report, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals* by the Institute of Laboratory Animal Resources, National Research Council, in accordance with the stipulations mandated for an AAALAC accredited facility.

The day prior to exposure, the guinea pigs had their fur clipped off their left side. The day of exposure they were weighed and assigned randomly into groups. Prior to VX exposure, a double-sided carpet tape strip with a hole punched through the assembly was placed on the left flank of the animal. This tape was used to hold a 14 mm plastic disposable vial cap (Evergreen Scientific, Inc., Los Angeles, CA) in place so that agent would not be spread after cutaneous application.

The animals were restrained inside a chemical agent fume hood by the buddy of the agent operator. The animals were held by their front shoulders and rear legs with their left flank flat facing up.

VX was applied in a 10% VX/90% methylene chloride solution. Use of a carrier solvent was needed to determine the percutaneous LD_{50} because the volumes of neat VX necessary to establish a LD_{50} curve are too small (<0.05 µL) to be accurately applied (1; 2). The VX solution was applied using either a 1 or 2 micro-L Hamilton syringe with applied volumes varying between 0.05-1.5 µL. Immediately after agent application, a plastic cap was placed over the exposure site and held in place by the carpet tape. A self adhesive elastic bandage was wrapped several times around the animal covering the exposure site, carpet tape and plastic cap. This bandage was used to keep the animal from removing the plastic cap and exposing the application site. Six hours after agent application, the bandage was cut and the carpet tape and plastic cap were removed, exposing the application site. Survival rates were determined after 24 hr. Euthanasia was carried out on the remaining animals, and all the bodies were decontaminated using excess bleach.

2.3.4 Results

The results of exposing un-anesthetized guinea pigs to VX and covering the application site with a cap are shown in Table 4.

Table 4. LD_{50} of VX in Un-Anesthetized and Capped Animals

Dose (mg/kg)	# Animals Exposed	# Animals Dead
0.01	1	0
0.03	2	0
0.05	4	0
0.07	2	0
0.09	2	0
0.10	2	1
0.11	1	0
0.12	2	1
0.14	2	2
0.16	1	1
0.20	1	1

2.3.5 Conclusions

The LD_{50} in un-anesthetized animals for the cutaneous application of 10% VX/90% methylene chloride with the application site covered with a plastic cap is 0.12 mg/kg. This result was not significantly different from the LD_{50} in animals that were anesthetized and the application site capped (2) or where animals were anesthetized and the application site was not capped (1).

2.3.6 Literature Cited

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2.4 Evaluation of the Time Course of Cholinesterase (ChE) Inhibition Following Sub-Lethal Percutaneous Exposure to VX

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2.4.1 Introduction

Chemical warfare nerve agents (CWNAs), including VX, are irreversible inhibitors of acetylcholinesterase (AChE). This inhibition of AChE causes an accumulation of acetylcholine (ACh) at central and peripheral sites, resulting in increased secretions, seizures, respiratory distress, and death. While the "G-CWNAs" are volatile and primarily an inhalation threat, the CWNA, VX, has low volatility, is readily absorbed through the skin and is primarily a percutaneous (PC) threat.

There are different physiochemical properties between V- and G-agents. V-agents are hydrolyzed more slowly than G-agents and some of their hydrolysis products are toxic (1). We previously compared the kinetics of exposure to sub-lethal doses of G-agents with exposure to VX. We observed that subcutaneous (SC) exposure to 0.1 and 0.4 LD_{50} VX resulted in lower area under the curve, lower maximum concentration, and higher time to maximum concentration, relative to exposure to 0.1 and 0.4 LD_{50} of GB or GF when using the fluoride ion regenerated method (see section 2.2). Furthermore, the half-life ($t_{½}$) for ethyl methylphosphonofluoridate (VX-G) in plasma was four or five times as long, respectively, compared to fluoride regenerated GF and fluoride regenerated GB. Others (2, 3) also reported that VX is more persistent in vivo relative to other nerve agents. The toxicokinetics of exposure to 1.0 intravenous with PC 1.0 LD_{50} VX was compared in anesthetized and atropinized hairless guinea pigs (2). While VX greatly inhibited AChE, VX inhibition of butyrylcholinesterase (BChE) was small. This is consistent with findings in guinea pigs exposed subcutaneously to VX (4; 5) and has also been observed in rats. Our current findings in percutaneously exposed guinea pigs provide further support for VX binding less to BChE than do G-agents. Data on the toxicology and toxicokinetics of VX are limited and have primarily been evaluated in anesthetized animals. Because anesthesia may affect the PC absorption of VX (6), it is important to evaluate kinetics in un-anesthetized animals.

In the current study, we evaluated the time-course of ChE inhibition in blood and tissue following a single sub-lethal and asymptomatic dose (0.4 LD_{50}) of VX, administered percutaneously, to un-anesthetized guinea pigs. The guinea pigs had repeated blood draws via an arterial catheter for measurement of blood levels of AChE and BChE at a range of time points (10 min to 48 hr) after VX exposure. Blood and tissues were collected to evaluate regenerated VX (as the G-analog or VX-G) and free VX, but are not included in this report; these data will be reported elsewhere. These data will be used in mathematical models of the absorption, distribution and elimination of CWNAs to improve toxicity estimates, extrapolate to humans and predict response to a range of nerve agent exposures.

2.4.2 Materials and Methods

2.4.2.1 Animals

Male Duncan Hartley guinea pigs (*Cavia porcellus*) (Crl: (HA) BR COBS; Charles River Labs, Kingston, NY) with carotid artery catheters pre-implanted by the vendor were used in this study. Upon arrival, the animals were quarantined for five days. The guinea pigs were individually housed in polycarbonate cages in temperature (21 ± 2 °C) and humidity (50 ± 10%) controlled animal quarters maintained on a 12 hr light-dark cycle with lights on at 0600. Guinea pig laboratory chow and water were available *ad libitum*. At the time of VX exposure, the animals weighed between 300-400 g. This study complements other in-house and extramural nerve agent study protocols in which data were generated with the use of guinea pigs. The guinea pig is the best approximation to non-human primate sensitivity to CWNA among rodent species (7).

2.4.2.2 Chemicals and Reagents

S-[2-(diisopropylamino) ethyl]-O-ethyl methylphosphonothioate (VX; CAS No. 50782-69-0; also known as EA 1701; mw = 267) was used in this study. Before use, chemical agent standard analytical reagent material-grade VX (lot # VX-U-5055-CTF-N) was verified by quantitative 31P-NMR to be 95.7% pure and stored in sealed ampoules containing nitrogen. Sealed VX ampoules of 1 mg agent/mL in saline were provided to MRICD where the agent was diluted for injection. Triethyl phosphate ([TEP] 99.9% purity; Aldrich Chemicals, Milwaukee, WI) was used as the internal standard (IS) for VX purity assays. Chemical analysis and agent transfer records are kept on file with all chemical analysis records associated with animal exposure records for VX. Also, 2H5-VX-G with 93% deuterium purity was procured from ECBC and used as GC-MS ISs.

Analytical grade solvents used in this study were obtained from Sigma-Aldrich (St. Louis, MO). Gases for the analytical instruments were obtained from Messer, Inc. (Chattanooga, TN) and had a minimum purity of 99.999%. All chemicals, solvents, and gases were used as obtained with no further purification.

2.4.2.3 PC VX Exposures

These VX studies were conducted at MRICD. On the day of exposure, the body weights of the guinea pigs were taken and the fur from their left (site for PC exposure) and right sides (unexposed site) was clipped off. A double-sided carpet tape strip with a hole punched through the assembly was then placed on the left flank of each animal. This tape was used to hold a plastic disposable vial cap (14 mm, Evergreen Scientific, Inc., Los Angeles, CA) in place so that the agent would not be spread after cutaneous application. The unanesthetized guinea pig was restrained inside a chemical agent fume hood by the buddy of the agent operator. The animals were held by their front shoulders and rear legs with their left flank facing up. The LD_{50} for the cutaneous application of 10% VX/90% isopropyl alcohol (IPA) with the application site covered with a plastic cap was previously determined in anesthetized guinea pigs to be 0.15 mg/kg (8); this dose was used in the current study in unanesthetized guinea pigs. Across several studies, Clarkson *et al.* (8, section 2.3) had previously determined the LD_{50} VX in methylene chloride to be similar (range 0.12 to 0.15 mg/kg) regardless of whether the animals were anesthetized or not. In addition, the LD_{50} data generated with IPA and methylene chloride as solvents gave similar results to each other within 0.01 mg/kg (only evaluated in anesthetized guinea pigs). In the current study, 0.4 LD_{50} (0.06 mg/kg) 10% VX/90% IPA solution was applied to the left flanks of unanesthetized guinea pigs. IPA was selected as the carrier because other chemical agents to be evaluated in future studies would require use of IPA as solvent, and comparisons of different agents with the same solvent will be preferred. A carrier solvent had to be used because the volumes of neat VX for the PC 0.4 LD_{50} were too small (<0.05 µL) to be accurately applied. The VX solution was applied using a 1 µL Hamilton syringe with applied volumes varying between 0.18-0.23 µL. Immediately after agent application, a plastic cap was placed over the exposure site and held in place by the carpet tape. A self adhesive elastic bandage was wrapped several times around the animal covering the exposure site, carpet tape and plastic cap. This bandage was used to keep the animal from removing the plastic cap and exposing the application site. Six hours after agent application the bandage was cut and the carpet tape and plastic cap were removed, exposing the application site. During the study, the guinea pigs were observed for clinical manifestations of agent toxicity such as localized muscle fasciculations, tremors, convulsions, respiratory distress, lacrimation and salivation; however, at the sub-lethal concentrations used in this study overt clinical signs of agent toxicity were not observed.

2.4.2.4 Blood and Tissue Collection

Prior to VX exposure, blood was drawn from the carotid artery via a catheter and evaluated for baseline levels of ChE. Blood (0.5 – 1.0 mL) was collected at various time points (range 10 min to 72 hr) following exposure to 0.4 LD_{50} VX and analyzed for blood ChE activity and for regenerated agent. The total volume removed from each animal was no more than 1% of its body weight. Blood was removed at each time interval via the carotid catheter and collected in BD Microtainer® plastic tubes with dipotassium EDTA additive to prevent clotting (BD, Franklin Lakes, NJ). Heparinized saline was administered following the blood draw.

Whole blood (80 µL) was diluted in 560 µL distilled water, snap frozen on dry ice and stored at -20 °C until assayed for ChE activity. For determination of agent regeneration, blood was separated into red blood cells and plasma fractions by centrifugation (15,000 x g for 5 min) and used for the regeneration assays (data reported elsewhere).

Heparinized arterial blood was used to examine various physiological parameters. Blood sodium, potassium, pH, pCO_2, pO_2, O_2 saturation, glucose, lactate, bicarbonate, hematocrit and hemoglobin were determined using an i-STAT analyzer equipped with EC4+ and CG4+ analysis cartridges or CG8+ analysis cartridges (9). In this study, the physiological values obtained using i-STAT analysis were within normal parameters for the guinea pig, even following 0.4 LD_{50} VX.

At the end of blood collection (range of time points 2 hr to 72 hr), guinea pigs were administered Nembutal® (0.7 mL into the carotid artery; 50 mg/mL) and euthanized by exsanguination. Following saline perfusion, samples of brain and eye, peripheral tissues (heart, lung, kidney, diaphragm and liver), as well as the VX injection/applied skin site and skin from the contralateral control side, were removed, wrapped in foil, and snap frozen in liquid nitrogen. A sample from each organ was collected (except skin) and diluted and homogenized in 1% Triton-X 100 solution (in normal saline). Homogenates were centrifuged at 4 °C for 10 min at 15000 x g, and the supernatant saved for analysis. The tissue was kept frozen at -80 °C until analysis for AChE activity.

2.4.2.5 ChE Determination Methods

AChE and BChE activity in whole blood (using acetylthiocholine and butyrylthiocholine, respectively, as the substrates) was determined by an automated method using a SPECTRAmax Plus microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA). The analytical procedure was based on the manual method of Ellman *et al.* (10) and modified for the microplate spectrophotometer. (11; 12)

For AChE in tissue samples were thawed and three 7 µL replicates of each were pipetted into a 96-well UV star microplate (Greiner, Longwood, FL, USA). Twenty microliters of deionized water was added to each well containing brain, eye and peripheral tissue samples. Following the addition of water, 200 µL of DTNB (0.424 M, pH 8.2) was added to each sample well. Each microplate was then incubated at 37 °C on a shaker before being placed in the SPECTRAmax Plus microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) and 30 µL of the substrate acetylthiocholine iodide (51.4 mM) was added to each well. The samples were read at 412 nm (at 20 s intervals) for 3.5 min, and the activity (µmol/mL/min) was determined using Softmax 4.8 LS software (Molecular Devices Corporation).

Protein levels in the tissue samples were determined by a BCA protein assay. The standard curve was created using a bovine serum albumin at the following concentrations: 0.5, 0.75, 1.0, 1.5, and 2.0 mg/mL. Three replicates of 10 µL for each brain, eye and peripheral tissue sample were added to individual microplate wells. Working reagent (BCA protein reagent kit; 200 µL) was added to each well of the brain and eye samples. Three replicates of 5 µL for each peripheral tissue sample were added to individual microplate wells. The peripheral tissue samples were further diluted by adding 5 µL of deionized water before adding 200 µL of working reagent. The microplates were incubated at 37 °C on a shaker for 30 min. The microplates were allowed to cool to room temperature before being read using the SPECTRAmax Plus microplate reader and Softmax Plus 4.8 LS software as described above. Tissue AChE activity was expressed as µmol/mg protein/min.

2.4.2.6 Data Analysis

For ChE activity (Units/mL) in blood, differences in pre-exposure *versus.* post-exposure VX were evaluated using a one way analysis of variance (ANOVA) with time of collection as an independent variable. For ChE activity in tissue, differences in saline control *versus.* post-exposure VX were evaluated using a one-way ANOVA with post-exposure time of collection as an independent variable. Significance level was $p \leq 0.05$.

2.4.3 Results

For purposes of this report, only the ChE data in blood and tissue are reported. Evaluation of the regenerated VX and free VX data will be reported in a subsequent report.

We observed significant inhibition of blood AChE activity 80 min after PC VX exposure; levels had not returned to baseline by 72 hr after exposure (Figure 5). Inhibition of BChE activity was delayed until 16 hr after exposure and was short-lived, returning to baseline by 30 hr after exposure. Figure 5 illustrates a logarithmic scale of the time-course change in ChE activity following exposure to 0.4 LD_{50} VX (PC). AChE was significantly inhibited 80 min after VX exposure and remained inhibited for at least 72 hr after exposure. BChE was significantly inhibited 960 min (16 hr) after exposure, but returned to baseline by 1800 min (30 hr) after exposure. AChE (*$p \leq 0.05$); BChE (+ $p \leq 0.05$).

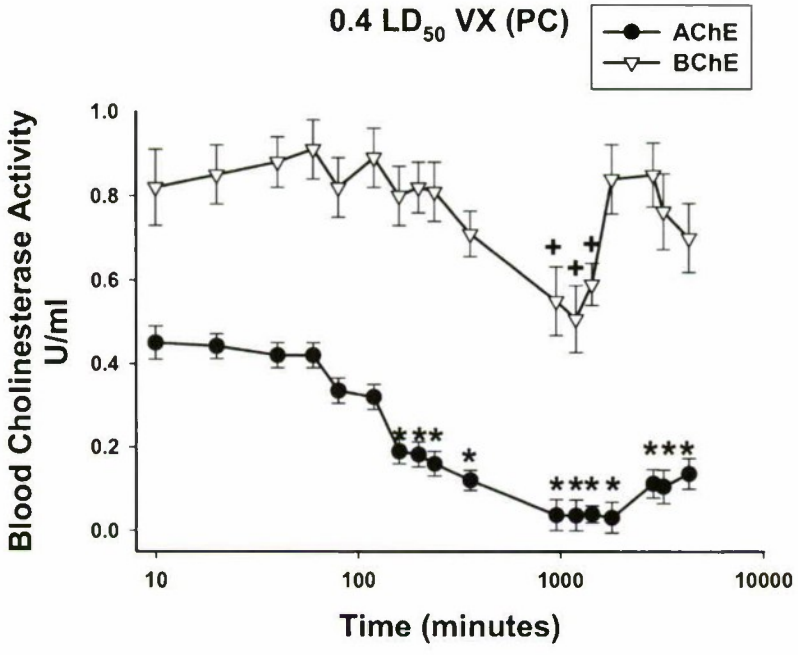

Figure 5. Logarithmic Scale of Time-Course Change in ChE Activity Following PC Exposure to 0.4 LD_{50} VX

Tissue levels of AChE activity were determined between 2 hr and 48 hr after PC exposure. Compared to saline control rats, we observed significant inhibition in brain (24 hr; Figure 6), heart (6 hr, 16 hr, 20 hr, 24 hr; Figure 7) and lung (20 hr, 24 hr). Additional control samples are needed for comparison. There was a non-significant trend for reduced AChE activity in diaphragm at 24 hr. There was no significant inhibition observed in the eye or in the kidney. For the liver, there were only two data points available for the saline control, so additional samples are needed for this organ.

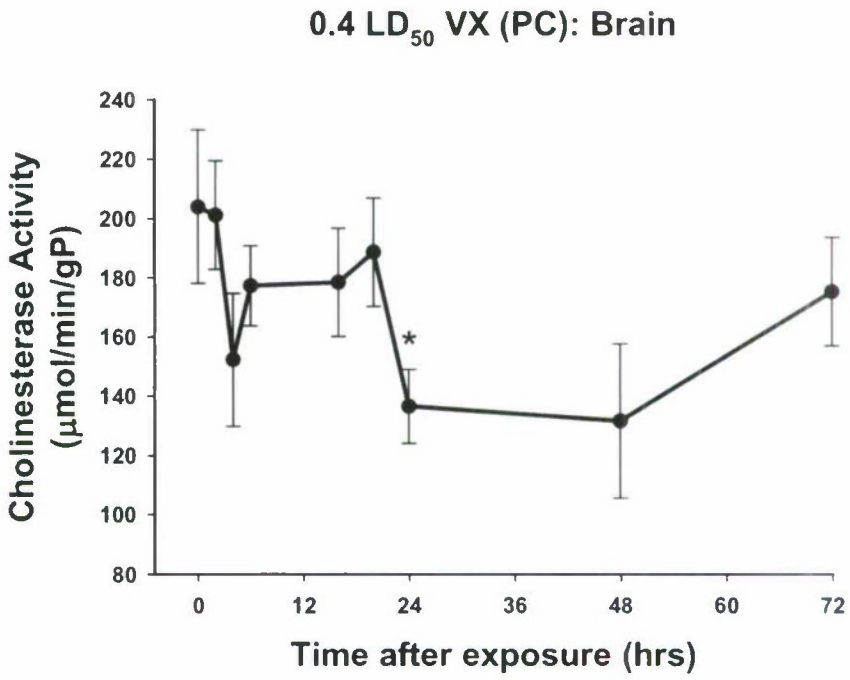

Figure 6. PC Exposure to 0.4 LD_{50} VX Significantly Reduced ChE Activity in Brain 24 hr after Exposure*

*gP = Grams of protein as determined by the BCA assay.

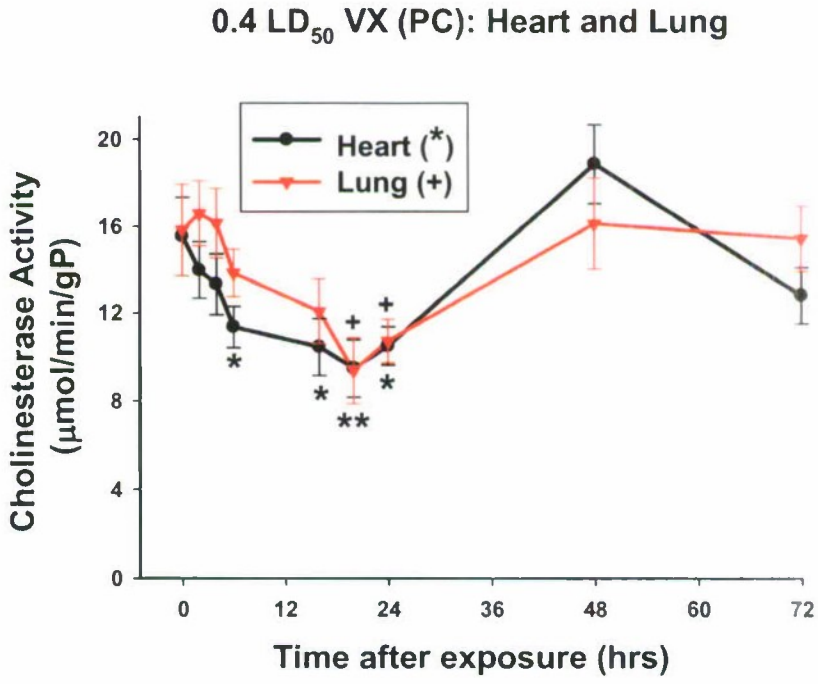

Figure 7. ChE Activity Significantly Inhibited in Heart Tissue between 6 to 24 hr (*p ≤ 0.05; **p ≤ 0.01) and in Lung Tissue 20 to 24 hr (+ p ≤ 0.05) after Exposure*

*gP = Grams of protein as determined by the BCA assay.

2.4.4 Conclusions

PC exposure to 0.4 LD_{50} VX depressed AChE activity in blood markedly (~90-95%) with slow recovery. AChE activity was inhibited at 80 min (Figure 5) post-exposure and remained suppressed 72 hr after exposure. This delayed inhibition following PC exposure contrasts with findings following SC exposure to VX, in which inhibition of AChE activity occurs within the first 10 min (see section 2.2).

Inhibition of BChE activity was delayed, short-lived and not as robust as AChE inhibition. BChE activity showed ~30% inhibition at 16 hr after exposure and returned to baseline by 30 hr post-exposure. These findings are consistent with previous observations that SC exposure to VX had minimal effects on BChE activity (see section 2.2; 4).

ChE activity was significantly inhibited in brain 24 hr after exposure to 0.4 LD_{50} VX (PC), as well as in heart from 6-24 hr and lung from 20-24 hr after exposure. ChE activity in diaphragm was more variable, and there were non-significant trends for inhibition. More control samples are needed before effects in liver can be determined.

These currently obtained experimental data will be fed into a physiologically based pharmacokinetic model to improve toxicity estimates, extrapolate to humans and predict response to a range of nerve agent exposures.

2.4.5 Acknowledgements

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2.5 VX Studies in Rabbits

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2.5.1 Introduction

These studies are the initial chapter in a comprehensive study involving another species (Göttingen Minipig) and other percutaneously hazardous chemical agents. The ultimate goal is to establish defensible human toxicity estimates from which contact hazards can be predicted.

For this purpose, contact hazard can be defined as any adverse effect resulting from PC contact with a contaminated [or formerly contaminated] surface. "Contact" can result from skin touching a surface or a surface (*e.g.*, contaminated clothing) touching skin. Endpoints other than lethality are required, and these data do not exist.

Rabbits were selected as the initial test species because they are one of the more sensitive species to VX (1). In addition, they are large enough to permit multiple blood samples for acetylcholinesterase (AChE) determinations and agent regeneration assays.

2.5.2 Materials and Methods

The goal was to elucidate the dose-response curve from no observed adverse effects—including no statistically significant AChE inhibition, to lethality. The studies were comprised of two parts: IV and PC dosing. Each part was divided into two phases. Phase I was designed to establish the LD_{50} and $ED_{50(severe)}$. Phase II was designed to determine threshold effects, the ED_{50} for AChE inhibition, and the lowest doses of agent from which the parent material could be regenerated from blood. Unless otherwise noted, the procedures for the two phases of each part were identical.

2.5.2.1 Test Agent

VX [sample VX-U-5251-CTF-N] was obtained from the Chemical Transfer Facility. It was analyzed by the Agent Chemistry Team; the purity was 94.6% +/- 0.39 by NMR.

2.5.2.2 Animals

Young, adult male, New Zealand White rabbits (NZW) [*Oryctologus cuniculus*] were obtained from Millbrook Breeding Labs, Amherst, Mass. Testing began after a 7-day quarantine, at which time they weighed 9-13 kg.

2.5.2.3 Blood Sampling

Blood samples were collected by pricking the lateral ear vein with a sterile needle and collecting the flowing blood in a 50 µL micropipette. To establish pre-exposure baseline AChE levels, blood samples were collected on three consecutive days before the animals were exposed to VX. Post-exposure samples for AChE determination and agent regeneration assays were similarly collected at 5, 10, 20, 30, and 60 min; then at 2, 4, 24, 48, and 27 hrs; and then at 7 and 14 days following dosing. (In the IV studies, samples were taken from the ear that had not been injected with VX.)

2.5.2.4 Dosing

PC Exposures

The afternoon before testing, about 150 cm^2 of the dorsum of each rabbit was clipped free of hair. On the day of the test, a 1 in. circle was drawn with an indelible marking pen to define the area to which the agent was to be delivered. For agent application, the rabbits were placed in neck-

collaring stanchions in a fume hood with their heads facing outward; they remained there until they were decontaminated at 24 hr post-exposure (any animals dying before 24 hr were removed and decontaminated immediately unless death occurred overnight). The agent was applied by "touching off" a droplet onto the skin with a blunted needle attached to an "Agla" microsyringe. For the lethal studies "neat" agent was used. For the sub-lethal studies, when it was not possible to deliver a small enough droplet with the microsyringe, the agent was diluted in IPA.

IV Exposures

For the IV studies, the agent was initially diluted in polyethylene glycol-200 (PEG-200). Subsequent dilutions were made with normal saline. Injection volumes were ≤1.0 mL. The agent solution was delivered into the marginal ear vein.

2.5.2.5 Clinical Signs

Clinical signs were recorded continually, to the nearest half minute until the animal died or until the end of the work day. Not all signs were observed for the different routes of exposure. [Some signs could not be observed because of how the exposures had to be performed (*i.e.*, neck-collaring stanchions for PC exposures precluded the observation of ataxia).]

2.5.3 Experimental Design

The range-finding studies employed 1-4 animals. Exposure groups within a phase typically consisted of 4-8 animals.

For Phase I of the PC exposures (neat agent) dosages ranged from 3.2 to 63 µg/kg. [Sixty-four rabbits were equally divided among 8 separate exposure groups.] For Phase II, PC exposures (dilute agent) dosages ranged from 0.25 to 5.0 µg/kg. [Thirty-two rabbits were equally divided among 8 separate exposure groups, plus one range-finding group of 4 rabbits.] In order to compare the toxicity of neat and IPA-diluted agent, dosages of 3.2 and 5.0 µg/kg were used in both phases (Table 5).

Table 5. Dosing Schedule for PC Studies

Phase	Test #	Dosage (µg/kg)	Agent	Date	# Animals
lethal	1	40.0	VX	2-13-07	8
	2	25.00			8
	3	15.8			8
	4	12.6		2-21-07	8
	5	7.9			8
	6	**5.0**			**8**
	7	**3.2**		2-22-07	**8**
	8	63			8
sub-lethal	**9**	**3.2**	VX/IPA	3-6-07	**4**
	10	2.0			4
	11	1.26			4
	12	0.79			4
	13	**5.0**		3-7-07	**4**
	14	3.2			4
	15	0.5			4
	16	0.32			4

Pink shading and bolding denote overlapping VX and VX/IPA doses

For Phase I of the IV exposures, dosages ranged from 2 to 20 µg/kg. [Sixty-four rabbits were equally divided among 8 separate exposure groups.] For the Phase II, IV exposures dosages

ranged from 0.10 to 4.0 µg/kg. [Thirty-two rabbits were equally divided among 8 separate exposure groups (Table 6).]

Table 6. Dosing Schedule for IV Studies

Phase	Test #	Dosage (µg/kg)	Date	# Animals
lethal	1	20.0	1-16-07	8
	2	12.6	1-17-07	8
	3	8.0		8
	4	6.4		8
	5	5.0		8
	6	9.2		8
	7	2.0	1-18-07	8
	8	3.2		8
sub-lethal	9	0.25	1-30-07	4
	10	0.5		4
	11	1.0		4
	12	2.0		4
	13	4.0	1-31-07	4
	14	3.2		4
	15	0.158		4
	16	1.0		4

2.5.4 Statistical Analysis

The two sets of data (PC and IV) could not be analyzed in an identical manner, so the analyses for each exposure route are presented individually. For IV exposures, the clinical effect observed at the lowest dosages was red blood cell (RBC) AChE inhibition; so AChE depression was used to characterize effects at the lowest dosages. All rabbits with overt clinical signs had AChE activity <50% of baseline. For PC exposures there were some instances in which mild clinical effects were observed, but there was no significant AChE inhibition. The observed toxic signs were not the same for the two routes of exposure.

Minitab®, Version 15 (Minitab, Inc., State College, PA) was used for all statistical tests. [Printouts of the Minitab® analyses are given in the Appendix.]

Ordinal logistic regressions[a] (2-4) were used to fit ordinal responses (presence or absence of a clinical sign) and to investigate how the probability of effect (ED_{50}) varied as a function of three factors: dosage, agent (neat *versus* dilute for PC exposures), and test day[b]. [Indicator[c] variables were used to test for the statistical significance of agent and test day.] [The following are examples of such factors: animals from different shipments, health changes in animals, different animal handlers (Mr. Smith in first half of study and Ms. Jones in the second half), differences in agent preparation (new dilutions must be prepared for each test day), time of day (it is not possible to do all the exposures at the same time of day), *etc.*] If either factor is statistically significant, its impact can be properly accounted for by the use of indicator variables in the model. The following model was used to fit the data.

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[a] With a normit link function.

[b] It was not possible to perform all the tests on the same day, so it was necessary to determine if ED_{50}s were influenced by test day.

[c] These are also called dummy or binary variables and are used to convert categorical information (e.g., gender, type of agent or material, *etc.*) into a format suitable for use in a regression analysis (4). Typically, an indicator variable only has two possible integer values (*i.e.*, -1 and 1, or 0 and 1).

$$Y_N = k_0 + k_D(\log_{10} D) + k_{Agent}(Agent) + \sum_{i=2}^{N} k_{Day,i}(Day_i) \qquad [1]$$

where

Y_N is a normit
ks are fitted coefficients
D is the dosage (µg/kg)
Agent [indicator variable to test for effect of agent composition][d] equals -1 for neat VX or 1 for VX/IPA
Day_i [indicator variable to test for cumulative effect (on a day-by-day basis) of uncontrolled and/or unknown factors] equals 1 when modeling the i^{th} test day and -1 for all other test days[e]

The intercept, k_0, is dependent on the toxicological endpoint. In the case of a binary response, k_0 serves as the traditional model intercept. The fitted coefficient, k_D, is the estimate for the probit slope. Y_N equals -1, 0 and 1, at the 16, 50, and 84% response levels, respectively. When using Equation [1] to model ordinal responses, two different scoring systems were investigated. One system was based upon clinical signs; the other also included AChE inhibition. The second system did not work for the PC data, so the data were scored based on clinical signs, and the AChE inhibition data were analyzed separately.

Score = 0 to 5:

0 No Observable Clinical Signs
1 Local Effects [slight fasciculations at dosing site—PC exposures only]
2 Mild Systemic Effects
3 Moderate Effects
4 Severe Effects
5 Lethality

All scores were based upon observed signs during a 24 hr post exposure period. Similarly, blood samples for AChE assays were drawn within this time period. Toxic signs associated with each category of effect (mild, moderate, *etc.*) were somewhat a function of the route of exposure [See Toxic Signs under 2.4.5 PC Studies and 2.4.6 IV Studies].

An analysis of variance (ANOVA)[f] was used to determine if an individual animal had statistically significant AChE depression. For each rabbit, the geometric mean of either three (PC exposures) or four (IV exposures) AChE determinations[g] was compared to the control[h]. The analysis was performed using the logarithm (base 10) of the percent of baseline AChE activity. If the p-value of the comparison was not less than 0.05, the null hypothesis (AChE depression had not occurred) was not rejected. ANOVA was used to investigate group differences in the AChE activity as a function of dosage group and time post-exposure. AChE depression was classified as either, "below threshold", "threshold"

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[d] Used for only PC exposures.

[e] Example: if there are four test days, three *Day* indicator variables are used (Day_2, Day_3 and Day_4). For a rabbit tested on Day 3, the indicator variables are Day_2 = -1, Day_3 = 1 and Day_4 = -1. For a rabbit tested on Day 1, all three variables will equal -1.

[f] The general linear model routine of Minitab® was used for the ANOVA.

[g] AChE activity was the geometric mean of samples taken at 2, 4, and 24 hrs post-exposure [PC exposures] or at 5, 10, 30, and 60 min post-exposure [IV exposures]. For PC exposures, if death occurred prior to 2 hours post-exposure, the lowest post-exposure level was used.

[h] The geometric mean of the AChE activity determinations made 1, 2, and 3 days pre-exposure.

or "moderate"[i]. "Threshold" is more a statistical measure than a physiological one—it is the point below which the mean AChE activity of an exposed rabbit can be considered statistically different from that of the control group. For PC exposures, the threshold was 76% of baseline activity; for IV exposures, it was 83.6% of baseline. Exposed animals having AChE activities above these thresholds were classed as NSI (no significant inhibition). Significant AChE inhibition and the presence or absence of clinical signs are summarized in Table 7.

Table 7. Summary of AChE Inhibition and Clinical Signs

Route	Agent	Dose (µg/kg)	Significant AChE Inhibition	Clinical Signs
			(# responding/# tested)	
PC	VX/IPA	0.25	0/1	0/1
		0.32	0/4	0/4
		0.50	0/5	3/5
		0.79	1/4	3/4
		1.00	0/1	1/1
		1.26	0/4	4/4
		2.00	1/5	5/5
		3.20	**4/8**	**8/8**
		5.00	**4/4**	**4/4**
	VX (neat)	**3.20**	**8/8**	**8/8**
		5.00	**8/8**	**8/8**
		7.90	8/8	8/8
		12.60	8/8	8/8
		15.80	8/8	8/8
		25.00	8/8	8/8
		40.00	8/8	8/8
		63.00	8/8	8/8
IV	VX	0.100	1/4	0/4
		0.158	2/4	0/4
		0.25	2/4	0/4
		0.50	4/4	0/4
		1.00	8/8	0/8
		2.00	12/12	0/12
		3.20	12/12	3/12
		4.00	4/4	4/4
		5.00	8/8	5/8
		6.40	8/8	8/8
		8.00	8/8	8/8
		9.20	8/8	8/8
		12.6	8/8	8/8
		20.0	8/8	8/8

KEY: blue shading indicates AChE inhibition in the absence of clinical signs or *vice versa*; pink shading and bolding indicate overlapping dosages for VX and VX/IPA.

[i] AChE inhibition $\geq$ 50%.

2.5.5 PC Studies

2.5.5.1 Toxic Signs

Based upon any observed signs and/or RBC AChE inhibition within the 24-hr post-exposure period the rabbits were placed into one of the following toxicity categories [categories were defined by time of onset (more severe effects occur later) and underlying physiological mechanisms]:

0 No Observable Clinical Signs

1 Local Effects: fasciculations at or around the site of dosing.

2 Mild Systemic Effects: salivation or the onset of miosis

3 Moderate Effects: chewing, head jerking, rapid respiration

4 Severe Effects: collapse, prostration, opisthotonos, running salivation, body spasms, pinpoint miosis, nystagmus, mydriasis, exopthalamos

5 Lethality (absence of a heart beat as determined by palpation).

(The use of the 0 to 7 ordinal score [see 2.5.3 Experimental Design] was also considered for analysis of the PC exposure data. [The additional two categories correspond to different levels of post-exposure AChE activity, with no other observable clinical signs.] However, for this ordinal score to work, the normal progression of toxic signs must have AChE depression appearing before any other observable clinical sign. Because there were many instances of PC exposure to VX/IPA when a rabbit had clinical signs, but no statistically significant AChE depression [see Summary Data from PC Exposures], a 0 to 7 ordinal score was not used.)

2.5.5.2 Summary Data from PC Exposures

Table 8 summarizes the data for clinical signs and AChE depression.

Table 8. Summary PC Data

Dosage (µg/kg)	Study Phase	Agent (mg/ml IPA)	AChE Activity (% baseline)			Observable Signs					
			NSI++	76-50	<50	none	local	mild	moderate	severe	lethal
			(# responding/# tested)								
0.25	sub-lethal	dilute	1/1	0/1	0/1	1/1	0/1	0/1	0/1	0/1	0/1
0.32			4/4	0/4	0/4	4/4	0/4	0/4	0/4	0/4	0/4
0.50			1/1	0/1	0/1	0/1	1/1	0/1	0/1	0/1	0/1
			4/4	0/4	0/4	2/4	2/4	0/4	0/4	0/4	0/4
0.79			3/4	1/4	0/4	1/4	3/4	0/4	0/4	0/4	0/4
1.00			1/1	0/1	0/1	0/1	1/1	0/1	0/1	0/1	0/1
1.26			4/4	0/4	0/4	0/4	0/4	4/4	0/4	0/4	0/4
2.0			4/5	1/5	0/5	0/5	3/5	2/5	0/5	0/5	0/5
3.2			3/4*	1/4*	0/4*	0/4*	0/4*	3/4*	1/4*	0/4*	0/4*
			1/4+	3/4+	0/4+	0/4+	1/4+	3/4+	0/4+	0/4+	0/4+
5.0			0/4	3/4	1/4	0/4	0/4	4/4	0/4	0/4	0/4
3.2	lethal	neat	0/8	2/8	6/8	0/8	5/8	3/8	0/8	0/8	0/8
5.0			0/8	0/8	8/8	0/8	2/8	6/8	0/8	0/8	0/8
7.9			0/8	0/8	8/8	0/8	0/8	8/8	0/8	0/8	0/8
12.6			0/8	3/8	5/8	0/8	0/8	5/8	3/8	0/8	0/8
15.8			0/8	0/8	8/8	0/8	0/8	0/8	2/8	2/8	4/8
25.0			0/8	0/8	8/8	0/8	0/8	0/8	0/8	2/8	6/8
40.0			0/8	0/8	8/8	0/8	0/8	0/8	1/8	2/8	5/8
63.0			0/8	0/8	8/8	0/8	0/8	0/8	0/8	0/8	8/8

++ no significant inhibition (see Section 2.4.4)
* 0.5 mg VX per 1 ml IPA
+ 1.0 mg VX per 1 ml IPA
pink and blue shading indicate overlapping dosages

Comparing the 3.2 and 5.0 µg/kg doses

For **neat VX**, the more salient observations from the above table are as follows:

- 56/56 animals had some sort of clinical sign(s); 49/56 had systemic effects
- at the lowest dosage (3.2 µg/kg), 8/8 rabbits had local or mild effects
- 100% lethality occurred at the highest dosage (63.0 µg/kg)
- 56/56 animals had significant AChE depression, 51/56 had AChE depression to 50% of baseline.

For VX/IPA solutions, the more salient observations are:

- 31/32 animals had <mild effects
- 5/5 animals at dosages below 0.50 µg/kg had no clinical signs
- 22/22 animals at dosages above 0.79 µg/kg had clinical signs
- only 10/36 animals had statistically significant AChE depression, and of these ten, only one was depressed below 50% of baseline
- 18/36 animals had mild or moderate signs without having statistically significant AChE depression.

ED_{50}s and Probit Slopes

Comparison of neat and dilute VX at the 3.2 and 5.0 µg/kg doses indicates that the VX/IPA solution was two times more potent than the neat agent for the $ED_{50(mild)}$ and the $ED_{50(moderate)}$, with 95% confidence limits (CL) of 1.5 to 2.8. [For more detailed discussion see "*Comparison of Neat VX and VX/IPA*".] The ED_{50} was not affected by the day on which the test was done.

ED_{50}s for observed effects are given in Tables 9 and 10. The probit slope is 5.2, with 95% CL of 4.0 to 6.5. The ED_{50}s and the $ED_{2.3}$ to $ED_{97.7}$ range,[a] as a function of severity of effects from Tables 9 and 10, are presented in Figure 8.

[a] The $ED_{2.3}$ to $ED_{97.7}$ range represents a spread of 4 normits (standard deviation units) from Y_N equals -2 to 2 (with zero corresponding to the ED_{50}). The effective doses (for a particular endpoint) of 95% of the rabbits are estimated to fall within this range.

Table 9. PC Phase I (Neat VX)

Effect(s)	ED_{50} (µg/kg)	95% CL		
		Lower	Upper	Upper/Lower*
Mild	3.4	2.6	4.3	1.67
Moderate	12.7	10.4	15.4	1.48
Severe	18.0	14.7	22.0	1.50
Lethal	23.4	18.9	29.1	1.54
Dosage Ratio		**95% CL**		
		Lower	**Upper**	**Upper/Lower***
lethal : mild	6.9	5.0	9.7	1.95
lethal : moderate	1.8	1.4	2.4	1.66
lethal : severe	1.3	1.1	1.6	1.48
severe : mild	5.3	3.9	7.3	1.87
severe : moderate	1.4	1.2	1.7	1.50
moderate : mild	3.8	2.8	5.0	1.78

*CLs for the ED_{50}s can be misleading when expressed on a linear basis because the ED_{50}s are actually calculated on a log basis. The proper metric for evaluating the precision of ED_{50} estimates is the ratio of the upper and lower CLs; lower ratios indicate greater precision.

Table 10. PC Phase II (VX/IPA)

Effect Level	ED_{50} (µg/kg)	95% CL		
		Lower	Upper	Upper/Lower*
Local	0.52	0.39	0.71	1.84
Mild	1.7	1.3	2.1	1.65
Moderate	6.3	4.6	8.6	1.86
Dosage Ratio		**95% CL**		
		Lower	**Upper**	**Upper/Lower***
moderate : local	12.0	7.8	18.4	2.36
moderate : mild	3.8	2.8	5.0	1.78
mild : local	3.2	2.2	4.7	2.15

*CLs for the ED_{50}s can be misleading when expressed on a linear basis because the ED_{50}s are actually calculated on a log basis. The proper metric for evaluating the precision of ED_{50} estimates is the ratio of the upper and lower CLs.

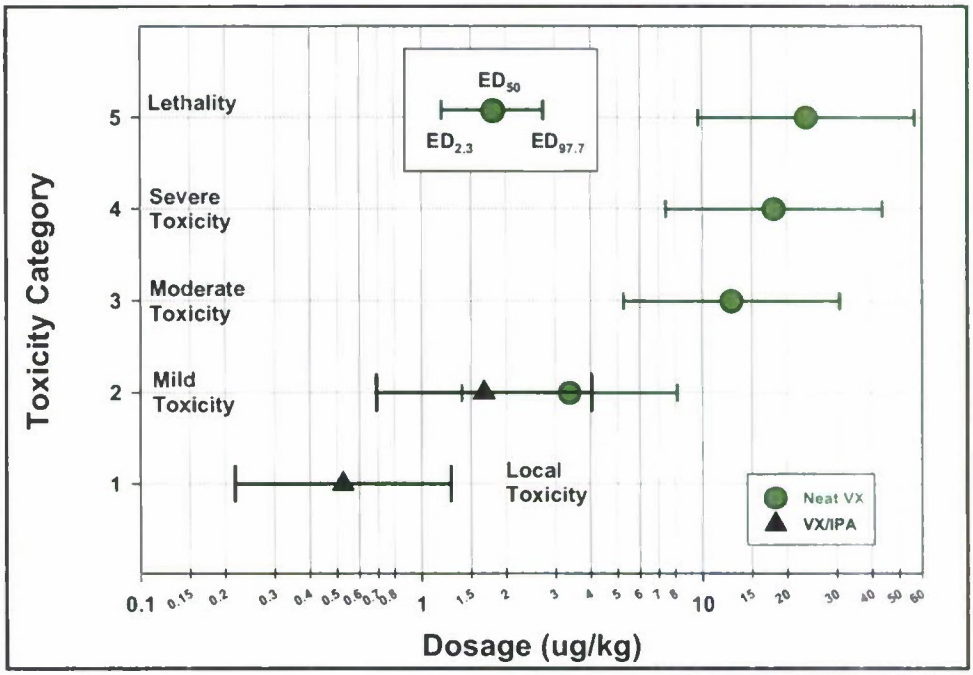

Figure 8. ED_{50} and $ED_{2.3}$ to $ED_{97.7}$ Range, as a Function of Severity of Effects

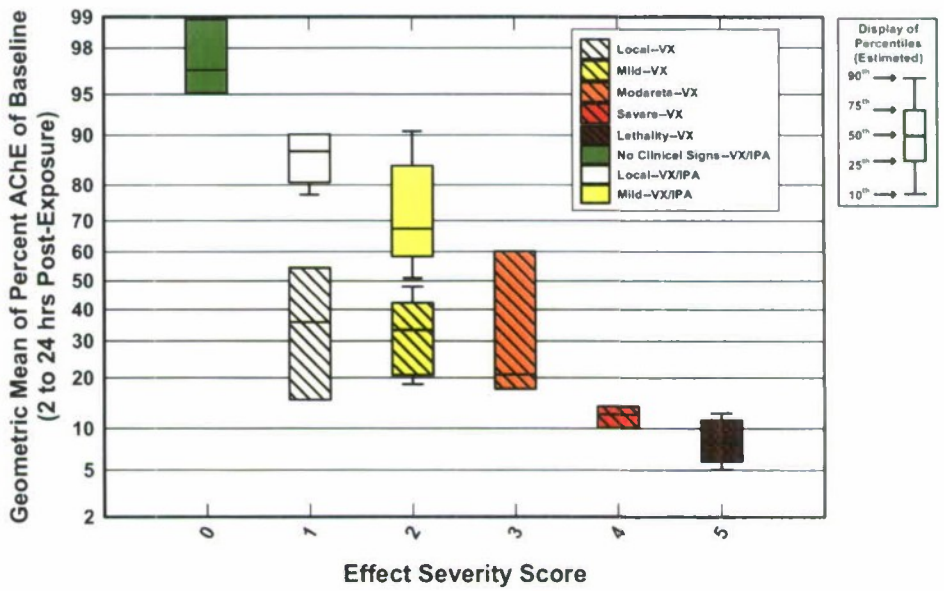

Figure 9. Boxplot[b] of AChE Activity as a Function of Severity of Effects–PC VX

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[b] Boxplots (also known as box-and-whisker plots) were invented by Tukey (5) and are a graphical summary of the distribution of values within a dataset. The horizontal lines on the boxes correspond to the 25th, 50th and 75th percentiles. The "whiskers" correspond to the 10th and 90th percentiles and are only shown if enough points exist within a dataset to permit the calculation of these percentiles.

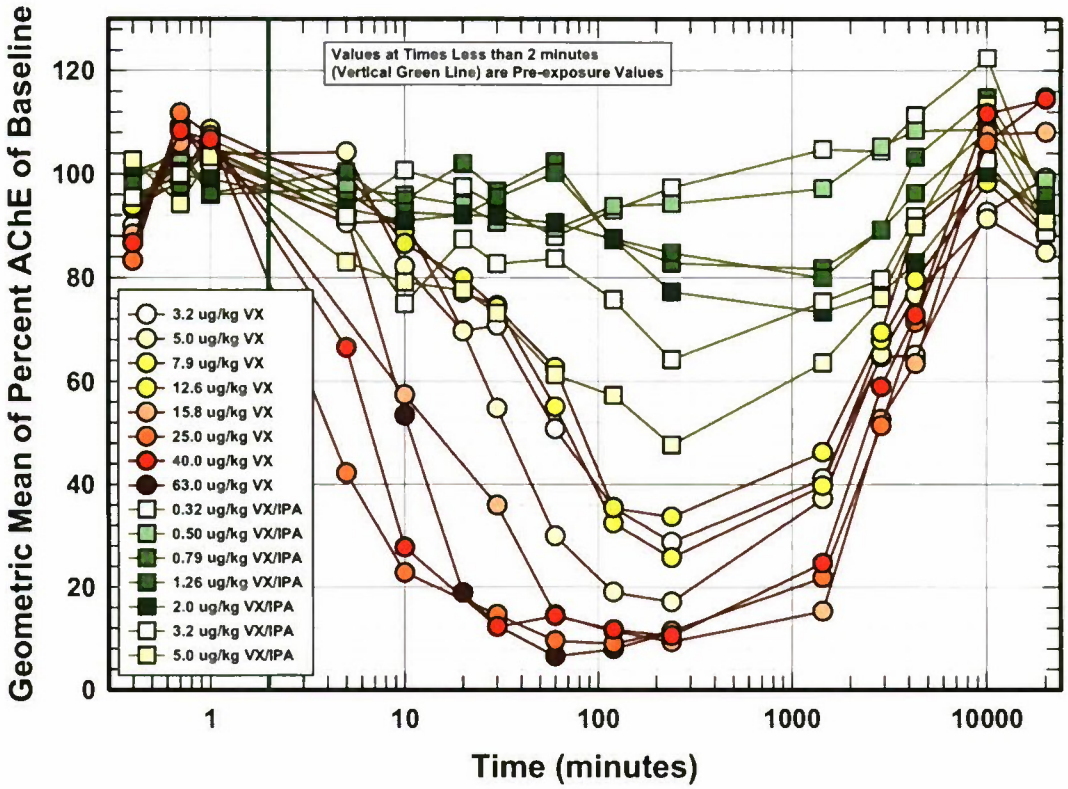

Figure 10. AChE Activity as a Function of Time Post-Exposure, Type of Agent, and Dosage—PC VX

AChE Inhibition

AChE activity as a function of dosage and severity of effects (using score system [0 to 5]) is presented in Figure 9. There is a statistically significant correlation of AChE depression with dosage and severity of effect. AChE activity as a function of time post-exposure, agent (VX *versus* VX/IPA), and dosage is given in Figure 10.

Comparison of Neat VX and VX/IPA

For dosages of 3.2 µg/kg and 5.0 µg/kg, both neat VX and VX/IPA were used. The direct comparison of the two agent preparations yields some very interesting results—neat VX was the more potent AChE inhibitor but more rabbits had clinical signs with VX/IPA (Table 11): 16/16 animals dosed with neat VX had at least threshold AChE inhibition (activity <76% of baseline) *versus* 8/12 animals dosed with the VX/IPA solution 14/16 animals dosed with neat VX had AChE inhibition of <50% *versus* 1/12 animals dosed with the VX/IPA solution. However, for observable signs, the reverse was true: 11/12 animals dosed with the VX/IPA solution had mild (or more severe) clinical signs—versus 9/16 animals dosed with neat VX had mild (or more severe) clinical signs (Table 11).

Table 11. Comparison of Quantal Responses for Neat VX and VX/IPA

Dosage (µg/kg)	Agent	AChE Activity (% baseline)			Observable Signs					
		NSI[++]	<76-50	<50	none	(local	mild	moderate	severe	lethal
		(# responding / # tested)								
3.2	VX/IPA	4/8	4/8	0/8	0/8	1/8	6/8	1/8	0/8	0/8
5.0		0/4	3/4	1/4	0/4	0/4	4/4	0/4	0/4	0/4
3.2	Neat VX	0/8	2/8	6/8	0/8	5/8	3/8	0/8	0/8	0/8
5.0		0/8	0/8	8/8	0/8	2/8	6/8	0/8	0/8	0/8

[++] no significant inhibition (see Section 2.5.4)

These results are also shown in the following 2 x 2 contingency tables [Table 12A and 12B]. A comparison of AChE activity and time post-exposure for VX *versus* VX/IPA is given in Figure 11.

Table 12. Contingency Tables for Quantal Response and AChE Depression

A

	AChE Activity (%baseline)		Subtotal
	>50	≤50	
	# of rabbits		
VX/IPA	11	1	12
Neat VX	2	14	16
Subtotal	13	15	28

B

	Severity of Signs		Subtotal
	< mild	> mild	
	# of rabbits		
VX/IPA	1	11	12
Neat VX	7	9	16
Subtotal	8	20	28

A chi-square analysis (6) was performed on each contingency table. The null hypothesis was that the number of rabbits in each cell is independent of the categorical variables, while the alternative hypothesis was that they were dependent. It was found (Table 12A) that the null hypothesis could be rejected with over 99.9% confidence; for Table 12B, it could be rejected with 96% confidence. Therefore, it is concluded that IPA does change the characteristics of the PC toxicity of VX, and in subsequent data analyses, VX and VX/IPA were treated as two different agents.

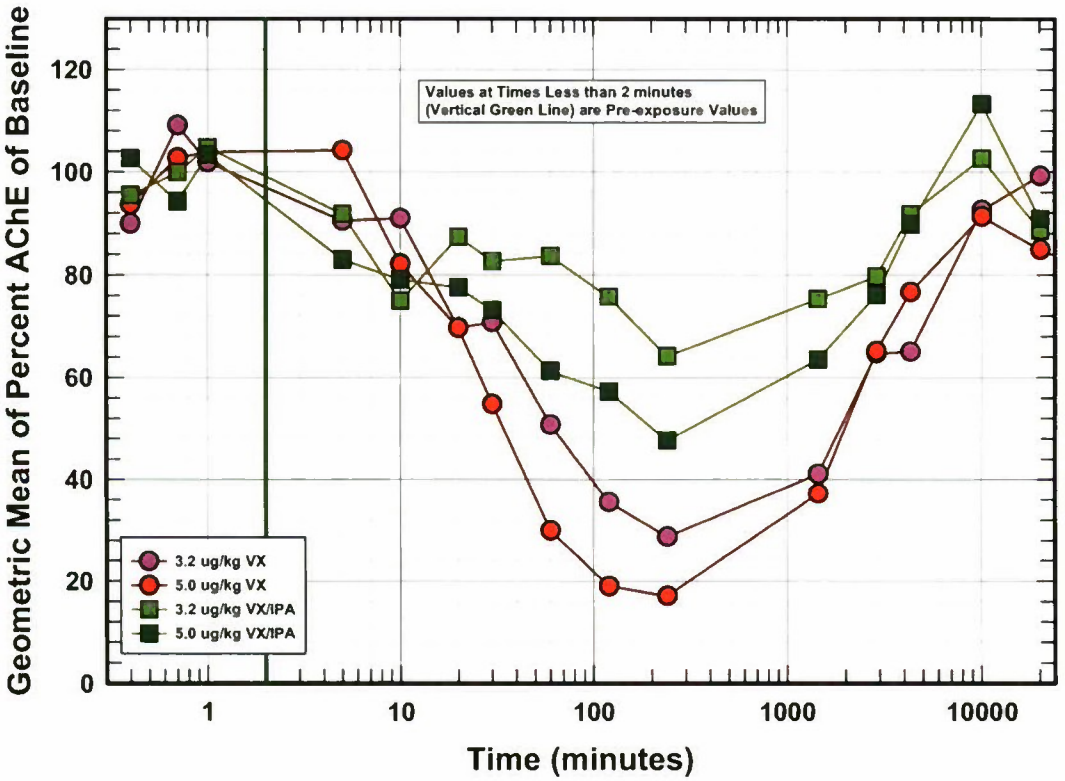

Figure 11. AChE Activity as a Function of Time (Post-Exposure) for PC Exposure to Neat VX or VX/IPA

The PC toxicities of the two agent preparations probably differ due to one or more factors (either acting singularly or in combination): differing amounts of liquid spreading on the skin (with VX/IPA spreading more than neat VX) and IPA changing the characteristics of VX skin absorption and penetration. However, it is not known if these differences would still be present outside of the 3.2 and 5.0 µg/kg dose range. Whatever the reason, the difference is enough to recommend that IPA not be used for any future VX PC liquid studies.

VX Regenerated from Blood Samples

A limited number of blood samples were drawn two weeks post-exposure and analyzed for VX [*via* agent regeneration techniques, (7)]. Regenerated agent was measured in both plasma and RBCs. The data are summarized in Table 13.

Table 13. Regenerated VX
(Blood Drawn 2 Weeks Post PC Exposure)

Dosage (µg/kg)	Agent	Regenerated VX+ (ng/g)	
		Plasma	RBC
3.2	Neat VX	BDL*	BDL*
		BDL*	0.1484
		BDL*	0.3125
5.0		0.1646	BDL*
		0.1940	BDL*
		0.2016	0.2179
3.2	VX/IPA	0.1108	BDL
		0.1226	BDL
		0.1330	BDL
		0.1410	0.0708
5.0		0.1782	0.1031
		0.3634	0.1582

+Bound VX is converted to the GE analog in the process of the regeneration process.
*Below detection limit ([BDL] 0.0500 ng/g).

An ANOVA[q] with three factors: (1) dosage, (2) agent composition and (3) fluid compartment (plasma *versus* RBC), was performed on the regenerated VX data in Table 13. For the purposes of this analysis, half the detection limit (or 0.0250 ng/g) was substituted for the nine BDL results. (Using half the detection limit for left-censored data[r] has been shown to be a reasonable approach to this type of problem (8).) It was found that regenerated agent was significantly correlated ($p = 0.025$) with dosage. The amounts of regenerated agent recovered from the two different blood compartments (plasma) versus RBCs (compartment) was borderline significant ($p = 0.092$); more regenerated agent was recovered from plasma than RBCs. There were two statistically significant two-way factor interactions: (1) agent x compartment ($p = 0.027$) and (2) dosage x compartment ($p = 0.032$).

There is more regenerated agent in plasma than in RBC for VX/IPA, but for neat VX there is no statistically significant difference between agent amounts in plasma and RBC (Figure 12). There is no statistically significant difference between the two dosages for regenerated agent in RBC. Yet, for regenerated agent in plasma, the higher dosage produced a larger amount of regenerated agent than the lower dosage (Figure 13).

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[q] Using the general linear model routine in MINITAB®.

[r] Censored data exist when the exact value of a measured response is not known but a range of possible values is known [*e.g.*, the length of an object is 90 cms (exact) versus the length being less than 100 cms (censored)]. If the range of possible values has a maximum value but an unknown minimum, then the data are left-censored (*e.g.*, length is less than 100 cms). If a minimum is known but not the maximum, then the data are right-censored (*e.g.*, length is greater than 200 cms). If both a maximum and minimum are known, then the data are arbitrarily censored (*e.g.*, length is less than 200 cms but greater than 100 cms).

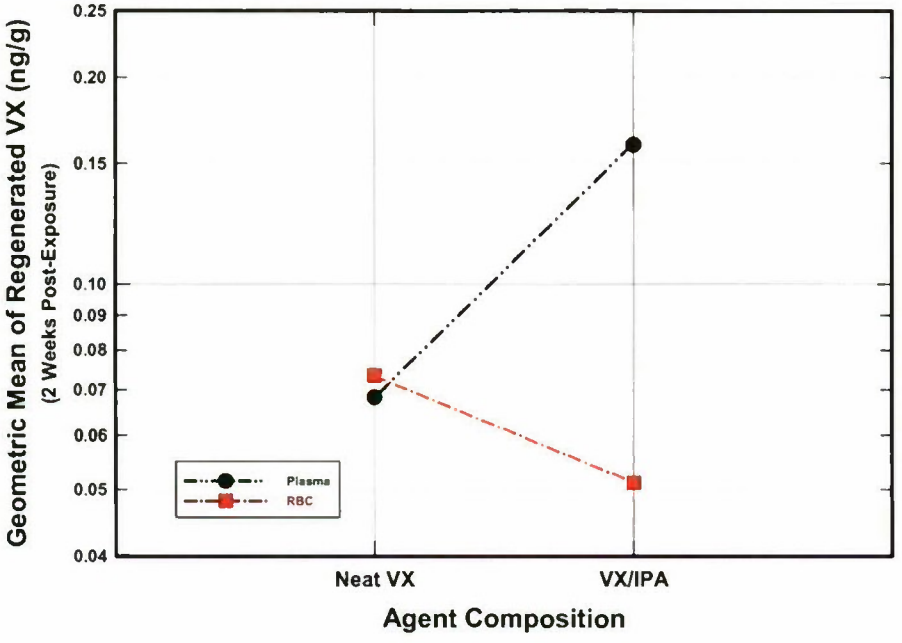

Figure 12. Regenerated VX as a Function of Blood Compartment and Agent Composition

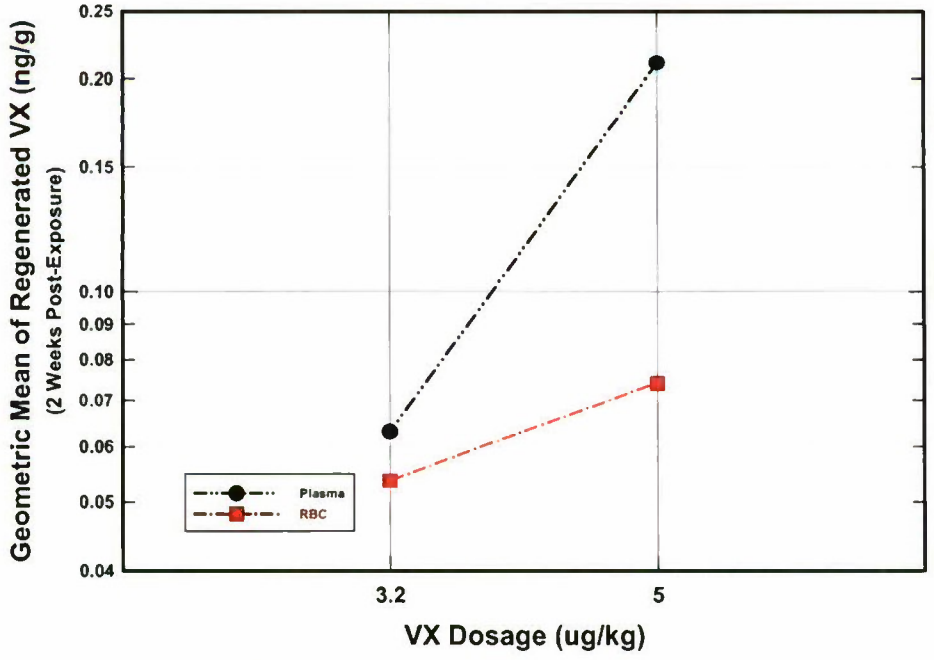

Figure 13. Regenerated VX as a Function of VX Dosage and Blood Compartment

The regenerated agent data are consistent with what was observed in the AChE data. For rabbits exposed to VX/IPA, less regenerated agent was found in the RBC in comparison to that found in the plasma.

The reverse was true for rabbits exposed to neat VX. The corresponding reduction in AChE activity was minimal with VX/IPA in comparison to what was observed with neat VX.

Discussion and Conclusions from PC Studies

1) The experimental dosage range of 0.25 to 63 µg/kg for PC permitted the estimation of ED_{50}s for effects ranging from threshold to lethal.
2) Dilution of VX in IPA—in order to deliver small doses—altered the ED_{50}s and the toxicokinetics of the agent. The following was observed for doses of 3.2 and 5.0 µg/kg: a) AChE inhibition is less pronounced with VX/IPA than with comparable doses of VX; b) clinical signs were more pronounced with VX/IPA than with comparable doses of VX; and c) VX diluted in IPA is more toxic than neat VX for PC exposures.
3) The regenerated agent data were consistent with what was observed with respect to AChE inhibition and clinical signs for doses of 3.2 and 5.0 µg/kg: a) for VX/IPA, less regenerated agent was found in the RBC in comparison to that found in the plasma; and b) for VX, the reverse was true.
4) The minimum AChE activity occurred from 2 to 24 hr post exposure. For the rabbits which survived the larger doses, full recovery of AChE activity took about a week.
5) AChE activity post-exposure is roughly correlated with the severity of clinical signs. However, due to the larger scatter in AChE measurements, this correlation is only useful for qualitative applications.

2.5.6 IV Studies

2.5.6.1 Toxic Signs

Based upon any observed signs and/or AChE activity in blood samples drawn within 24 hr post exposure period, all exposed rabbits were placed into one of the seven following toxicity categories, yielding a (0 to 6) scoring system:

0 No Observable Clinical Signs (no significant AChE inhibition and no toxic signs)
1 Threshold Effects: significant post-exposure AChE inhibition but not exceeding 50% depression and no other toxic signs
2 Covert Toxicity: >50% AChE inhibition and no other toxic signs
3 Mild Toxicity: ataxia or mild tremors/jerks of the head
4 Moderate Toxicity: miosis, salivation, rapid respiration, lacrimation, "circling", chewing, rapid respiration, "jumping"
5 Severe Toxicity: "opisthotonos", splayed legs, nystagmus, "rolling", "running", collapse, prostration, cyanosis, tonic-clonic movements, gasping, cyanosis
6 Lethality (absence of a heart beat as determined by palpation).

For some tables and figures, ChE inhibition was eliminated from the above seven categories and the following (0 to 4) scoring system was used:

0 No Observable Clinical Signs

1 Mild Toxicity: ataxia or mild tremors/jerks of the head

2 Moderate Toxicity: miosis, salivation, rapid respiration, lacrimation, "circling", chewing, rapid respiration, "jumping"

3 Severe Toxicity: "opisthotonos", splayed legs, nystagmus, "rolling", "running", collapse, prostration, cyanosis, tonic-clonic movements, gasping, cyanosis

4 Lethality (absence of a heart beat as determined by palpation).

2.5.6.2 Summary Data from IV Exposures

This portion of the study focused on collecting sufficient (quantal) data to estimate ED_{50}s for lethal and sub-lethal effects in rabbits exposed intravenously to VX. The data were used to predict dose-response relationships and dose-related probabilities, of effects. The results of the data analysis are described below. Table 14 summarizes the results obtained as a function of two different scoring systems ([0 to 4—clinical signs only] and [0 to 6—clinical signs and AChE inhibition]):

- 0.10 µg/kg (the lowest dosage) produced statistically significant AChE inhibition in 1/4 animals
- <2.0 µg/kg produced no effects other than statistically significant AChE depression
- 3.2 µg/kg, produced mild clinical signs in 3/12 rabbits
- 12.6 µg/kg produced 100% lethality (8/8)
- 20.0 µg/kg produced 100% lethality (8/8).

Table 14. Summary IV Data

Dosage (µg/kg)	Study Phase	AChE Activity (% Baseline) [no clinical signs]			Observable Signs				
		NSI*	(83-50)	(<50)	none	mild	moderate	severe	lethal
		(# responding/# tested)							
0.1	sub-lethal	***3/4***	***1/4***	***0/4***	***4/4***	***0/4***	***0/4***	***0/4***	***0/4***
0.158		***2/4***	***2/4***	***0/4***	***4/4***	***0/4***	***0/4***	***0/4***	***0/4***
0.25		2/4	2/4	0/4	4/4	0/4	0/4	0/4	0/4
0.5		0/4	4/4	0/4	4/4	0/4	0/4	0/4	0/4
1		0/4	4/4	0/4	4/4	0/4	0/4	0/4	0/4
2	lethal	0/8	0/8	8/8	8/8	0/8	0/8	0/8	0/8
2	sub-lethal	0/4	0/4	4/4	4/4	0/4	0/4	0/4	0/4
3.2	lethal	0/8	0/8	7/8	7/8	1/8	0/8	0/8	0/8
3.2	sub-lethal	***0/4***	***0/4***	***2/4***	***2/4***	***2/4***	***0/4***	***0/4***	***0/4***
4		***0/4***	***0/4***	***0/4***	***0/4***	***0/4***	***3/4***	***1/4***	***0/4***
5	lethal	0/8	0/8	3/8	3/8	5/8	0/8	0/8	0/8
6.4		0/8	0/8	0/8	0/8	0/8	3/8	5/8	0/8
8		0/8	0/8	0/8	0/8	0/8	1/8	6/8	1/8
9.2		0/8	0/8	0/8	0/8	0/8	0/8	2/8	6/8
12.6		0/8	0/8	0/8	0/8	0/8	0/8	0/8	8/8
20		0/8	0/8	0/8	0/8	0/8	0/8	0/8	8/8

*no significant inhibition (see Section 2.5.4)
pink shading designates overlapping dosages
bolded italics denote outliers—all tested on 31 January 2007

ED_{50}s and Probit Slopes

It was found that the results from the exposures conducted on the second day of the sub-lethal phase (2dSp) were statistically different from those of the rest of the study. These outlier dosages were 0.100, 0.158, 3.2 and 4.0 µg/kg. Using the following form of Equation [1], an indicator variable was used to account for the effect of this group on the ED_{50}s otherwise calculated.

$$Y_N = k_0 + k_D(\log_{10} D) + k_{Day}(Day) \qquad [2]$$

Where

Y_N is a normit
*k*s are fitted coefficients
D is the dosage (µg/kg)
Day is an indicator variable
Equals 1 for exposures on the second test day for the sub-lethal phase and -1 for all other test days

ED_{50}s based on the 2dSp dosages (Day = 1) are a factor of 1.5 times lower (with 95% CL of 1.3 to 1.7) than those based on the other 12 exposures (Day = -1) based upon the ordinal regression fit of Equation [2]. This difference could be due to the unknown contributions of a variety of factors (see 2.4.3 Experimental Design). LD_{50}s and ED_{50}s can be calculated based on one of three assumptions:

1) The quantal results from the 2dSp dosages better reflect the true VX toxicity; thus Equation [2] should be used with Day = 1;
2) The quantal results from the other 12 dosages in the study better reflect the true VX toxicity, thus Equation [2] should be used with Day = -1;
3) The true VX toxicity is best represented by taking an average of the two dosage groups, thus Equation [2] should be used with Day = 0;

Of the three assumptions, the second was used in the calculation of the LD_{50} and the ED_{50}s (sub-lethal effects) listed in Table 15. The second assumption is more defensible for several reasons. First, the resulting LD_{50} is in better agreement with the historical LD_{50}s, which typically range from 8 to 10 µg/kg (1). The LD_{50} based on the first and third assumptions equal 5.8 and 7.2 µg/kg, respectively, both of which fall outside the historical range. Second, observational errorss are less likely to occur as the effects become more severe. None of the 2dSp dosages produced any deaths and only one rabbit exhibit severe effects.

The probit slope equals 14.1, with 95% CL of 10.2 to 17.9. The ED_{50}s (and the $ED_{2.3}$ to $ED_{97.7}$ range) as a function of severity of effects from Table 15 are presented in Figure 14.

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[s] Observational errors can either be *via* commission (mischaracterization of a clinical sign) or omission (missing a sign).

Table 15. ED_{50}s and Dosage Ratios+

Effect(s)	ED_{50} (µg/kg)	95% CL		
		Lower	Upper	Upper/Lower*
threshold AChE	0.23	0.19	0.27	1.39
< 50% AChE	1.36	1.04	1.77	1.71
Mild	4.42	3.92	5.00	1.28
moderate	5.49	4.99	6.05	1.21
Severe	6.54	5.98	7.16	1.20
Lethal	8.91	8.10	9.80	1.21
Dosage Ratio		**95% CLs**		
		Lower	**Upper**	**Upper/Lower***
lethal : threshold AChE	39.5	32.7	47.8	1.5
lethal : <50% AChE	6.6	4.9	8.7	1.8
lethal : mild	2.0	1.7	2.4	1.4
lethal : moderate	1.6	1.4	1.9	1.3
lethal : severe	1.4	1.2	1.5	1.3
severe : threshold AChE	29.0	24.3	34.6	1.4
severe : < 50% AChE	4.8	3.6	6.4	1.8
severe : mild	1.5	1.3	1.7	1.3
severe : moderate	1.2	1.1	1.3	1.2
moderate : threshold AChE	24.4	20.5	29.0	1.4
moderate : <50% AChE	4.1	3.1	5.4	1.8
moderate : mild	1.2	1.1	1.4	1.3
mild : threshold AChE	19.6	16.3	23.6	1.4
mild : <50% AChE	3.3	2.4	4.4	1.8
<50% ChE : threshold AChE	3.3	2.4	4.4	1.8

+Rating scale 0-6–Indicator variable, Day, set equal to -1.

*CLs for the ED_{50}s can be misleading when expressed on a linear basis because the ED_{50}s are actually calculated on a log basis. Thus, the proper metric for evaluating the precision of ED_{50} estimates is the ratio of the upper and lower CLs.

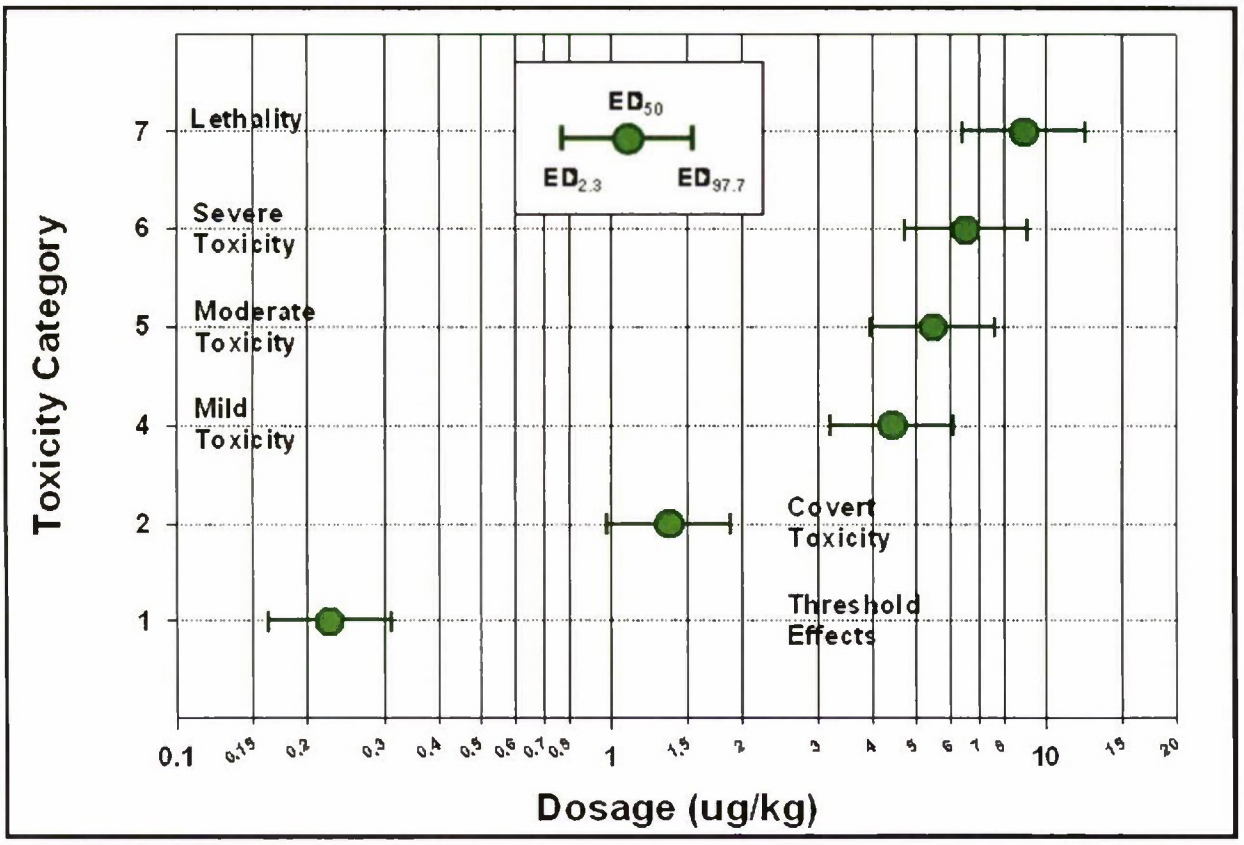

Figure 14. ED_{50}s as a Function of Endpoint for IV Exposures

AChE Inhibition

The percent depression of AChE activity as a function of dosage and severity of effects (using score system [0 to 6]) is presented in Figure 15. Boxplots of the percent depression as a function of severity of effects (using score system [0 to 4]) are presented in Figure 16. There is a statistically significant correlation of AChE depression on the dosage and severity of effect. AChE activity as a function of time post-exposure and dosage is given in Figure 17.

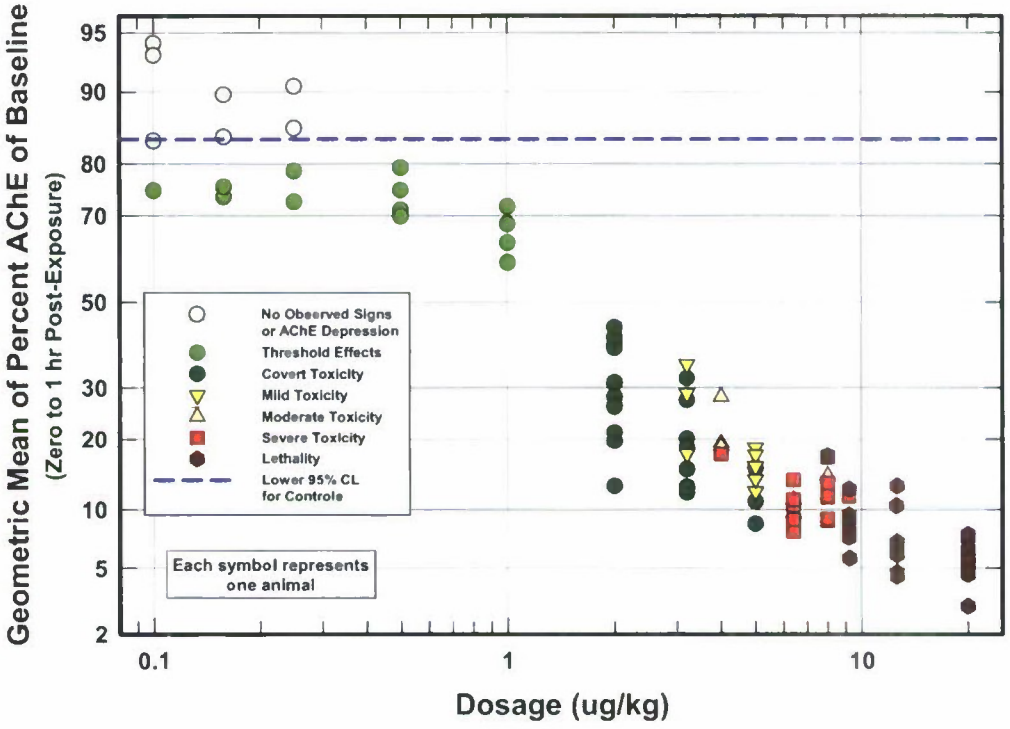

Figure 15. VX-Induced AChE Depression (0 to 1 hr Post-Exposure) as a Function of Dosage and Severity of Effects—IV

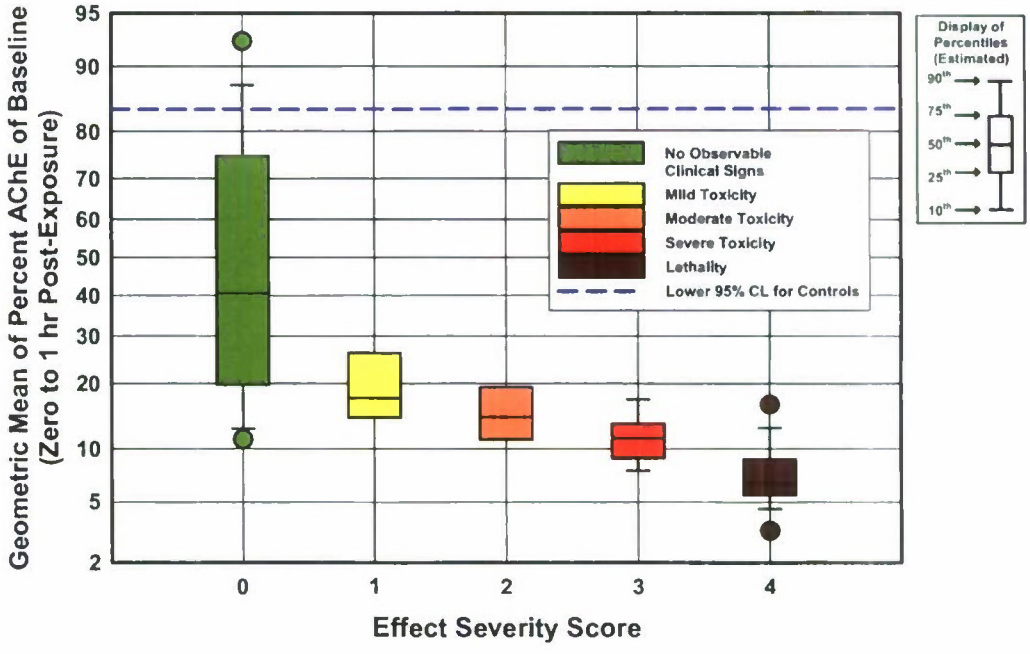

Figure 16. Boxplot of VX-Induced AChE Depression as a Function of Severity of Effects—IV

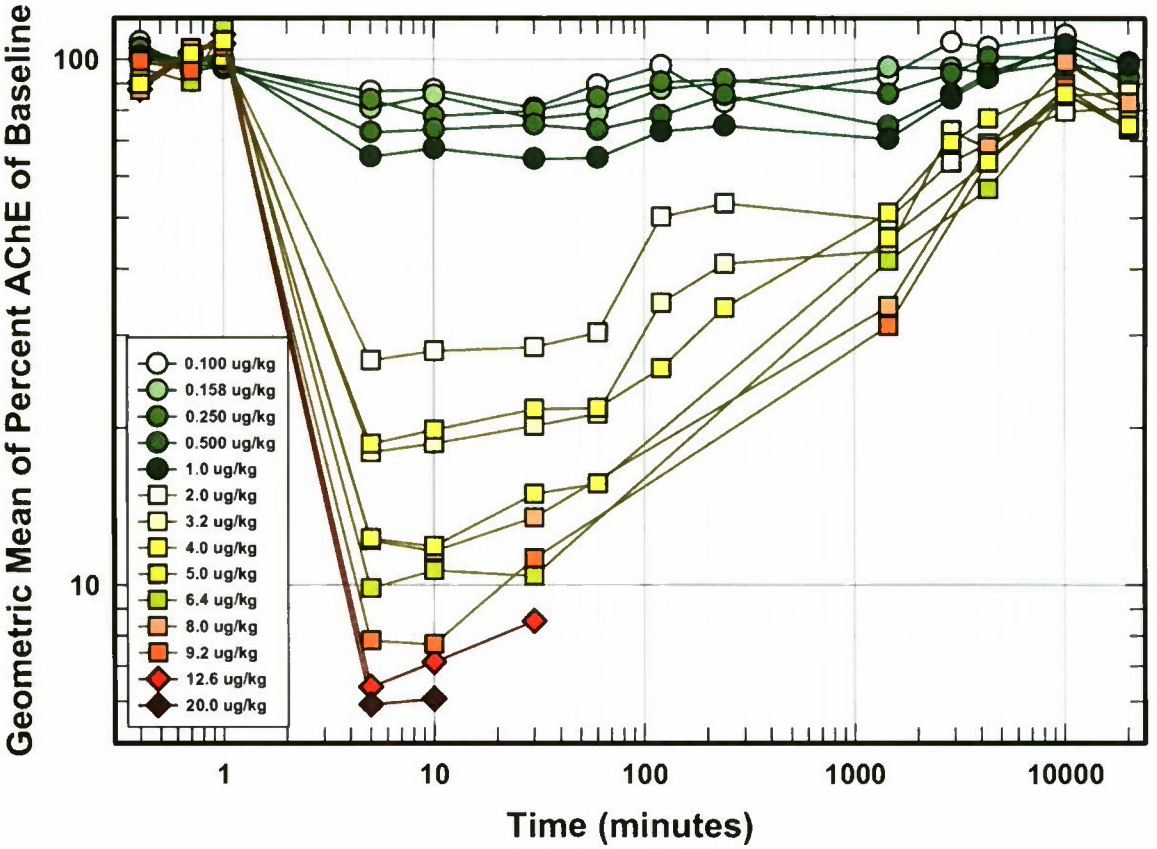

Figure 17. AChE Activity *versus* Time Post-Exposure and VX Dosage—IV

Discussion and Conclusions from IV Studies

1) The experimental dosage range of 0.1 to 20 µg/kg permitted the estimation of the ED_{50}s for effects ranging from lethality to threshold AChE depression (with no observable clinical signs).

2) The dose-response curve (for percent affected) is steep for IV exposures. The probit slope is 14.1 (with 95% CLs of 10.2 to 17.9) for all endpoints.

3) The dose-response curve (for severity of effects) is shallow at the lower doses. (AChE depression is the first noticeable sign;[t] for doses ranging from 0.1 to about 2 µg/kg [a factor of 20] only AChE depression is observed.) However, at larger doses, the severity of effects and number of animals affected rapidly increases. (A small percentage of animals had mild effects at 3 µg/kg, and 98% receiving a dosage of 10 µg/kg [a factor of 3.3 increase] died [Figures 14 and 15]).

4) No clinical signs were observed in any rabbit having AChE activity of 40% or more of baseline (within 1 hr post-exposure). The lowest post-exposure AChE activity in the absence of any other signs was 9% of baseline (at a dose of 5 µg/kg). Statistically significant AChE depression, with no other signs, was observed at dosages of 2.0 µg/kg or less.

5) Maximum AChE depression was observed within 5 min of exposure. Once maximum

[t] In contrast to some rabbits exposed percutaneously to VX/IPA, where clinical signs were observed in absence of AChE depression--see Section 2.5.5.

depression was reached, the AChE activity stayed relatively constant up to 1 hr post-exposure (for surviving rabbits) followed by a slow rate of recovery. For the higher doses, it took about 7 days for a full recovery.

Comparison of PC and IV Exposures

Two findings are noted: (1) There is no statistically significant difference between the PC to IV ratios for moderate, severe and lethal effects. The weighted average of the three ratios equals 2.55 with 95% confidence interval of 2.22 to 2.92 (Table 16).[u] (2) The PC to IV ratio for mild (systemic) effects is different (with over 95% confidence) from the ratios for moderate, severe, and lethal effects.

Table 16. Ratio of PC to IV ED_{50}s for Rabbit VX Exposures

Level of Effect	Route	ED_{50} (µg/kg)	log(ED_{50})	SE on log(ED_{50})	PC to IV Ratio of ED_{50}s				
					log(Ratio)	SE on log(Ratio)	Ratio	95% CI on Ratio	
								Lower	Upper
Mild	PC	3.60	0.5567	0.057	-0.0932	0.063	0.81	0.61	1.07
	IV	4.47	0.6499	0.027					
Moderate	PC	12.5	1.0967	0.045	0.3752	0.050	2.37	1.89	2.98
	IV	5.27	0.7215	0.022					
Severe	PC	17.7	1.2471	0.044	0.4308	0.049	2.70	2.16	3.36
	IV	6.55	0.8163	0.020					
Lethal	PC	23.0	1.3615	0.047	0.4106	0.052	2.57	2.04	3.25
	IV	8.93	0.9510	0.021					
Weighted Average of log(Ratio)s from Moderate, Severe and Lethal					0.406	0.030	2.55	2.22	2.92

2.5.6.3 Future Directions and Recommendations

- Augment the rabbit data with analyses of similar studies in Göttingen minipigs
- Validate different micropippetters for delivery of smaller doses of neat agent than were possible in this study.
- Do not use IPA as a diluent for future work.
- If possible, assay agent dilutions before and after each study.

2.5.7 Literature Cited

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[u]The weighted average was performed on the three log(Ratio)s using the inverse of the squares of the individual standard errors (SE) as the weights.

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2.5.8 Appendix—Ordinal Regression Analysis of Dataset from both Neat VX and VX/IPA PC Exposures of Rabbits

Tabulated statistics: Dosage, Score, Dilute

Tabulated statistics: Dosage, Score, Dilute

Results for Dilute = -1 (Neat VX)

Rows: Dosage Columns: Score

	0	1	2	3	4	5	All
3.20	0	5	3	0	0	0	8
5.00	0	2	6	0	0	0	8
7.90	0	0	8	0	0	0	8
12.60	0	0	5	3	0	0	8
15.80	0	0	0	2	2	4	8
25.00	0	0	0	0	2	6	8
40.00	0	0	0	1	2	5	8
63.00	0	0	0	0	0	8	8
All	0	7	22	6	6	23	64

Results for Dilute = 1 (VX/IPA)

Rows: Dosage Columns: Score

	0	1	2	3	4	5	All
0.25	1	0	0	0	0	0	1
0.32	4	0	0	0	0	0	4
0.50	2	3	0	0	0	0	5
0.79	1	3	0	0	0	0	4
1.00	0	1	0	0	0	0	1
1.26	0	0	4	0	0	0	4
2.00	0	3	2	0	0	0	5
3.20	0	1	6	1	0	0	8
5.00	0	0	4	0	0	0	4
All	8	11	16	1	0	0	36

Cell Contents: Number of Rabbits

Score
0: No observable effects
1: Local
2: Mild
3: Moderate
4: Severe
5: Lethal

Dosage: Applied dosage of VX (µg/kg)
logD: log base 10 of the agent dosage (µg/kg)

Ordinal Logistic Regression: Score versus logD, Dilute

Link Function: Normit

Response Information

Variable	Value	Count
Score	0	8
	1	18
	2	38
	3	7
	4	6
	5	23
	Total	100

Logistic Regression Table

Predictor	Coef	SE Coef	Z	P
Const(1)	-0.663326	0.378898	-1.75	0.080
Const(2)	1.95244	0.360422	5.42	0.000
Const(3)	4.95558	0.609605	8.13	0.000
Const(4)	5.74038	0.671161	8.55	0.000
Const(5)	6.33930	0.722873	8.77	0.000
logD	-5.21018	0.635739	-8.20	0.000
Dilute	-0.795507	0.230042	-3.46	0.001

Log-Likelihood = -71.353
Test that all slopes are zero: G = 171.570, DF = 2, P-Value = 0.000
Goodness-of-Fit Tests

Method	Chi-Square	DF	P
Pearson	37.3566	78	1.000
Deviance	42.7734	78	1.000

Variance-Covariance Matrix

0.143564	0.004110	-0.008572	-0.011647	-0.013374	-0.006557	-0.0261192
0.004110	0.129904	0.159181	0.174731	0.187803	-0.177387	-0.0419142
-0.008572	0.159181	0.371618	0.382616	0.401397	-0.357255	-0.0714570
-0.011647	0.174731	0.382616	0.450457	0.460210	-0.393567	-0.0762239
-0.013374	0.187803	0.401397	0.460210	0.522545	-0.423563	-0.0810827
-0.006557	-0.177387	-0.357255	-0.393567	-0.423563	0.404164	0.0997174
-0.026119	-0.041914	-0.071457	-0.076224	-0.081083	0.099717	0.0529195

[DRS] The ratio of ED_{50}s for neat and diluted VX liquid equals 2.02 with 95% confidence interval of 1.47 to 2.77 (based upon calculations from the variance-covariance matrix).

[DRS] For examples on how to calculate from MINITAB printouts the ED_{50}s, ratios of ED_{50}s and their associated standard errors, see the appendix of Benton, BJ, *et al.*, *Effects of Whole-Body VX Vapor Exposure on Lethality in Rats*; ECBC-TR-525; US Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2007;UNCLASSIFIED Report (AD-A462960).

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Ordinal Regression Analysis of Dataset VX IV Exposures of Rabbits

Tabulated statistics: Dosage, Score, Day4

Results for Day4 = -1
(Runs on days other than 31 January 2007)

Rows: Dosage Columns: Score

	0	1	2	3	4	5	6	All
0.250	2	2	0	0	0	0	0	4
0.500	0	4	0	0	0	0	0	4
1.000	0	4	0	0	0	0	0	4
2.000	0	0	12	0	0	0	0	12
3.200	0	0	7	1	0	0	0	8
5.000	0	0	3	5	0	0	0	8
6.400	0	0	0	0	3	5	0	8
8.000	0	0	0	0	1	6	1	8
9.200	0	0	0	0	0	2	6	8
12.600	0	0	0	0	0	0	8	8
20.000	0	0	0	0	0	0	8	8
All	2	10	22	6	4	13	23	80

Results for Day4 = 1
(Runs on 31 January 2007)

Rows: Dosage Columns: Scores

	0	1	2	3	4	5	6	All
0.100	3	1	0	0	0	0	0	4
0.158	2	2	0	0	0	0	0	4
3.200	0	0	2	2	0	0	0	4
4.000	0	0	0	0	3	1	0	4
All	5	3	2	2	3	1	0	16

Cell Contents: Count

Score
0: No observable effects and AChE activity greater than 83% baseline
1: No observable effects and AChE activity between 50 and 83% baseline
2: No observable effects and AChE activity below 50% baseline
3: Mild
4: Moderate
5: Severe
6: Lethal

Dosage: Applied dosage of VX (µg/kg)
logD: log base 10 of the agent dosage (µg/kg)

Ordinal Logistic Regression: Score versus logD, Day4

Link Function: Normit

Response Information

Variable	Value	Count
Score	0	7
	1	13
	2	24
	3	8
	4	7
	5	14
	6	23
	Total	96

Logistic Regression Table

Predictor	Coef	SE Coef	Z	P
Const(1)	-10.3908	1.47388	-7.05	0.000
Const(2)	0.572092	0.863884	0.66	0.508
Const(3)	7.78957	1.23784	6.29	0.000
Const(4)	9.11468	1.33075	6.85	0.000
Const(5)	10.1788	1.42542	7.14	0.000
Const(6)	12.0660	1.64224	7.35	0.000
logD	-14.0614	1.97071	-7.14	0.000
Day4	-1.29021	0.292922	-4.40	0.000

Log-Likelihood = -49.922
Test that all slopes are zero: G = 251.393, DF = 2, P-Value = 0.000

Goodness-of-Fit Tests

Method	Chi-Square	DF	P
Pearson	44.3265	82	1.000
Deviance	26.2452	82	1.000

Variance-Covariance Matrix

2.17231	-0.158950	-1.66859	-1.82500	-1.95953	-2.25474	2.76296	0.294959
-0.15895	0.746296	0.14752	0.16071	0.17676	0.21439	-0.21812	0.016819
-1.66859	0.147523	1.53226	1.57754	1.67731	1.92499	-2.34333	-0.237447
-1.82500	0.160710	1.57754	1.77089	1.84992	2.10707	-2.56307	-0.260361
-1.95953	0.176755	1.67731	1.84992	2.03181	2.27178	-2.75145	-0.275263
-2.25474	0.214395	1.92499	2.10707	2.27178	2.69696	-3.16451	-0.305483
2.76296	-0.218118	-2.34333	-2.56307	-2.75145	-3.16451	3.88370	0.419910
0.29496	0.016819	-0.23745	-0.26036	-0.27526	-0.30548	0.41991	0.085803

2.6 A Proposed Procedure for Determining Performance Impact from Contact Agent Exposure in African Green Monkeys[v]

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2.6.1 Introduction

The evaluation of the performance impact of sub-lethal percutaneous (PC) exposure to chemical warfare nerve agents (CWNAs) in non-human primates presents particular challenges. These compounds are typically potent and have steep dose-effect functions. Furthermore, the inherent variability in absorption from PC application presents further complications. We present here a proposed model to conduct such evaluations in non-human primates in a standardized fashion. The paradigm employs three general and independent evaluative dimensions to enable the collection of dependent measures. First, we use scored observations to quantify the appearance of subjective signs of toxicity. Second, we use a serial probe recognition (SPR) task that evaluates general performance and specifically, cognitive performance. Lastly, we sample blood for an assay of parent compound or analogues (e.g., using a regeneration assay), or of pharmacological effects of the exposure (e.g., cholinesterase assay). All three measures are relatively noninvasive and are collected in unanaesthetized subjects. Data can be collected before and after exposure to capture relatively small changes in individual subjects. Furthermore, data can be collected over moderately long time periods to address the persistence or delayed onset of effects.

We have used this basic model previously to evaluate the effects of parenteral exposure to VX (1) and sarin (GB) (2) in African green monkeys. More recently, we have used a similar model to evaluate the effects of whole-body vapor exposure to GB (3) and soman (in press 4). The latter two evaluations also included threshold determinations for the occurrence of miosis. Our current objective is to extend the model for use with a PC exposure to a CWNA such as VX.

2.6.2 Materials and Methods

2.6.2.1 Subjects

African green monkeys ([*Chlorocebus aethiops*] [Primate Products, Inc., Miami, FL]) will be used. Monkeys will be adults weighing 3-5 kg and individually housed and maintained in a temperature (20-22 °C) and humidity (50 +/-10%) controlled vivarium under a 12 hr light-dark cycle (lights on at 0600). Water will be available ad libitum and all monkeys will be fed commercial primate rations supplemented with fresh fruit, nuts, and vegetables. The diet will also include banana-flavored nutritional pellets (190 mg, Bio-Serv, Frenchtown, NJ) earned during behavioral sessions. The monkeys will be mildly food restricted to maintain performance motivation by regulating food intake outside of that earned during behavioral sessions. Additionally, all monkeys will be weighed at least once every two weeks to confirm a stable body weight and to make minor adjustments to daily food allotments.

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[v] The views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense, (para 4-3, AR 360-5). Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. All procedures were reviewed and approved by the Institutes' Animal Care and Use Committees, and performed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

2.6.2.2 PC Exposure Procedure

An area of approximately 25 cm^2 on the upper shoulder, approximately 12 cm from the vertebral column, of each monkey will be prepared as the exposure site by removal of hair and inspection for any skin breaks. On the day of exposure, the monkeys will be chaired and moved to a surety room. An applicator will be prepared by affixing a stainless steel disc, measuring approximately 15 mm in diameter, in the center of an adhesive wrap measuring approximately 20 cm^2. Measured agent will be applied to the stainless steel disc using a calibrated digital syringe. Subsequently, the applicator will be pressed onto the exposure site on the monkey. For the duration of the exposure, the monkey will be observed to insure that full contact of the applicator with the skin is maintained. Following the exposure period, the applicator will be removed from the monkey and can subsequently be assayed for agent quantity. Additionally, the exposure site can be swabbed before decontamination and the swabs can also be assayed for agent quantity.

2.6.2.3 Subject Observation Procedure

All monkeys will be specifically monitored for clinical signs of toxicity. Sign evaluation can be customized for each agent if particular signs are anticipated to occur or are associated with the agent. For example, for organophosphorus agents such as VX, monkeys can be evaluated for the presence of a number of signs including: ataxia, tremors, convulsions, salivation, fasciculation, hyperactivity, rhinorrhea, lacrimation and prostration, which are associated with an increase in cholinergic activity. Data collection for subject observation is implemented through a scoring checklist and can correspond to evaluation at different post-exposure intervals. Section 2.6.5 Appendix includes an example of a score sheet suitable for use for the evaluation of PC VX exposure.

2.6.2.4 Blood Sampling and Assay Procedure

For blood sample collection, the monkeys will be moved from the home cage using a pole and collar technique (5) and placed in a primate restraining chair. Blood samples will be collected from the lateral saphenous vein at multiple time points relative to the PC exposures and can be taken for several months following the exposure as needed. Additionally, blood will be collected after a control exposure occurring prior to the agent exposure for each subject.

Assays for acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity, indicated for PC exposure to VX, will be conducted using whole blood. Details of the procedure, which is a modification of the Ellman method (6), have been presented previously (7). Additionally, blood samples can be assayed for regenerated agent to estimate body burden. In the case of PC VX exposures, assays can be performed to quantify VX (S-[2-(diisopropylamino)ethyl]-O-ethyl methylphosphonothioate) (8) and/or the VX-G analogue (O-ethyl methylphosphonofluoridate) (9).

2.6.2.5 Behavioral Testing Procedure

All monkeys will be tested behaviorally using a SPR task, which is a list memory task wherein sequences of stimuli (sample stimuli) are presented. Subsequently, the subject is presented with a choice stimulus (probe) and, based upon extensive training, responds differentially depending upon whether or not the probe stimulus appeared in the previous sequence. Behavioral sessions will be conducted with monkeys, unrestrained in their home cages, using a custom-manufactured, computer-controlled behavioral test panel that contains an interactive touch screen monitor for the presentation of visual stimuli and the detection of subject responses (Figure 18). Details of the apparatus and our implementation of the SPR have been published previously (2). SPR sessions consist of a series of discrete trials. Each trial contains an observation period and a choice period, and a sample trial is illustrated in Figure 19. In the observation period, sequences of up to three stimuli (alpha-character pairs) are presented individually in the center of the screen. The stimuli are chosen randomly, without replacement, within the trial from a set of 197 stimuli.

After all list stimuli are displayed, the choice period begins. In the choice period, two stimuli are presented side by side on the screen. One choice stimulus is always a white square. The

other stimulus is one from the pool of all stimuli, and thus, may have been presented in the previous list ($p = 0.50$). If the latter stimulus were presented in the previous list, then touching it on the screen is considered a correct choice and is reinforced with a brief 1000 Hz tone and the presentation of a banana-flavored food pellet. If the character stimulus during the choice screen was not among those presented in the previous stimuli list, touching the white square is considered a correct choice and is reinforced in the same manner. Sessions can be conducted daily and, ideally, at approximately the same time of day, and normally consist of 240 trials or until 120 min elapse. Sessions normally can be conducted on exposure days, beginning shortly after the exposure concludes.

Figure 18. African Green Monkey Working on the SPR Test in Home Cage

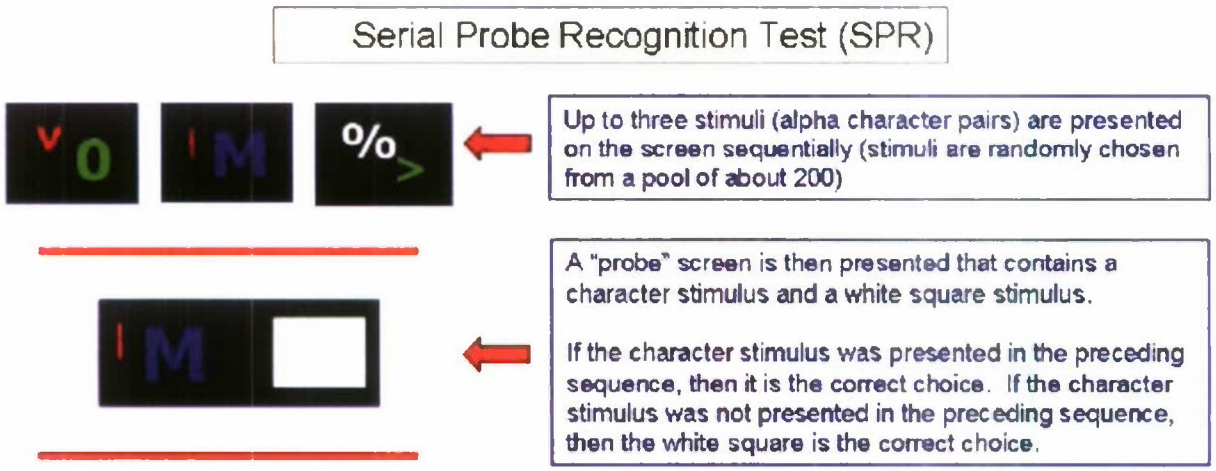

Figure 19. Single Trial on the SPR Test

Performance on the SPR is characterized by three major indices: accuracy, trials completed, and response time. Accuracy is defined as the percentage correct for trials that include a choice response. Trials completed are defined as the number of trials where a choice was made, with 240 normally being the maximum. Response time is defined as the length of the interval between presentation of the choice stimuli and a subject's touch on one of the choice stimuli, and is only calculated on trials during which a choice was made.

2.6.3 Conclusions

A paradigm for the systematic evaluation of PC exposure to CWNA is described. The procedures include systematic scoring of subjective signs of toxicity, evaluation of general and cognitive behavioral performance, and blood assay. The model has been successfully used previously to evaluate parenteral and inhalation (whole-body vapor) exposure to CWNA. We are currently extending the model to include PC exposure to VX.

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2.6.5 Appendix—Subjective Signs Score Sheet

Protocol #:	**Route:** Percutaneous	**Compound:**	**Date:**
Species/ Strain: African Green Monkey	**Group:**	**Applied Concentration (µg):**	**Start Time:**
Exposure Bldg./Room:	**T (°F):** **R.H.(%):**	**Exposure Duration (min):**	**Stop Time:**
Post-Exposure Bldg./Room:	**T (°F):** **R.H.(%):**		

Subject ID _____		Ataxia	Tremors	Convulsions	Salivation	Fasciculation	Hyperactivity	Rhinorrhea	Lacrimation	Prostration	Lethargy	Diarrhea	Miosis		
0-60 min															
1-4 h															
5-12h															
13-24 h															
25-48 h															
49-72 h															

Study Director:	**Observer:**

3 BIOAVAILABILITY AND KINETICS

3.1 Biomarker Methods for Nerve Agents

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Biomarker methods for nerve agents, traditional and non-traditional, will depend on the stability and metabolism of the agents in a particular biological model. Depending on the structure of the agent and the chemical environment, it may be stable enough to be measured unchanged. This may be especially true with agent pools in the skin after contact exposure. However, nerve agents owe their biological activity to their ability to react with certain proteins; therefore, breakdown and metabolism is inevitable.

The traditional chemical warfare nerve agents (CWNAs), such as soman (O-pinacolyl methylphosphonofluoridate, GD, CAS Registry Number: 96-64-0), sarin (O-isopropyl methylphosphonofluoridate, GB), cyclosarin (O-cyclohexyl methylphosphonofluoridate, GF), and VX (S-[2-(diisopropylamino)ethyl]-O-ethyl methylphosphonothioate), are extremely toxic organophosphorous compounds that inhibit the enzymatic activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). AChE and BChE can be found in the blood; however, a more physiological relevant pool of AChE resides in the nervous system. Nervous system signs of exposure include muscle twitches, tremors, convulsions, seizures, salivation, miosis, and death attributable to respiratory failure (1). At lower doses, the majority of these toxic signs are not typically observed necessitating the development of alternative exposure detection methods. While blood cholinesterase (ChE) inhibition has been shown to be markers of CWNA vapor exposure, they are not optimum indicators of the severity of exposure as well as the subsequent onset of clinical signs (*i.e.*, salivation, tremors, and convulsions)(2).

Nerve agents can also undergo hydrolysis independent of their reaction with ChEs. Some of these reactions may be facilitated by enzymes present in blood and tissues. In all cases of hydrolysis, the products of the reaction are an alkyl methylphosphonate and a fluoride ion, or the sulfur sidechain in the case of V-agents (3, 4, 5). Both reaction products have been used to verify exposure and are biomarkers; however, they are rapidly eliminated in the urine (3).

To better examine the degree of nerve agent exposures, a fluoride ion-induced nerve agent regeneration method has been utilized for the retrospective detection of CWAs n blood and tissues from exposed individuals that is both specific and quantitative (6). The regeneration method is dependent upon incubation of nerve agent-exposed tissues with fluoride ions, after which, the phosphonyl moiety of the nerve agent is released, resulting in regeneration of either the original CWA or a fluoro derivative in the case of VX (O-ethyl methylphosphonofluoridate; VX-G). A variation of this fluoride reactivation assay for detecting VX-G has been previously applied as a biomarker for the presence of VX in spiked rat and human serum (7).

The following sections will highlight the available methods used to study the biomarkers of nerve agent exposure with the exception of ChE inhibition, which has been covered extensively in the literature. These methods will include an alkyl methylphosphonate method for GF in plasma (8), a method for free VX (9), a regeneration method for GD (10), a regeneration method for VX applied to an accidental human exposure (11), and a regeneration method for VX applied to known exposure levels in the minipig (12).

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3.2 Quantification of Sarin and Cyclosarin Metabolites Isopropyl Methylphosphonic Acid and Cyclohexyl Methylphosphonic Acid in Minipig Plasma Using Isotope-Dilution and Liquid Chromatography-Time of Flight Mass Spectrometry

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3.2.1 Introduction

The study of the toxicological effects of chemical warfare agents (CWAs) and organophosphorous (OP) pesticides are of increasing importance due to the changing political climate and continued threats from terrorist organizations. The acute toxicity, simplicity of manufacture, and ease of delivery of nerve agents such as sarin (GB, isopropyl methylphosphonofluoridate, CAS Registry Number 107-44-8) and cyclosarin (GF, cyclohexyl methylphosphonofluoridate, CAS Registry Number 329-99-7) make them prime candidates for use in terrorist attacks and under battlefield conditions. GB and GF are part of a group of OP nerve agents that are highly toxic due to their ability to inhibit cholinesterases (ChEs). ChEs, such as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), are inactivated by phosphonylating the serine hydroxyl group at the active site of the enzyme. This inhibition renders AChE unable to hydrolyze acetylcholine released during the transmission of nerve signals used to control a myriad of physiological functions. As a result, the primary symptoms of nerve agent exposure include miosis, loss of bodily functions, convulsions, excessive mucous secretions, asphyxia, and ultimately death (1).

In determining the potential exposure of a subject to this class of nerve agents, it is important to develop analytical methods that are sensitive and selective enough to provide dose-metric information at low levels, even when clinical signs are absent. The dose-metric information obtained in the laboratory setting may allow soldiers or emergency responders to assess the risks and safety measures required for working in potentially contaminated environments.

Previous studies have investigated blood esterase activity as a measure of the exposure level a subject has received. These esterase activity methods have high variability within a population and have limited utility as dose-metric biomarkers (2). The practice of measuring ChE activity in blood samples is based on activity depression, which requires knowledge of individual pre-exposure baseline levels. Even with baseline values, these assays may also lack the sensitivity required for low level exposure and the specificity to determine the cause of the inhibition.

The use of fluoride ion reactivation assays have shown better utility as an indicator of exposure because the original agent is released from the ChE and quantified (3-6). These methods have the unique ability of specifically detecting the agent for a period up to two weeks post-exposure, and have been successfully applied to red blood cell (RBC), plasma, and tissue samples (6). However, the fluoride ion reactivation assay does have certain limitations. Specifically, ChE bound agents may undergo a process known as "aging" in which the O-alkyl group of the bound agent is removed by hydrolysis rendering the enzyme unable to undergo reactivation (1,7). The rate at which aging occurs is dependent on the agent that is bound, and is particularly rapid with soman (8). Methods have been developed to analyze for the aged products; however, they are difficult and time consuming to perform as they require isolation and digestion of the ChE enzymes followed by mass spectrometric (MS) analysis (7).

Nerve agents entering the body may be metabolized by OP hydrolases to alkyl methlyphosphonic acids, which can account for a significant portion of the overall agent exposure (9). GB is hydrolyzed to isopropyl methylphosphonic acid (IMPA) and GF is hydrolyzed to cyclohexyl methylphosphonic acid through the loss of the fluoride moiety (Figure 20). Methods have been developed to quantify hydrolysis products in urine, saliva, and plasma by gas chromatography (GC) (10-15) and liquid chromatography (LC). In recent years, the emergence of electrospray ionization (ESI) LC-MS methods has been shown to be as sensitive as previously reported GC techniques, without extensive

sample preparation or derivatization (16-21). Limit of detection (LOD) in blood samples has been reported to be 30 ng/mL by GC/MS (22). The LOD in serum was demonstrated to be 1 ng/mL by LC/ESI/MS/MS in an analysis of blood samples taken within 90 min of GB exposure from the Tokyo subway terrorist attack victims. In this study the authors reported concentrations of IMPA of 2 ng/mL or greater for 15 of 18 subjects studied (23). Urinary detection limits have been shown to be 0.1 ng/mL by GC tandem ion trap MS (24), and as low as 30 pg/mL by LC/ESI/MS/MS (25).

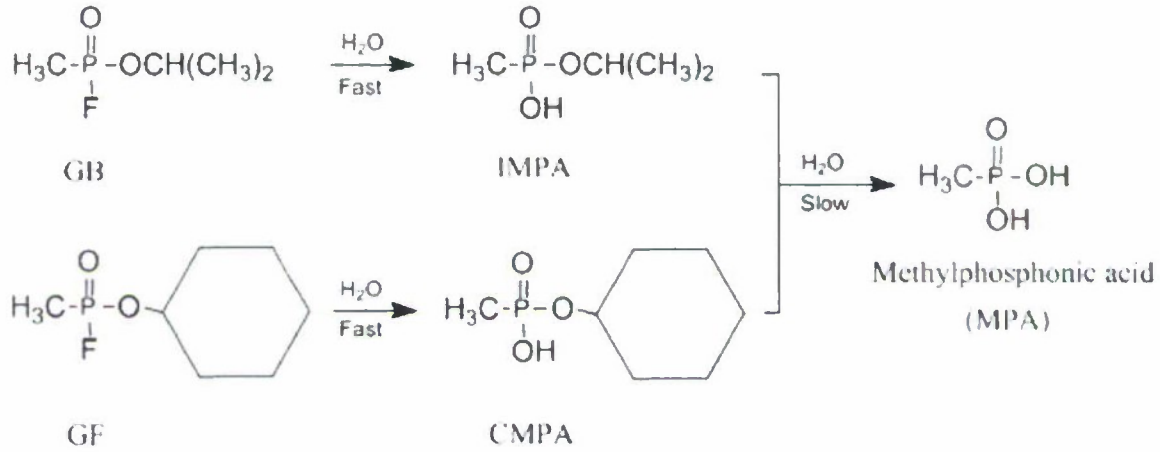

Figure 20. Hydrolysis Pathway for Nerve Agents GB and GF

This paper presents a method for the quantification of the hydrolysis products of GB and GF, IMPA and CMPA, in minipig plasma. This method uses LC/ESI/MS with time of flight (TOF) detection and isotope dilution to achieve the sensitivity of comparable methods without extensive sample preparation while utilizing a relatively inexpensive bench top instrumentation. Furthermore, the TOF data provides virtually high resolution (above 4000 at tuning m/z 112.98) mass spectral information which can eliminate potential interferences. The method validation samples and exposure samples presented here utilized blood samples collected from Gottingen minipigs before, during, and following whole body GB and GF nerve agent inhalation exposure.

3.2.2 Materials and Methods

3.2.2.1 Chemicals and Materials

High Performance Liquid Chromatography (HPLC) grade methanol was obtained from the Fisher Scientific Corporation (Hampton, NH). Deionized water (18.2 MΩ) was obtained using a Millipore Milli-Q purification system (Billerica, MA). Reagent grade formic acid was purchased from Sigma Aldrich Corporation (St. Louis, MO). GB, GF, and IMPA were procured from ECBC. CMPA, $^{2}H_{7}$-IMPA, and $^{13}C_{6}$-CMPA were purchased from Cerilliant Corporation (Red Rock, TX). All chemicals and solvents were used as obtained without further purification.

3.2.2.2 Standard Preparation and Characterization

IMPA was weighed and diluted in deionized water to create a stock solution with a concentration of 1.609 mg/mL in deionized water. CMPA, internal standard (IS) 2H7-IMPA, and IS 13C6-CMPA were purchased at a concentration of 1 mg/mL. Working solutions of 1 µg/mL for each of the analytes were prepared and 10 µg/mL for the IS in deionized water. Standards were serially diluted in deionized water from the working solutions to yield analyte concentrations of 0.4, 1, 2, 5, 10, 15, 20, and 25 ng/mL and an IS concentration of 20 ng/mL.

3.2.2.3 Minipig Exposure

Pigs were surgically prepared under anesthesia with jugular cannulae for serial blood sampling during and following exposure. Whole blood samples drawn from pre-exposed Gottingen minipigs (9-12 kg, Marshall Farms, North, NY) were used as control samples. Whole body exposures of minipigs were conducted in a 1,000 L chamber using concentrations of native GB or native GF ranging from 0.69 to 24.3 mg/m^3 for periods of 10, 60, or 180 min. In order to simulate realistic exposure conditions no anesthetic, pretreatment, or post-treatment drugs were administered to test animals. Blood samples (7-10 mL) were collected in ethylenediaminetetraacetic acid tubes before, during, and after agent exposures.

3.2.2.4 Sample Preparation

Collected whole blood samples were separated into RBC and plasma fractions by centrifugation (Micromass, Thermo IEC, Needham Heights, MA) for 5 min at a relative centrifugal force of 21,000 g, with plasma samples stored frozen at -20 °C. Samples (100 µL) were pre-weighed prior to sample preparation. The plasma was diluted with 500 µL of deionized water, acidified with 20 µL 1M HCl, and spiked with 1 µL of 10 µg/mL IS to achieve a final concentration of 20 ng/mL in the final extract. Following vigorous mixing, the solution was placed onto a conditioned (2 mL methanol, 2 mL deionized water) solid phase extraction cartridge (Varian Bond Elut Large Reservoir Capacity C18, 100 mg) for sample clean-up. Samples were eluted with 1 mL methanol, heated to 75 °C, and evaporated to near dryness under a stream of nitrogen. Samples were reconstituted in a final volume of 500 µL of 0.1% formic acid in water for analysis.

The RBC samples analyzed by the fluoride ion reactivation method were extracted using C18 SPE columns (200 mg Sep-Pak, Waters Associates, Millipore Corp., Milford, MA) which were first conditioned with 1 mL each of ethyl acetate, followed by isopropanol, and then pH 3.5 acetate buffer. A portion of the RBC samples was weighed in a tared centrifuge micro-vial with sample sizes ranging from 0.2 to 0.5 g. To the RBC samples was added 1 mL of acetate buffer, 200 µL of KF solution (6 M), and a corresponding deuterated IS. The mixture was vortexed followed by centrifugation for 5 min at a relative centrifugal force of 21,000 g. The combined reaction mixture was passed through the conditioned SPE column and washed with 500 µL of acetate buffer and allowed to dry using a light vacuum to pull air through the column for 3 min. The analytes were eluted with 1 mL of ethyl acetate that was collected and dried over anhydrous sodium sulfate. The ethyl acetate was removed from the collection tube and filtered through a 0.2 µm nylon Acrodisc syringe filter (Pall Gelman Laboratory, Ann Arbor, MI) into a GC autosampler vial for isotope dilution GC/MS analysis.

3.2.2.5 Instrumental Analysis

Samples were analyzed using an Agilent 1100 Series Liquid Chromatograph interfaced with an Agilent Series C TOF mass spectrometer (Agilent Technologies, Santa Clara, CA). Injections of 50 µL of extract were made with a constant flow rate of 0.8 mL/min through a Hichrome RPB C8/C18 LC column (250 mm x 4.6 mm, 5 µm df, Life Science USA, Paso Robles, CA) housed in a 40 °C compartment for chromatographic separation. The solvent gradient program was initially composed of 5% organic phase (0.1% formic acid in methanol) and 95% aqueous phase (0.1% formic acid in deionized water) and held for 5 min. This was followed by a linear gradient program increasing to 80% organic phase over a 10 min ramp, followed by a 1 min hold, and then returned to the initial conditions over a 2 min gradient. Lastly, there was a final 5 min post-run hold for a total run time of 23 min.

Detection was performed using negative ion ESI with reference mass correction to <5 ppm error during TOF analysis. MS parameters were adjusted using repeated direct injections of standards in column bypass mode to optimize for peak height. The conditions optimized for analysis were: fragmentor voltage (140 V), capillary voltage (4000 V), nitrogen drying gas temperature (350 °C), drying gas flow (13l pm), and skimmer voltage (60 V). Data were collected over a mass range of m/z 50-500.

3.2.2.6 Data Processing and Analysis

The Agilent software package "Analyst" provided with the MS was used to process and analyze the data. The software allowed automatic analysis of the IS method based on the analyte/IS area ratios of the peaks at their respective retention times. Automated peak selections were checked to ensure for the proper peak selection, peak shape, baseline evaluation, and presence of interferences.

3.2.2.7 Quantitation

Calibration standards were prepared in deionized water at concentrations of 0.4, 1, 2, 5, 10, 15, 20, and 25 ng/mL with an IS concentration of 20 ng/mL. The masses used for quantitation were m/z 137.04 for IMPA, m/z 144.08 for 2H7-IMPA, m/z 177.07 for CMPA, and m/z 183.09 for 13C6-CMPA with an extracted range of +/- 0.02 a.m.u. for each ion. The calibration curve was a plot of the ratio of analyte area to IS area plotted against the ratio of analyte mass on-column to IS mass on-column. The concentrations of unknown samples were determined using the slope and intercept calculated by linear regression analysis of the calibration curves. Matrix-spiked samples and calibration standards were analyzed in each group of unknown samples to verify calibration.

3.2.2.8 Method Validation

The method was validated by assessing the instrument reproducibility, selectivity, LOD, Limit of quantitation (LOQ), precision, accuracy, and recovery of IMPA and CMPA spiked into native male and female minipig plasma samples.

Instrument Reproducibility

The instrument reproducibility was demonstrated by using seven calibration concentrations and comparing the variance of the slope, intercept, and correlation over five consecutive weeks.

Selectivity

Three male and three female native samples were analyzed for selectivity by comparing true blank samples with those spiked near the limit of detection (1 ng/mL) for the analytes and at 20 ng/mL for the ISs. A comparison of the extracted ion chromatograms for each compound was conducted to assess potential background matrix interference.

LOD and Lower Limit of Quantitation (LLOQ)

Three concentrations were chosen to represent the methods lower, middle, and upper limits of quantitation. The LOD and LLOQ were determined by spiking unexposed plasma samples near the LLOQ and defining the calculated LOD as a peak-to-peak signal to noise ratio (p-p S/N) of 3 and a LLOQ p-p S/N of 10.

Precision and Accuracy

The precision and accuracy of the method were measured by spiking three to five replicate plasma samples near the LOD, at a medium quantitation concentration (MQC), and at a high quantitation concentration (HQC).

Method Recovery

The method recovery was computed by spiking analytes and ISs directly into LC mobile phase and comparing against extracted plasma samples with the same spiking amounts. The percent recovery for each compound was based on the area counts recorded from the LC/MS TOF analysis.

3.2.3 Results and Discussion

The isotope dilution method demonstrated the ability to detect low picogram amounts of IMPA and CMPA on-column despite the complexity of the plasma matrix. The reportable range was applicable to a range of 2 to 125 ng/mL of analyte in 100 µL of plasma. The relative variance of the slope and correlation of the instrument calibration over the reportable range was less than 15% over the five week span. The intercept did not change significantly over the study with an average ratio of intercept divided by the slope of 0.04. The $[M-H]^-$ ions were chosen as the best target ions to monitor for native IMPA (m/z 137.04) and native CMPA (m/z 177.07). Analogous ions were seen for the ISs. Figure 21 shows extracted ion chromatograms of a control minipig plasma extract compared to a plasma extract spiked near the LOD of the method for CMPA as a demonstration of method selectivity. There were no measurable amounts of IMPA or CMPA in any of the plasma control samples analyzed. The method LOD was determined to be 2 ng/mL and the LLOQ was calculated to be 5 ng/mL in plasma based on signal to noise measurements. The precision and accuracy of the method were evaluated during validation using the LOD, MQC, and HQC matrix-spiked samples. The results for the precision and accuracy are shown in Table 17. Daily quality control was accomplished by preparing and analyzing matrix blank and spiked samples to yield 10 ng/mL in plasma with each sample set (Figures 22 and 23). The 95 and 99% confidence limits of the matrix spike recoveries were calculated based on the mean and standard deviation of the spiking results, which were calculated to be 91.3 +/- 13.4 for IMPA and 104.2 +/- 9.6 for CMPA. Therefore, the 95% confidence limit was equal to 91.3 +/- 2(13.4) for IMPA and 104.2 +/- 2(9.6) for CMPA, and the 99% confidence limit was equal to 91.3 +/- 2.7(13.4) for IMPA and 104.2 +/- 2.7(9.6) for CMPA. The method recovery ranged from 65-83% for both the analytes and ISs.

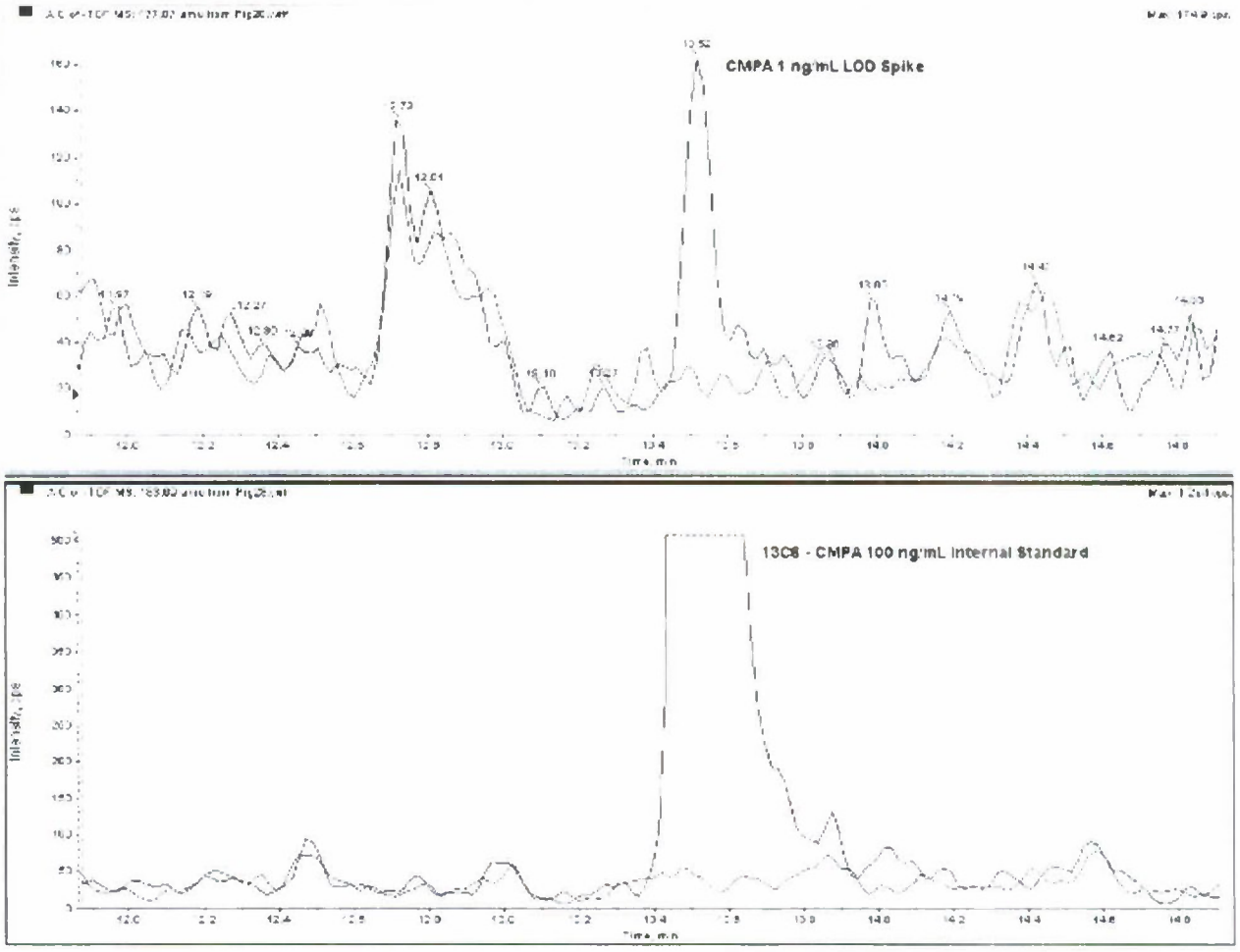

Figure 21. Extracted Ion Chromatograms for CMPA and $^{13}C_6$-CMPA

Overlaid chromatograms show the selectivity of a matrix spiked sample at the LOD (1 ng/mL) for CMPA (top) and at a concentration of 100 ng/mL of IS (bottom) as compared to a native minipig plasma control.

Table 17. Precision and Accuracy Data for IMPA and CMPA during Method Validation Using Control Minipig Plasma

IMPA (ng/mL)	Mean (ng/mL)	Std Dev (ng/mL)	% Std Dev	% Error
LOD = (1.96)[a]	2.2	0.2	8.0	8.2
MQC = (12.4)	12.2	0.4	3.1	1.8
HQC = (49.9)	47.4	1.2	2.5	-5.0
CMPA (ng/mL)				
LOD = (1)	1.1	0.4	33.6	14.2
MQC = (50)	50.3	3.2	6.3	0.6
HQC = (100)	97.4	6.6	6.8	-2.6

[a]Mean, n=3 (n= 5 for other concentration levels)

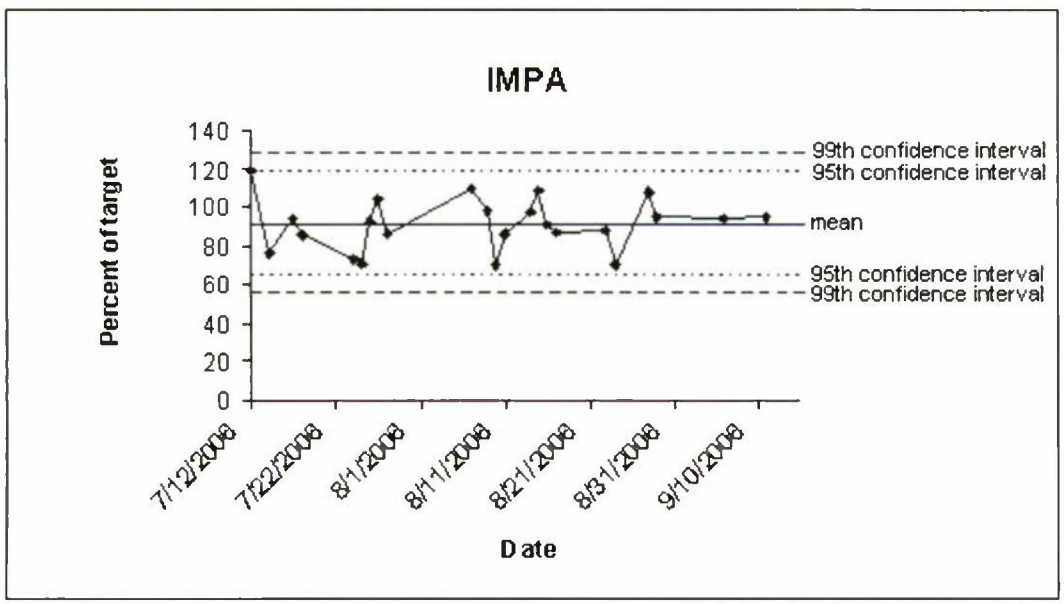

Figure 22. Plot of IMPA Quality Control Matrix Spikes at a Concentration Yielding 10 ng/mL in Plasma Showing the Mean, 95%, and 99% Confidence Limits

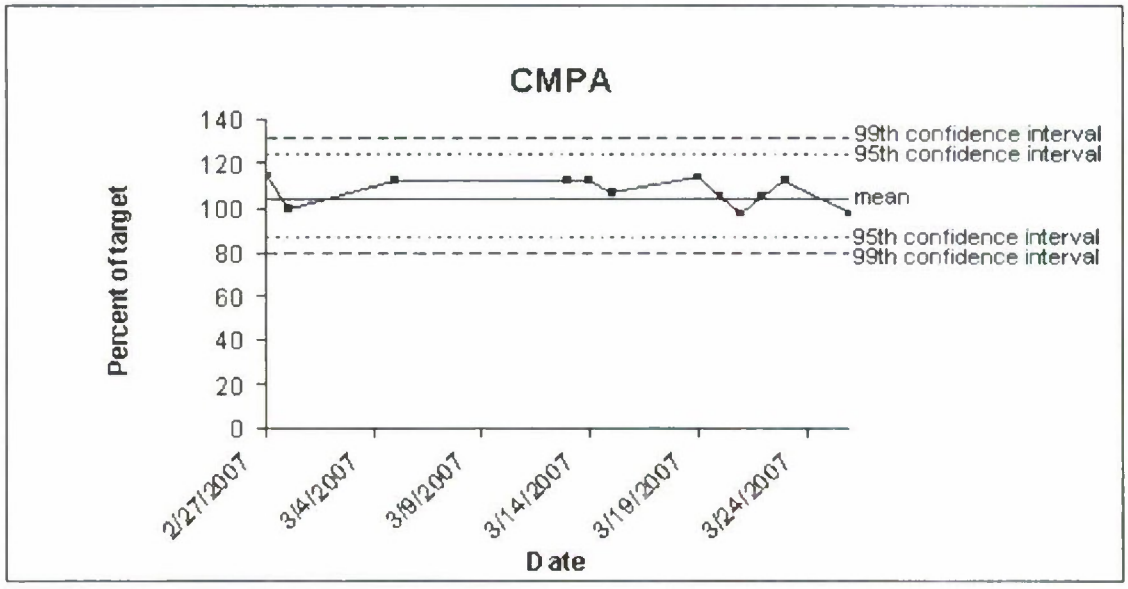

Figure 23. Plot of CMPA Quality Control Matrix Spikes at a Concentration Yielding 10 ng/mL in Plasma Showing the Mean, 95%, and 99% Confidence Limits

Alkyl methylphosphonate concentrations for the agent exposed minipig plasma samples were plotted versus time to generate a response profile of the hydrolysis products, as demonstrated in Figure 24, for a 10 min inhalation exposure at a GB concentration of 6.53 mg/m^3 and a GF concentration of 6.65 mg/m^3. There is a rapid linear increase in the agent biomarkers during the initial phase of the exposure for both GB and GF. The best fit linear regression lines for this initial agent exposure period were determined and the slopes of the regression lines for IMPA and CMPA are listed as response rates in Tables 18 and 19. For comparison, the RBC reactivation results for the agent, by the method described by Jakubowski, *et al.* (5), using fluoride ions to regenerate the original agent bound to the RBCs, are listed in the aforementioned tables. A comparison of the initial rates of increase of the hydrolysis products and the original agent recovered by fluoride ion regeneration (denoted R-GB, R-GF) in RBCs was calculated to assess the relative proportion of agent being directly bound to proteins such as AChE and BChE versus the amount being hydrolyzed immediately by the body. The percent response for hydrolysis product is obtained by taking the initial rate of increase of the hydrolysis product response and dividing by the sum of the hydrolysis product and regenerated agent response slopes. An estimate of the half life for the hydrolysis products in plasma was performed using a pharmacokinetic model estimation program (Winonlin, Version 5) using a first order elimination rate and the maximum value at the endpoint of exposures. From this, it was estimated that the t1/2 for IMPA is 44 min and the t1/2 for CMPA is approximately 61 min.

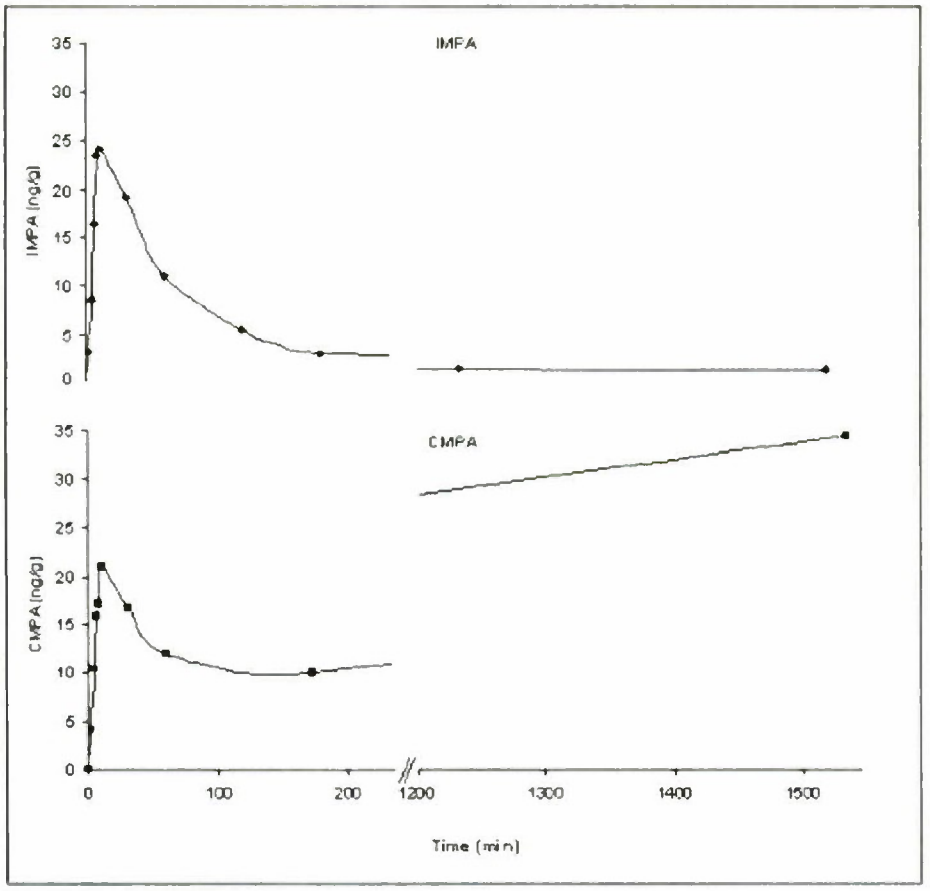

Figure 24. Time Response Profile for IMPA and CMPA in Plasma during and after a 10 min Inhalation Exposure to GB and GF in Minipigs

GB (6.53 mg/m^3), GF (6.65 mg/m^3)

Table 18. Dose-Response-Time Summary for Whole Body Inhalation Minipig Exposures

Pig Number	Exposure Time (min)	GB Exposure Concentration (mg/m^3)	Response Rate Plasma IMPA (ng/g)/min	Response Rate RBC R-GB (ng/g)/min	% Response IMPA	% Response R-GB
67	10	5.10	1.7776	0.8144	69	31
54	10	5.35	2.1912	1.1156	66	34
70	10	6.53	2.8415	1.254	69	31
59	10	5.90	3.0871	1.176	72	28
65	10	7.95	2.7205	1.9872	58	42
69	10	9.45	6.422	1.3463	83	17
66	60	1.28	0.1068	0.1964	35	65
46	60	1.68	0.1895	0.2064	48	52
75	60	1.71	0.1864	0.2282	45	55
61	60	1.82	0.1493	0.1493	50	50
51	60	1.64	0.2624	0.3007	47	53
55	60	1.78	0.3269	0.3269	50	50
53	180	0.80	0.0363	0.1029	26	74
71	180	0.90	0.0285	0.0834	25	75
58	180	0.98	0.0526	0.0236	69	31
64	180	0.84	0.0712	0.0989	42	58

Table 19. GF Dose-Response-Time Summary for Whole Body Inhalation Minipig Exposures

Pig Number	Exposure Time (min)	GF Exposure Concentration (mg/m^3)	Response Rate Plasma CMPA (ng/g)/min	Response Rate RBC R-GF (ng/g)/min	% Response CMPA	% Response R-GF
133	10	6.65	2.135	0.278	88	12
139	10	8.30	1.898	0.289	87	13
143	10	14.5	3.616	0.408	90	10
161	10	17.9	5.509	0.464	92	8
171	10	21.8	7.308	0.744	91	9
132	60	1.63	0.176	0.040	82	18
157	60	3.31	0.738	0.065	92	8
158	60	3.36	0.564	0.080	88	12
164	60	4.06	0.394	0.060	87	13
149	60	4.63	0.872	0.068	93	7
134	180	0.69	0.051	0.013	80	20
156	180	1.34	0.076	0.017	81	19
163	180	1.93	0.124	0.022	85	15
167	180	2.63	0.253	0.038	87	13
165	180	3.00	0.302	0.028	92	8

3.2.3.1 Discussion

This method was optimized for analyte recovery by testing several solid phase extraction cartridges, HPLC columns, and MS sensitivity. The Bond-Elut C_{18} cartridge proved to be the most

efficient solid phase extraction cartridge when compared to other brands of C_{18}, C_8, and C_2 extraction materials. The Hichrome C_8/C_{18} HPLC column improved the LOD by a factor of five compared to other C_{18} columns tested, possibly due to the end-capping process used in the production of the Hichrome column. The column size was selected based on the capacity to load biological extracts and use large injection volumes without chromatographic deterioration. The sample preparation routinely could be performed in less than 2 hr in sample sets of 20, followed by a sample run time of approximately 25 min per injection. The LC separation was optimized for selectivity rather than speed of analysis; however, the analysis time could be shortened, if necessary, by adjusting gradient and flow rates.

The precision and accuracy were demonstrated during the validation process with a mean relative error less than 15% for all sample levels tested and a relative standard deviation of less than 10% for samples above the LLOQ. The quality control chart had some values outside the 15% laboratory tolerance limit for control matrix spikes although no pattern of error could be determined. The outliers may have been the result of using microliter quantities of spiking solutions with multiple analysts involved in sample preparation.

Choosing the TOF MS has the advantage of being able to collect data over a wide mass range, making it possible to screen for multiple compounds in a sample, including compounds not yet considered at the time of data collection. It is likely that the LOD, linear dynamic range, and instrument precision and accuracy can be improved by utilizing tandem mass spectrometric instrumentation; however, structural information provided by the exact mass TOF measurements would be lost.

The time profiles observed for the two hydrolysis products show that there were differences in the manner that the precursor agents were introduced into the bloodstream. The maximum values for IMPA in plasma were typically seen at the endpoint of the exposure followed by a rapid decline in concentration over time. The CMPA profile was more complex and has an initial increase in concentration during the exposure with a period of elimination followed by a second spike that yields maximum concentrations between 6 and 24 hr post-exposure. Sample collection during this second spike period was not typically taken due to logistical reasons making an estimate of the time and concentrations of the maximum values in the plasma difficult. This effect was also observed in the GF agent regeneration data, which indicated a delayed infusion of GF from the whole body exposure as opposed to an additional mechanism for the hydrolysis of the agent. It is unknown why this difference occurs although differences in lipophilicity between the two agents may affect the way the agents are absorbed by the upper respiratory tract of the animals, or there is a significant percutaneous component to the GF exposure. The slopes of the uptake rates during exposure were compared to the regenerated agent data in RBCs to compare the relative rates that each was entering the bloodstream. The regenerated agents found in the plasma fraction, which would include both butyrylcholinesterase bound and unbound agent, accounted for less than 5% of the RBC concentration and was not considered in the slope response comparisons.

Plotting the initial rates of increase of the hydrolysis products and regenerated agents versus concentration of agent during exposure on a log-log basis shows that the relationship between uptake and exposure concentration is exponential as shown in Figures 25 and 26. An average of the 10 min exposure results show that approximately 70% of the recoverable GB that reaches the bloodstream is hydrolyzed to IMPA while the remaining 30% is bound to the blood proteins. Similarly, 90% of the recoverable GF that reaches the bloodstream is hydrolyzed to CMPA and the remaining 10% is bound to the blood proteins. The higher elimination rate may account for lower initial rates of increase of IMPA observed during the longer 60 and 180 min exposure periods when compared to the equivalent data for CMPA. The response profiles for IMPA and CMPA are in contrast to the R-GB and R-GF results which show the cumulative amounts of bound and unbound agent found in the RBC fraction. With the limited data points available during this study, it was difficult to determine if the estimated CMPA elimination rate may have been skewed by latent absorption of GF into the bloodstream from a currently undefined mechanism.

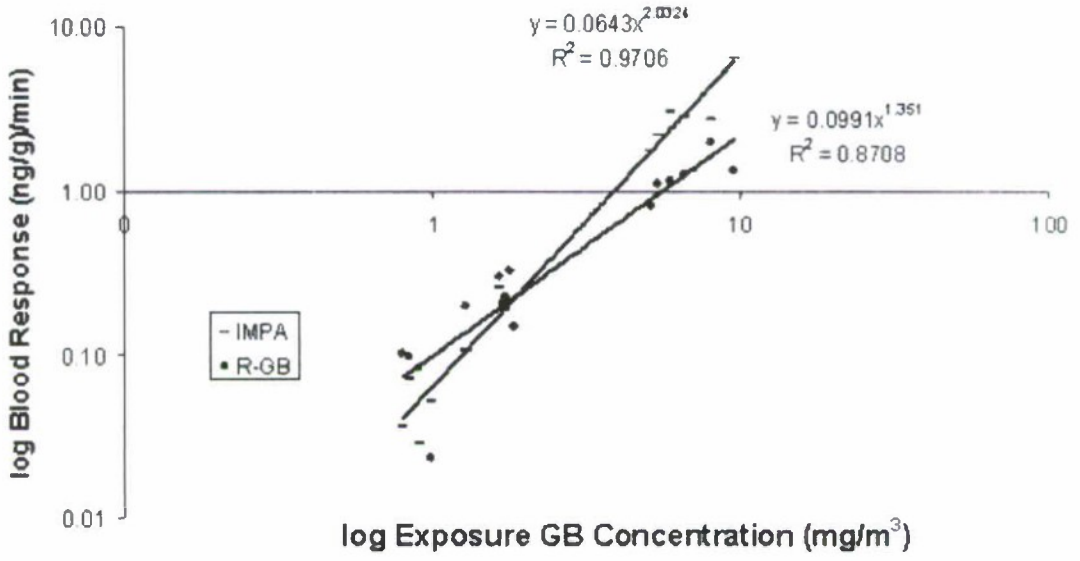

Figure 25. Log-Log Plot of the Dose-Response-Time Data for GB Exposures

The blood response data are the linear regression-derived slope of R-GB and IMPA during the exposure.

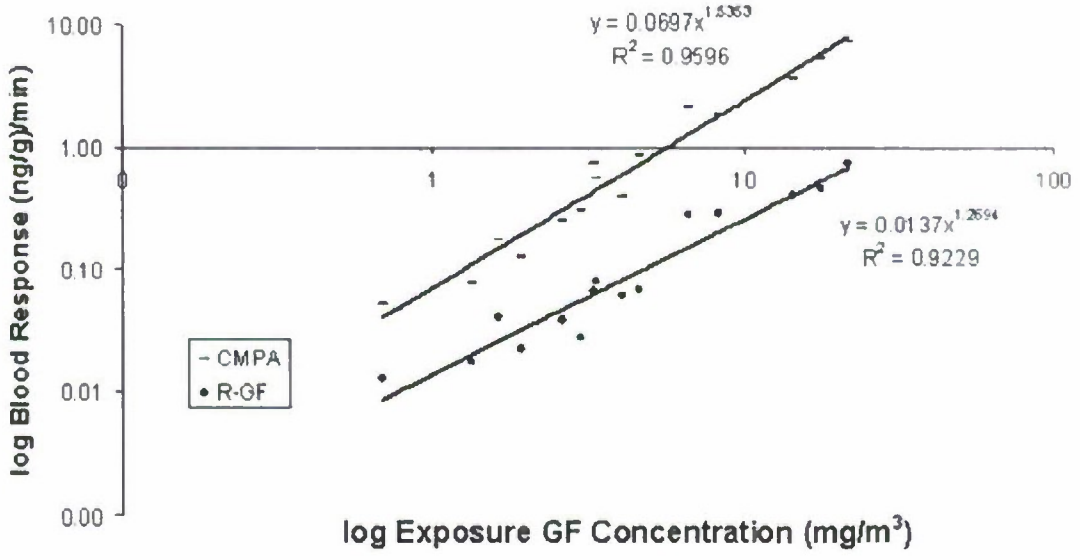

Figure 26. Log-Log Plot of the Dose-Response-Time Data for GF Exposures

The blood response data are the linear regression-derived slope of R-GF and CMPA during the exposure.

In determining the internal dosage received by a subject within a few hours of exposure, it would be ideal to obtain both agent-related and hydrolysis-related analyte concentrations over time and apply these to determine the overall quantity of agent absorbed into the bloodstream during the exposure. An alternative method would be to apply the elimination rate to the concentrations observed for the hydrolysis products in the plasma, provided that the time points of the exposure event and the collection of the blood samples are known. This method would not be as straightforward in the case of GF due to its slower absorptive pathway because GF reaches the bloodstream hours after initial exposure.

3.2.4 Conclusions

The method described herein for the quantification of the hydrolysis products IMPA and CMPA in minipig plasma can be used to estimate the internal dosage of agent received during an exposure. Utilizing LC/ESI/MSD/TOF and isotope dilution proved capable of achieving the selectivity and sensitivity of comparable methods without extensive sample preparation while utilizing bench top instrumentation. The method achieved detection levels of 2 ng/mL of hydrolysis product in plasma while utilizing a sample volume of 100 µL. Because the method is sensitive and the TOF collects full scan exact mass data, the method may be adapted towards economically screening for a wide range of OP nerve agents and their metabolites with minimal sample preparation. The hydrolysis product data collected in this study will aid in the construction of physiologically based pharmacokinetic animal models, in conjunction with agent regeneration data to provide information about the distribution of GB and GF and their metabolites in the body. This systematic approach will allow for a better understanding of the fate of OP nerve agents in the body and help to develop safety guidelines for these toxic compounds.

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3.3 A Rapid and Sensitive Technique for Assessing Exposure to VX via GC-MS/MS Analysis

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3.3.1 Introduction

S-[2-(diisopropylamino)ethyl]-O-ethyl methylphosphonothioate (VX, CAS Registry Number: 50782-69-9) is an extremely toxic organophosphate chemical warfare nerve agent (CWNA), which disrupts neurological regulation by binding with the enzyme acetylcholinesterase (AChE). Although the use of this nerve agent and others, such as tabun, sarin, and soman is forbidden by an international convention (1), documented cases of the use of these nerve agents exist (2-5). The extreme toxicity of these nerve agents, particularly VX, makes retrospective detection of potential chemical warfare agent (CWA) exposure an important issue both for the civilian population due to possible terrorist attacks or individuals involved with CWA stockpile demilitarization operations and military personnel who may encounter these agents in battlefield operations (6).

Currently, methods that could be used to assist in assessing the degree of exposure to VX in affected individuals include the identification and quantitation of the VX metabolite *O*-ethyl methylphosphonic acid in serum (5) or urine (7), and the fluoride-induced reactivated phosphonyl moiety of VX, *O*-ethyl methylphosphonofluoridate in serum (8) or plasma (9). However, these methods involve tedious sample preparation steps and/or derivatization of the isolated analyte prior to analysis. Recently, the quantification of VX in whole blood has been reported and applied to a toxicokinetic investigation in guinea pigs and marmosets (10).

In this paper, an alternate method for determining exposure to VX in plasma is described, which involves a simple extraction of residual VX followed by analysis using isotope-dilution gas chromatography-tandem mass spectrometry (GC-MS/MS). Application of the method is demonstrated in the toxicokinetic study of VX after intravenous (IV) bolus administration to male Göttingen Minipigs®.

3.3.2 Materials and Methods

3.3.2.1 Chemicals and Reagents

VX (Figure 27) of purity greater than 90% and $^{2}H_{5}$-*O*-ethyl-S-[2-(diisopropylamino)ethyl] methylphosphonothioate (deuterated VX, Figure 28) of isotopic purity greater than 90% were procured from ECBC. Sodium hydroxide (10.0 N) was obtained from LabChem, Inc. (Pittsburgh, PA). Anhydrous sodium sulfate and 2-propanol (both ≥99% purity) were purchased from Sigma-Aldrich (St. Louis, MO). Hexane (≥95% *n*-hexane, ≥99% hexanes) and saline (0.9% sterile and preservative free) were obtained from Fischer Chemicals (Fair Lawn, NJ) and Phoenix Pharmaceutical, Inc. (St Joseph, MO). Ammonia and methane were obtained from Sigma-Aldrich, while helium and argon were obtained from Messer, Inc. (Malvern, PA). All gases had purities >99.9%.

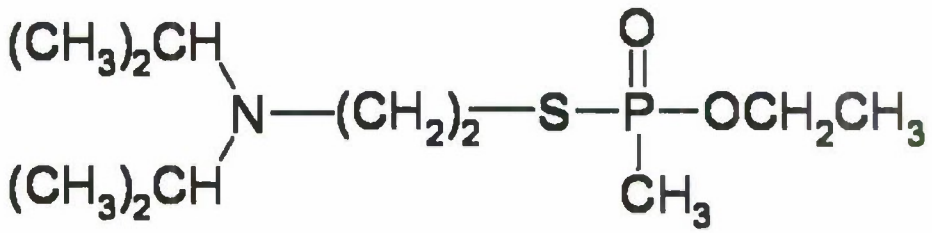

Figure 27. Structure of VX (CAS Registry Number: 50782-69-9)

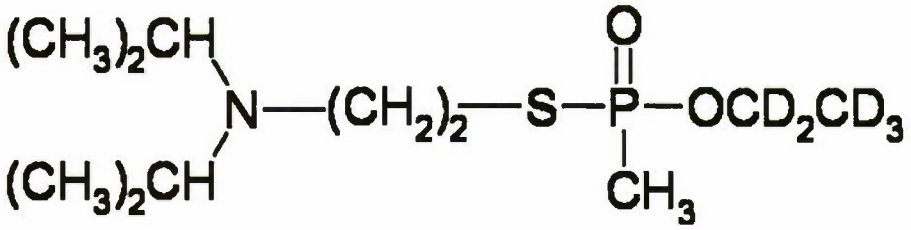

Figure 28. Structure of Deuterated VX

3.3.2.2 Animals

The experimental animals used were Göttingen Minipigs® obtained from Marshall BioResources (North Rose, NY). The animals were sexually mature males (3-4 months of age) weighing 11-13 kg. The pigs were injected with a dilute VX saline solution intravenously through a catheter placed in a leg vein. Neat VX was diluted in saline to the concentration desired in order to deliver the correct dose of nerve agent. IV injections were delivered as a single bolus in a total volume of 1 mL. All studies were approved by the ECBC Institutional Animal Care and Use Committee in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International. Venous blood draws were taken from a second catheter surgically implanted in a jugular vein prior to the start of the exposure and at periodic intervals throughout into BD Vacutainer® tubes containing K_3EDTA. The total volume of blood drawn did not exceed 1% of the animal's body weight over a 1 week span. An equivalent volume of Lactated Ringers Solution (a mixture of salts often used for fluid resuscitation after a blood loss) replaced drawn sample volumes. Samples were centrifuged at 4000 x g for 5 min at 4 °C. The plasma was removed and stored at -20 °C until analysis.

3.3.2.3 Stock Solutions and Calibration Standards Preparation

Stock solutions of VX and deuterated VX were prepared in 2-propanol at concentrations of 207.41 µg/mL and 984.35 µg/mL and stored at -20 °C until used. Individual working solutions (10.37 µg/mL for VX and 49.22 µg/mL for deuterated VX) were prepared by diluting stock solutions in 2-propanol for optimization of chromatographic and mass spectrometric conditions. Calibration standards of VX were prepared by diluting either the stock solution or working solution to obtain the following nine concentration points: 10, 25, 50, 100, 200, 400, 600, 800, and 1000 ng/mL. Each calibration standard also contained 1,000 ng/mL deuterated VX diluted from the stock solution. All of the calibration standards were also stored at -20 °C until analysis.

3.3.2.4 Analytical Method

The assay was performed using an Agilent Technologies GC Model 6890 (Wilmington, DE) interfaced to a Waters Micromass Quattro micro GC™ tandem quadrupole mass spectrometer (Milford, MA). Gas chromatographic separations were achieved using a Restek Rtx®-5MS column (Bellefonte, PA) 30 m X 0.25 mm i.d. with a 0.25 µm film thickness. The carrier gas was helium with a flow rate of 1 mL/min. Injections of 1.0-3.0 µL were made by autoinjector (ALS Model 7683B, Agilent Technologies) into a splitless injector port at a temperature of 250 °C. The initial oven temperature of 70 °C was held for 2 min, then ramped at 25 °C/min to 300 °C and held for an additional 2 min. Elution times for both VX and deuterated VX were typically 9.2 to 9.3 min as shown in Figure 29.

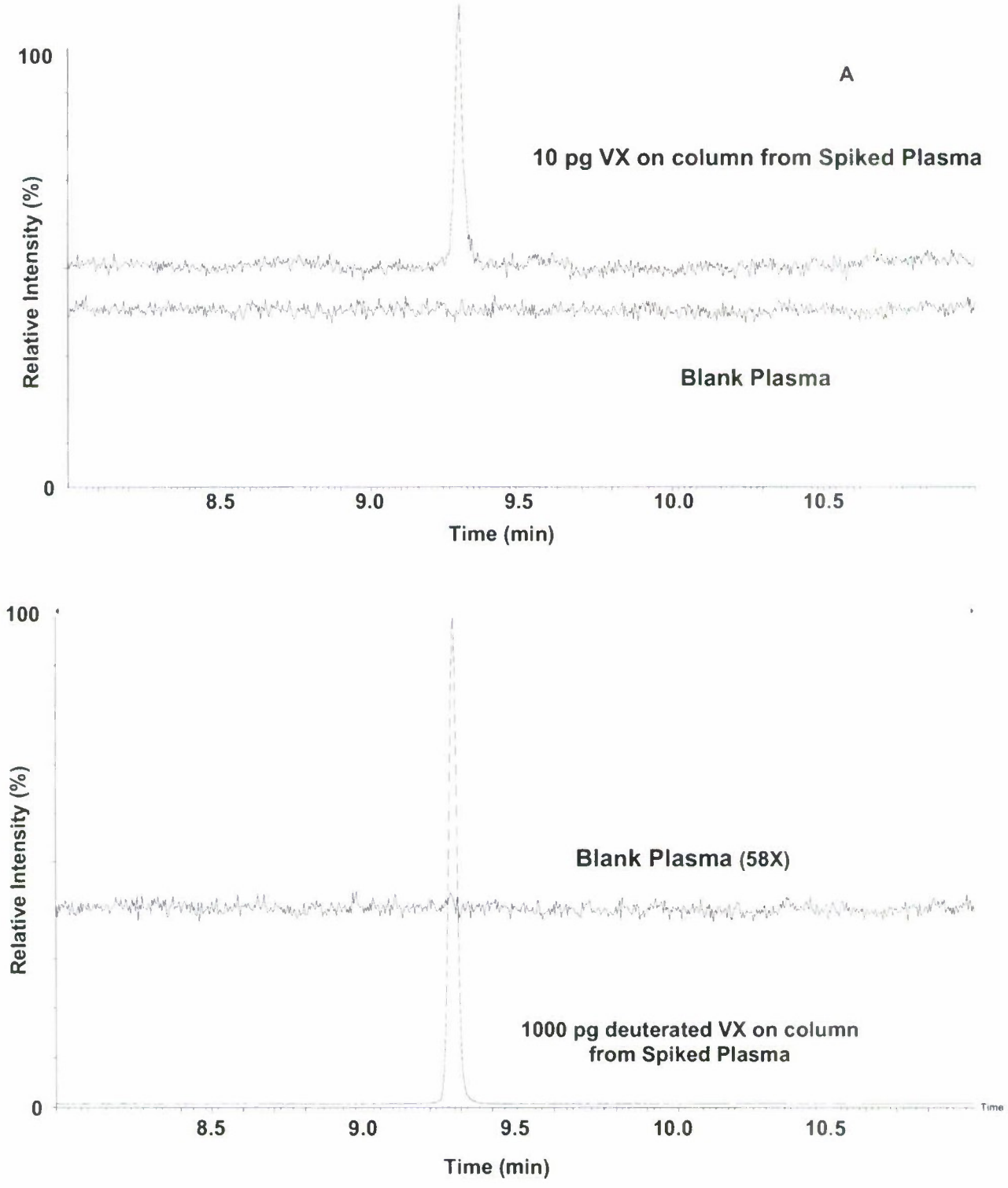

Figure 29. MRM Chromatograms for Monitoring VX and Internal Standard (IS) in Blank and Spiked Minipig

Multiple reaction monitoring (MRM) chromatograms obtained from 1µL injections of organic extracts. 268 > 128 transition for monitoring VX in blank and spiked minipig plasma (top), 273 > 128 transition for monitoring deuterated VX in blank and spiked minipig plasma (bottom).

Samples were ionized by positive-ion chemical ionization (CI) with ammonia reagent gas. CI source conditions were optimized using Fluoroether E3 (CAS Registry Number: 3330-16-3, Agilent Technologies) tuning compound with methane reagent gas. Mass spectra were obtained at a dwell time of 0.05 sec for each transition in the MRM mode. Argon was used as the collision gas at a pressure of ~2 mTorr, with a collision-induced dissociation (CID) energy of 15 eV. The CID energy was optimized for the m/z 268 > 128 ($C_8H_{18}N^+$) transition for VX and the m/z 273 > 128 transition for deuterated VX. These transitions were chosen based on earlier investigations into the electrospray ionization ion trap MS fragmentation of VX (11), which indicated that CID of the protonated ion of VX (m/z 268) showed two product ions with m/z values of 128 and 167 with an intensity ratio of ca. 20:1. Typical CID mass spectra obtained in our laboratory are shown in Figure 30. The MassLynx application software provided with the Quattro micro GC was used to process and analyze the data. The QuanLynx software provides automated peak detection, calibration, and quantification.

3.3.2.5 Sample Preparation

To a weighed sample (0.1-0.2 g) of blank, spiked or exposed plasma in a 1.5 mL microcentrifuge tube (Sigma-Aldrich, St. Louis, MO), 25 µL of 0.01 N sodium hydroxide was added and vigorously mixed. Immediately, 450 µL of *n*-hexane/2-propanol (90:10, v/v) was added and gently shaken. The organic layer was transferred to a 2.0 mL microcentrifuge tube containing 1 g of anhydrous sodium sulfate. A second extraction with an additional 450 µL of *n*-hexane/2-propanol (90:10, v/v) was done and added to the 2.0 mL microcentrifuge vial. The IS was added and vigorously mixed. The organic layer was withdrawn and filtered through a 0.2 µm nylon Acrodisc® syringe filter (Pall Gelman Laboratory, Ann Arbor, MI), after pre-wetting the filter with 1 mL of *n*-hexane/2-propanol (90:10, v/v), into a GC autosampler vial (Agilent Technologies, Wilmington, DE). The filtrate was concentrated for analysis under a gentle stream of nitrogen at a temperature of approximately 30 °C to a final volume of 50 µL for exposed plasma samples or 500 µL for spiked plasma samples.

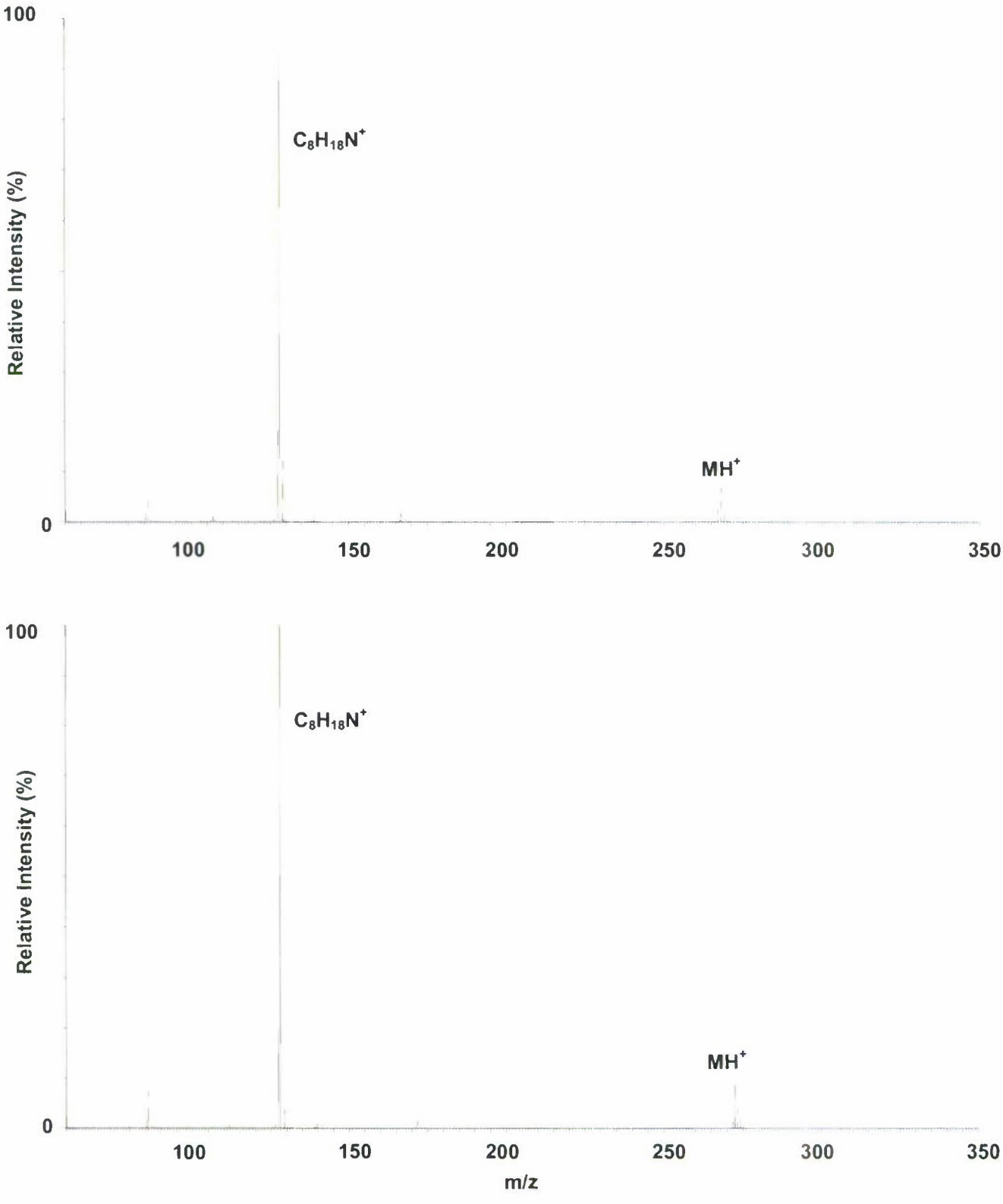

Figure 30. CID Mass Spectra of m/z 268 (MH+) from VX (top) and m/z 273 (MH+) from Deuterated VX (bottom)

Collision energy = 15 eV at 2.25 mTorr argon.

3.3.3 Results and Discussion

3.3.3.1 Method Development

While the method of van der Schans *et al.* (10) dealt with VX extraction from whole blood, we focused on only the plasma fraction because at physiological pH, VX would be expected to be protonated (with a dissociation constant, pKa of 9.4 as determined by van der Schans) and more soluble in the aqueous fraction. This necessitated a change in the sodium hydroxide concentration (from 0.1 M to 0.01 M) in order to maintain pH between 10.5 and 11. Also, the organic extraction solvent was optimized to be 10% 2-propanol in hexane rather than 5% methanol in hexane. This resulted in extraction recoveries for (±)-VX in saline at a concentration of 50-100 ng/mL of 95 ± 10% versus 84 ± 9% for (+)-VX and 89 ± 12% for (-)-VX in the van der Schans study. Finally, through the use of MS/MS, we were able to achieve lower absolute detection limits, specifically 0.4 pg of (±)-VX on column at a signal to noise root mean square, RMS of 3:1 versus ≥ 0.8 pg in the van der Schans method.

3.3.3.2 Method Validation

Linearity

Figure 31 shows the response of VX to the IS (deuterated VX), with response being defined as [Area $_{VX}$ X (Conc $_{IS}$/Area $_{IS}$)] as a function of pg VX injected onto the column. The data are an average taken over the course of five weeks with n = 7, the total number of injections at each standard level obtained over five weeks. The data show good correlation (r^2 = 0.9998) over two orders of magnitude with an average slope of 1.275 ± 0.037.

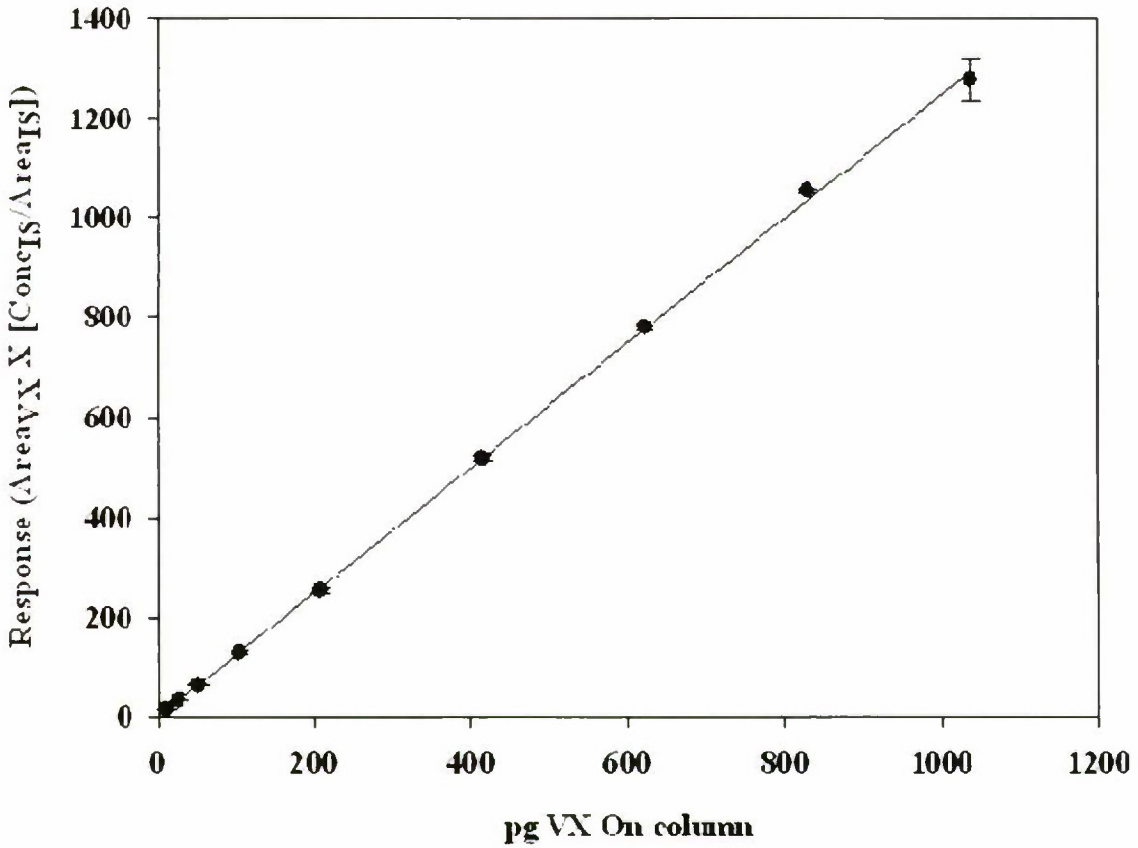

Figure 31. Standard Calibration Curve for VX Response

Data represent mean values (n = 7) with an average slope of 1.275 ± 0.037

Sensitivity and Selectivity

The limit of quantitation (LOQ) was determined as the minimum concentration that could be accurately and precisely quantified (lowest data point of the standard curve), while the limit of detection (on column) was defined as the amount that could be detected with a signal to noise RMS of 3:1. The selectivity of the assay for VX is shown in Figure 29. It was assessed by comparing the lowest concentration in the calibration curves (10 ng/mL, LOQ) spiked into plasma with VX-free plasma.

Accuracy, Precision, and Recovery

The precision (presented as relative standard deviation [RSD]) of the assay was determined using low quality control, medium quality control, and high quality control samples equivalent to the concentrations of VX on column. Results ranged from 18.36% RSD (low quality control) to 2.61% RSD (high quality control) and are given in Table 20. Accuracy (or bias) was determined from the percentage ratio measured quality control concentration over measured concentration of a standard prepared in 10% 2-propanol/hexane. The negative bias for all three quality control samples could be due in part to limited irreversible binding of the VX to plasma proteins because no effort was made to occupy these sites with an alternative CWA as was done in the van der Schans study (10).

Table 20. Precision and Accuracy of VX in Spiked Plasma Samples (n = 10)

QC Sample	Mean (ng/mL)	RSD (%)	Bias (%)
Low Quality Control 10	8.68	18.36	-9.9
Med Quality Control 200	174.44	12.02	-9.3
High Quality Control 1000	902.97	2.61	-9.0

Stability

To evaluate the room temperature and freeze/thaw cycle stability of the extracts, five replicates of the quality control samples at each of the three concentrations were processed and stored under autosampler conditions (room temperature with vial septum syringe punctures) for either 8 hr or 48 hr then assayed. The same samples were then subjected to three freeze/thaw cycles and assayed. Samples were stored at -20 °C for 24 hr and thawed unassisted at room temperature. When completely thawed, the cycle was repeated two more times with analysis following the third cycle. Stability was assessed by comparing the mean concentration of the stored samples with freshly prepared samples (Table 21). In general, there were no statistically significant differences at the 95% confidence interval between the short term, long term, and freeze/thaw stability assays, and the freshly prepared samples.

Table 21. Stability of VX Extracts in *n*-hexane/2-propanol (90:10 v/v) (n = 5)

Statistical variable	Theoretical concentration (ng/mL)		
	10	200	1000
Short Term (8 hr at room temperature)			
Mean	8.84	165.08	915.25
RSD (%)	12.26	4.67	2.59
Bias (%)	-12.9	-15.9	-7.7
Long Term (48 hr at room temperature)			
Mean	8.52	183.80	892.74
RSD (%)	24.84	14.51	2.46
Bias (%)	-9.3	-2.7	-10.3
Freeze/Thaw (3 cycles)			
Mean	8.95	159.43	902.31
RSD (%)	14.62	6.69	6.86
Bias (%)	-9.9	-10.6	-10.2

3.3.3.3 Toxicokinetic Study of VX in Minipigs

The method has been successfully applied to the toxicokinetic study of VX after an IV bolus administration (12 µg/kg) to male Göttingen Minipigs®. The toxicokinetic parameters were determined using WinNonlin Professional, Version 5.0 (Pharsight Corporation, Mountain View, CA) software and are shown in Table 22. The VX concentration-time profile conformed to a two-compartment model with first order elimination and is shown in Figure 32. The correlation between the observed and predicted values is 0.9883.

Table 22. Toxicokinetic Parameters of VX Obtained after IV Injection (12 µg/kg) to Minipigs (n = 3, mean ± SD)

V (L/kg)	3.33 ± 0.30
$T_{1/2\alpha}$ (min)	1.61 ± 0.27
$T_{1/2\beta}$ (min)	406 ± 134
CL (L/min/kg)	0.029 ± 0.008
AUC (min-ng/mL)	414 ± 112
MRT (min)	577 ± 190

V = Volume of distribution, $T_{½\alpha}$ = Half-life ($t_{1/2}$) of initial slope, $T_{½\beta}$ = $t_{1/2}$of terminal slope,
CL = Clearance,AUC = Area Under the Curve, MRT = Mean Residence Time

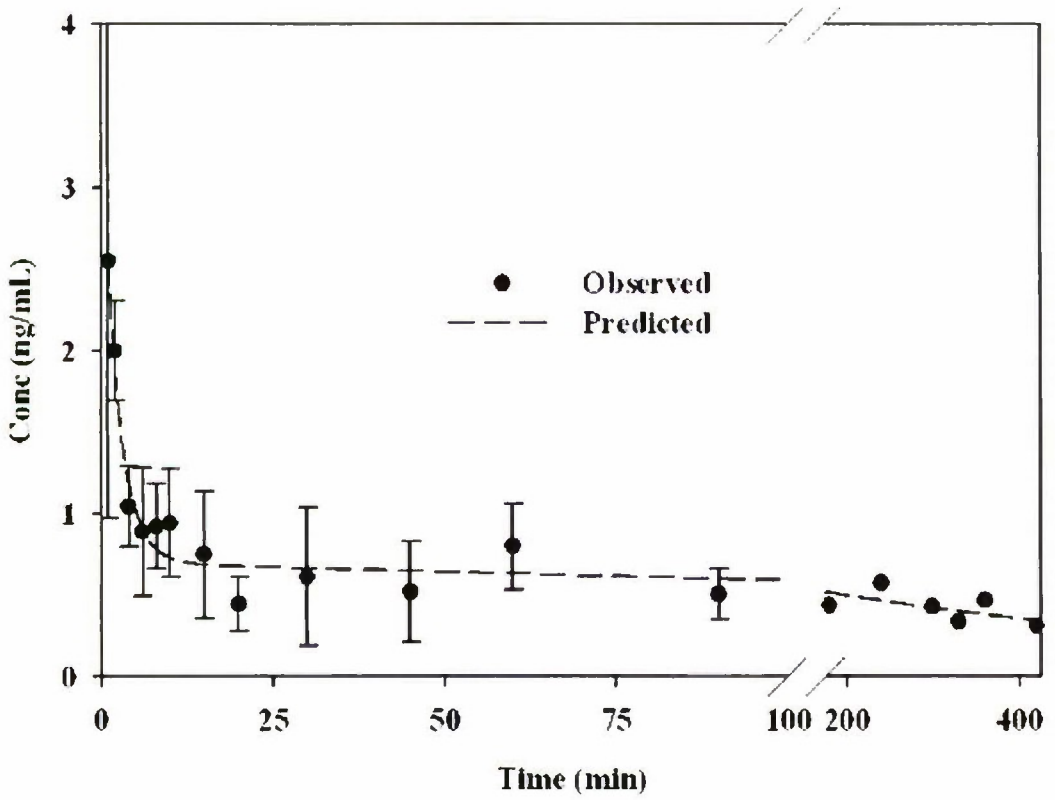

Figure 32. Plasma VX Concentration *versus* Time Profile in Minipigs after Administration of an IV Injection of VX at 12 µg/kg

Data at < 100 min represent mean values (n = 3), while data at > 100 min are only single points. Curve-fitting of the measured mean concentration-time course of VX was performed according to the two-exponential equation $[VX]_t = Ae^{-\alpha t} + Be^{-\beta t}$.

3.3.4 Conclusions

An alternate method for determining exposure to VX by the analysis of plasma samples was described which involved a simple liquid-liquid extraction of residual VX followed by analysis using isotope-dilution GC-MS/MS. Application of the method was demonstrated in the toxicokinetic study of VX after IV bolus administration to male Göttingen Minipigs®.

3.3.5 Literature Cited

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3.4 Validation and Application of a GC/MS Method for Determining Soman Concentration in Rat Plasma Following Low Level Vapor Exposure

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3.4.1 Introduction

The chemical warfare nerve agent (CWNA) O-pinacolyl methylphosphonofluoridate (Figure 33), also known as soman (GD, CAS Registry Number: 96-64-0) is an extremely toxic organophosphorous compound that inhibits the enzymatic activity of acetylcholinesterase (AChE). In the reaction of GD with AChE, GD loses a fluoride ion (1) and phosphonylates the serine residue (Figure 34) in the active site of AChE, thereby inhibiting its ability to hydrolyze the neurotransmitter acetylcholine (ACh). This results in the accumulation of ACh and overstimulation of cholinergic receptors (2). Signs of exposure to high levels of GD include muscle twitches, tremors, convulsions, seizures, salivation, miosis, and death attributable to respiratory failure (1). At lower doses, the majority of these toxic signs are not typically observed necessitating the development of alternative exposure detection methods.

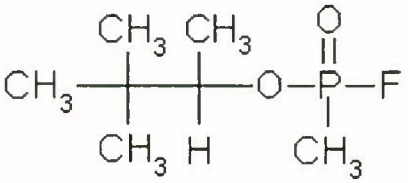

Figure 33. Structure of GD

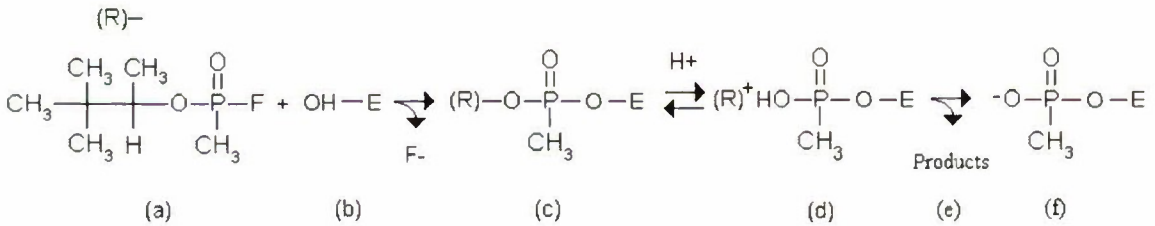

Figure 34. GD Binding to the Serine Residue of AChE and "Aging" of the GD – AChE Complex

(A) GD; (B) Serine Residue of AChE; (C) Phosphorylated Enzyme; (D) Carbocationic Intermediate; (E) Dealkylation Products; (F) Aged Enzyme

In today's world, the use of chemical warfare agents (CWAs) such as GD poses a significant threat. Despite efforts to end the use of such agents (3), several incidents involving the terrorist use of nerve agents have occurred (4, 5). In addition, nerve agent exposure is possible for military personnel on the battlefield. Therefore, the retrospective detection and filling of critical toxicological data gaps are of high importance in nerve agent research.

Currently, inhalation studies are being conducted to determine the toxic effects of low level GD vapor exposure using animal models. To gain a better understanding of the dose-response relationship in these models, an estimate of the levels of GD present in the blood is needed. Methods have been developed previously to quantify the internal dose of various nerve agents after exposure. Such methods quantitate the activity of AChE in blood (6), unbound nerve agent in blood ("free agent") (7-10), and hydrolysis products of the nerve agent in blood and urine (2, 11-13). In addition, methods for

the detection of GD specifically have been developed, including isotope dilution using liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the detection of GD hydrolysis products in urine (13) and isotope dilution with the use of gas chromatography (GC) MS/MS and derivatization of urinary metabolites of GD (2). Some of these methods involve tedious sample preparation due to derivatization of analyte prior to analysis, while others, such as the measurements of enzyme activity, do not achieve the sensitivity needed to detect low levels of GD (14). While the LC-MS/MS methods reach the sensitivity levels needed and do not require derivatizing agents, they focus only on the hydrolysis products of GD, mainly pinacolyl methylphosphonic acid.

In an attempt to overcome the limitations of previous techniques, a different method was developed for the quantification of GD in plasma and was applied to a toxicokinetic investigation in guinea pigs and rhesus monkeys (11). The method measures bound nerve agent using an excess of fluoride ions to shift the equilibrium of the GD-serine binding reaction that occurs in the active site of AChE to regenerate intact GD. Similar methods have been reported previously for the nerve agent sarin (15). Even though these methods have proven to be reliable and sensitive detection methods for the identification of nerve agent exposures (15, 17), they do not overcome GD specific difficulties attributed to its rapid rate of dealkylation ("aging") (Figure 34). Once the irreversible "aging" reaction of the GD-AChE complex has occurred, the method is no longer able to regenerate the parent compound. Adams *et al.* (11) report large measurement variations in levels of regenerated GD attributed to rapid "aging" and variability in the post-exposure analysis time, demonstrating the need for immediate sample processing post-exposure.

In the present study, a modified version of the fluoride regeneration assay and instrumental analysis method described previously (15) is validated and used immediately after GD vapor exposure to quantitate the concentration in rat plasma. This method uses solid-phase extraction of regenerated GD and a deuterated isotope internal standard ([IS] $^2H_4$-GD, Figure 35) followed by analysis using large volume injection GC with ammonia chemical ionization (CI) MS detection. This sensitive method was successfully applied to the analysis of GD in rat plasma, with the amount of GD regenerated from the blood correlating with the dose of GD to which the animals were exposed.

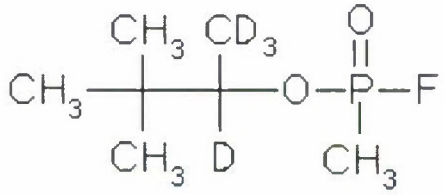

Figure 35. Structure of Deuterated GD (d_4-GD)

3.4.2 Materials and Methods

3.4.2.1 Chemicals and Reagents

GD and $^2H_4$-GD (d_4-GD) were procured through ECBC. Analytical grade ethyl acetate (99.5+% purity), 2-propanol (≥99.5% purity), sodium sulfate anhydrous granular (≥99% purity), potassium fluoride (99+% purity) were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO). Sodium acetate (99.4% purity) was obtained from Fisher Chemicals (Fair Lawn, NJ) and the glacial acetic acid (99.7+% purity) also used to make the buffer solution was obtained from Sigma – Aldrich Chemical Company. Ammonia anhydrous (99.99+% purity) and methane (99.0+% purity) were obtained from Sigma-Aldrich Chemical Company, and nitrogen compressed gas (ultra high purity) and helium compressed gas (ultra high purity) from GT&S, Inc.(Allentown, PA).

3.4.2.2 Animal Use

Groups consisting of five adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 225-300 g at the time of testing were exposed to GD vapor in a whole body dynamic airflow chamber for 60 min. A total of 25 male rats were exposed to concentrations ranging from 0.139 mg/m^3 to 0.401 mg/m^3, and doses, expressed as concentration x time (Ct), ranging from 8.3 mg-min/m^3 to 24.1 mg-min/m^3. Following exposure, 1000 µL of whole blood was collected from a tail snip for use in the regeneration assay described in the following sections. All experiments and procedures were approved by the ECBC Institutional Animal Care and Use Committee, and conducted in accordance with the requirements of Army Regulation 70-18 and the National Research Council's *The Guide for the Care and Use of Laboratory Animals* (18).

3.4.2.3 GD Vapor Generation

Whole body vapor exposures were conducted using a Rochester-style 750 L dynamic airflow inhalation chamber. GD vapor generation was accomplished using a glass saturator cell as described previously (19). Briefly, saturated GD vapor streams were generated by placing neat liquid GD into a glass multi-pass saturator cell containing a hollow ceramic cylinder and allowing the nitrogen carrier gas to flow through the saturator cell and over the GD wetted ceramic cylinder. The temperature of the saturator cell and the flow rate of nitrogen determined the amount of GD vapor generated. GD concentrations were then determined using GC with flame photometric detection (Agilent Technologies Inc., Wilmington, DE).

3.4.2.4 Stock Solutions and Calibration Standards Preparation

Stock solutions of GD and d_4-GD were prepared in hexane at concentrations of 1.100 mg/mL and 1.698 mg/mL, respectively. Intermediate levels used as working standards were prepared by diluting stock solutions in ethyl acetate at concentrations of 110 µg/mL, 11 µg/mL, and 1.1 µg/mL for GD and 3.396 µg/mL for d_4-GD. Calibration standards were prepared by spiking a known amount of GD and d_4-GD stock or working standards directly into ethyl acetate resulting in nine concentration points spanning the range of 10-1000 pg injected on-column of GD and a constant 998.424 pg on-column of d_4-GD. All stock and standard solutions were stored at -20 °C.

3.4.2.5 Sample Preparation

The sample preparation method follows the regeneration assay previously developed by Jakubowski *et al.* (15). Whole blood (1000 µL) from both control and exposed rats was collected from a tail snip in Microtainer K2EDTA-containing tubes (BD Vacutainer®, Franklin Lake, NJ) and then centrifuged at 15,000 rpm for 5 min (Thermal International Equipment Company, Micromax model, radius = 7.5 cm) to separate the plasma and red blood cell (RBC) fractions immediately post-exposure. For both control and exposed samples, a 200 µL aliquot of plasma was weighed. To each plasma sample, 1 mL of acetate buffer (pH 3.5), 200 µL of 6 M potassium fluoride, and a d_4-GD spike (yielding a concentration of 20 ng/mL in the final extract) were then added. Plasma samples from unexposed rats were spiked with a known amount of GD and d_4-GD and used as positive controls. Negative controls were also employed by using unexposed rat plasma and spiking only with the d_4-GD. Samples were vortexed, centrifuged at 15,000 rpm for 5 min, and the supernatant was then transferred to C18 solid phase extraction (SPE) cartridges (Sep-Pak, Waters Corporation, Milford, MA) pre-conditioned with 1 mL ethyl acetate, 1 mL 2- propanol, followed by 1 mL of acetate buffer. Samples were filtered through the C18 SPE cartridge and the filtrates discarded. The original sample pellet was re-suspended in 1 mL acetate buffer and 200 µL 6 M potassium fluoride, vortexed, and centrifuged. Supernatant was once again transferred to SPE cartridges and filtered. The cartridges were then washed with 500 µL acetate buffer, swabbed to remove residue, and dried under a vacuum. GD and d_4-GD were eluted by adding 1 mL of ethyl acetate to the cartridge. The eluent containing GD/d_4-GD was collected in glass tubes containing sodium sulfate to dry the extract. The extracts were then filtered through a 0.2 µm syringe microfilter (PN 4436, Pall Corporation Life Sciences, Ann Arbor, MI), followed by an additional sodium sulfate rinse with 500 µL of ethyl acetate. Final sample volume was dependent on exposure concentration, with higher

concentrations requiring dilution with ethyl acetate, and lower concentrations requiring concentration with nitrogen compressed gas.

3.4.2.6 Analytical Method

Analytical separation and detection was achieved using an Agilent Technologies GC Model 6890 (Wilmington, DE) coupled with a Mass Spectrometric Detector (Model 5973 MSD, Agilent Technologies). Injections of 50 µL of extract were made by autoinjector (Model 7673, Agilent Technologies) into the large volume injector port (PTV Inlet, Agilent Technologies). The inlet was cooled to -30 °C by liquid nitrogen and was held for 5.1 min. The temperature then ramped at a rate of 720 °C/min for a final inlet temperature of 225 °C. Solvent vent time was 5.0 min and the vent flow was 300 mL/min. Purge flow was 500 mL/min and purge time 8.7 min. An intermediate polarity deactivated guard column (Restek, Inc., Bellefonte, PA; 5 m x 0.32 mmID) and an Rtx-1701 analytical column (Restek, Inc.; 30 m x 0.32 mm x 1.0 µm film thickness) were used with helium as the carrier gas with a constant flow rate of 3 mL/min. The GC had an initial oven temperature of 35 °C for 5 min which then ramped to 164 °C at rate of 25 °C/min. The temperature was then increased to 170 °C at a rate of 2 °C/min. Finally, the temperature was ramped to 270 °C at a rate of 50 °C/min and held at this temperature for 3 min for a total runtime of 18.16 min. Elution times for the diastereomeric mixtures of GD and d_4-GD were typically ~11.4 min as shown in Figure 36.

Mass spectrometric detection was accomplished using positive ion ammonia CI in the selected ion monitoring mode with a deuterated stable isotope as an IS. CI source conditions were optimized using Fluoroether E3 (CAS Registry Number: 3330-16-3, also known as PFDTD; Agilent Technologies) tuning compound and methane as a reagent gas. An ammonia tune was conducted before each sequence to optimize ionization parameters. The ammonia adduct ion ratio of m/z ions 200/204 (GD/d_4-GD) was used for quantitation. Mass spectra were obtained at a dwell time of 50 msec for each ion (3.77 cycles/sec). After manually integrating the characteristic double peaks of GD together, quantitation of unknown samples was determined by using the slope and intercept calculated by linear regression analysis of calibration curves (Enhanced Chemstation Data Analysis: MSD Chemstation D.01.02.16, Agilent Technologies, Wilmington, DE).

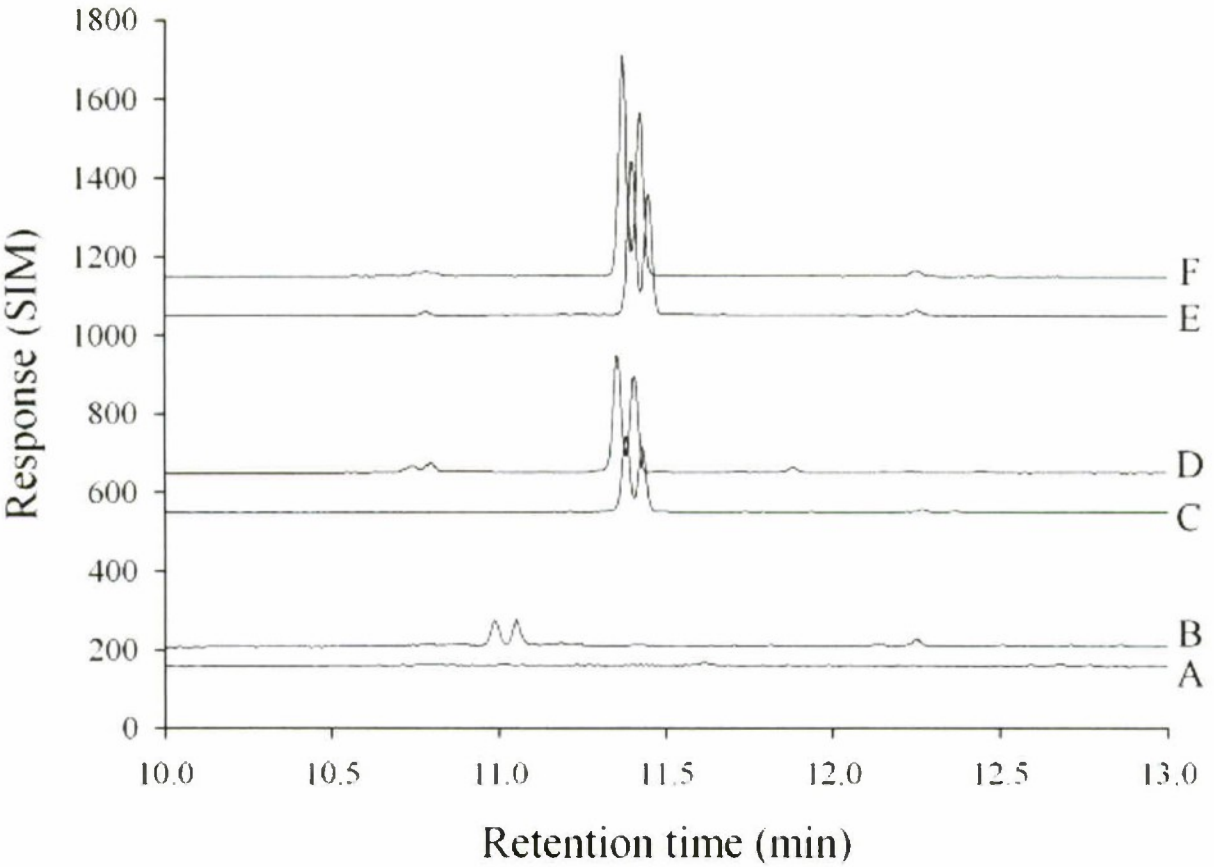

Figure 36. Representative GC-MS Extracted Ion Chromatograms of Plasma Samples Extracted for GD

(A) Plasma Blank at m/z =200; (B) Plasma Blank at m/z = 204; (C) Recovered GD at m/z =200 from Rat Plasma at the medium quantitation concentration (MQC) (401.5 pg on – column); (D) Recovered d_4-GD at m/z 204 from Rat Plasma at 1000 pg on-column; (E) GD Standard in Ethyl Acetate at m/z 200 at the MQC; (F) IS d_4-GD in Ethyl Acetate at m/z = 204 at 1000 pg on-column. The double peaks were manually integrated together for all quantitation.

3.4.3 Results and Discussion

3.4.3.1 Method Validation

Method validity was assessed by determining the linearity, sensitivity, selectivity, accuracy, precision, recovery, and stability of GD spiked into control rat plasma samples. Initial testing showed that a larger amount of GD was recovered from plasma than RBCs (data not shown) suggesting that there may be more binding sites or more unbound agent in the plasma (14). Alternatively, it may be an indication that "aging" occurs to a lesser extent or at a slower rate in plasma than RBCs. RBCs dry out when stored for long periods of time, making it difficult to recover the desired amount of sample. Given the greater amount of GD present in the plasma, and the poor recovery from older RBC samples, the regeneration method utilized in the present study was validated in plasma only.

Linearity

Figure 37 shows the response (peak area) ratio of GD to the IS (d_4-GD). Instrument linearity and reproducibility are demonstrated using no less than seven calibration concentration standards and comparing the slope, intercept, and correlation of five calibration curves over four consecutive weeks. The data show good correlation (r^2 = 0.9995) over two orders of magnitude with 0.08% variance and a slope variance of 3.76%. The intercept did not change significantly over the course of the study.

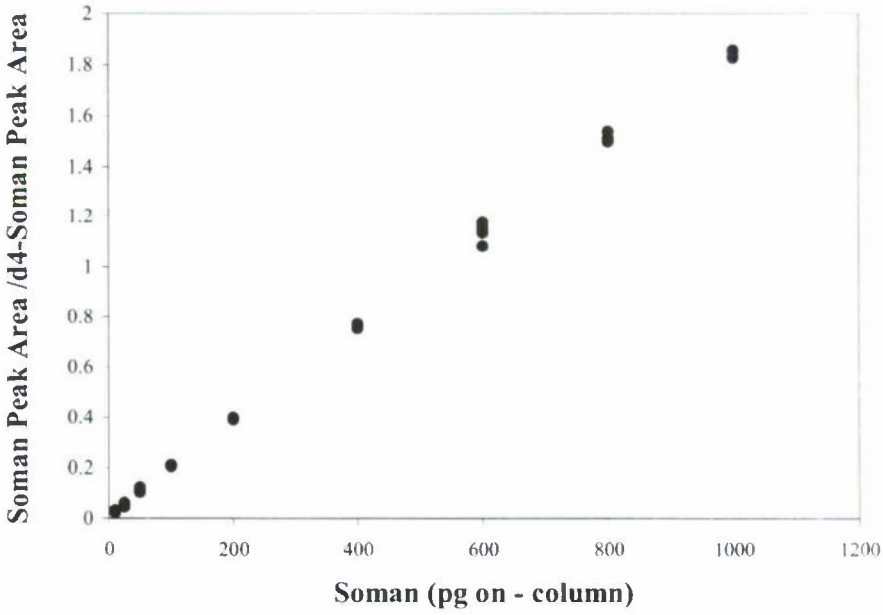

Figure 37. Peak Area Ratio of GD to d_4-GD *versus* pg GD Injected onto the Column

Each point represents the peak area ratio of GD to d_4-GD at each standard calibration concentration (10-1000 pg on-column). These points were obtained from five calibration curves over four consecutive weeks using seven or more standard concentrations for each curve.

Sensitivity and Selectivity

The sensitivity of this method was established by determining the limit of quantitation (LOQ) and the limit of detection (LOD). The LOQ is the minimum concentration of the analyte that can be accurately and precisely quantified with a peak-to-peak signal-to-noise (S/N) ratio of 10:1. The LOD is the smallest amount that can be detected with a peak-to-peak S/N ratio of 3:1. The average LOQ in plasma was calculated to be 18.83 pg on-column and the average LOD in plasma was 5.65 pg on-column. Selectivity was demonstrated by comparing six blank plasma samples and six spiked plasma samples, corresponding with the lowest point on the calibration curve (9.9 pg on-column). Each blank sample was analyzed for potential interferences and no interfering signals of consequence at the retention times of GD or d_4-GD were found as shown in Figure 36.

Accuracy, Precision, and Recovery

The accuracy and precision of the method was determined by injecting five replicate plasma samples at three known concentrations, LQC, medium quantitation concentration (MQC), and high quantitation concentration (HQC), over the expected range of the method (Table 23). The relative errors reported for the replicates were 8.69%, 1.50%, and 9.53% for the LQC, MQC, and HQC, respectively indicating that the method is both accurate and precise. Precision of the instrument, or

instrument repeatability, was measured by injecting the same three levels of known concentrations (n = 1 at each level) five times each and determining the relative standard deviation (RSD) for each series. The RSDs for the five repeated injections at each level were 3.0% or less. Method recovery was determined by comparing GD and d_4-GD extracted from plasma samples to those spiked directly into ethyl acetate (unextracted). The percent recovery for both GD and d_4-GD were based on the area count responses reported by the data analysis software. The recoveries for the LQC, MQC, and HQC were 101, 93, and 87%, respectively.

Table 23. Accuracy and Precision of the Regenerated GD Method

	Mean (pg on-column)	**% Relative Error**	**STD**
LQC (9.9 pg on-column)	9.04	8.69	0.76
MQC (401.5 pg on-column)	395.48	1.50	16.29
HQC (990 pg on-column)	895.69	9.53	29.43

Five rat plasma sample replicates were spiked with GD and d_4-GD at the LQC, MQC, and HQC. GD recovered was reported as pg on – column from which the mean was calculated.

Stability

A series of stability tests were conducted to assess the stability of GD at different points throughout the regeneration assay in order to determine how rapidly the steps of the assay must be completed following receipt of GD containing samples. The stability of GD in plasma stored at 4 °C and in plasma with the addition of 1 mL acetate buffer (pH 3.5) and 200 µL potassium fluoride solution stored at 4 °C, both intermediate steps in the regeneration assay, were determined. In addition, GD stability in ethyl acetate, the final matrix of the regeneration assay, was assessed.

Agent Stability in Plasma Matrix

To evaluate how stable GD is in the plasma matrix, five control rat plasma samples were spiked with GD at a concentration of 99 ng/g, which is equivalent to the HQC validation samples (990 pg on-column) in six sets (initial, 24 hr, week 1, week 2, week 3, week 4) for a total of 30 samples. The initial set was prepared immediately after spiking by adding the IS d_4-GD and then completing the regeneration assay while the other samples were stored at 4 °C until ready for preparation, with d_4-GD added at the time of extraction. The results show a rapid decrease in the average percent recovery of GD as time in storage increased (Figure 38A).

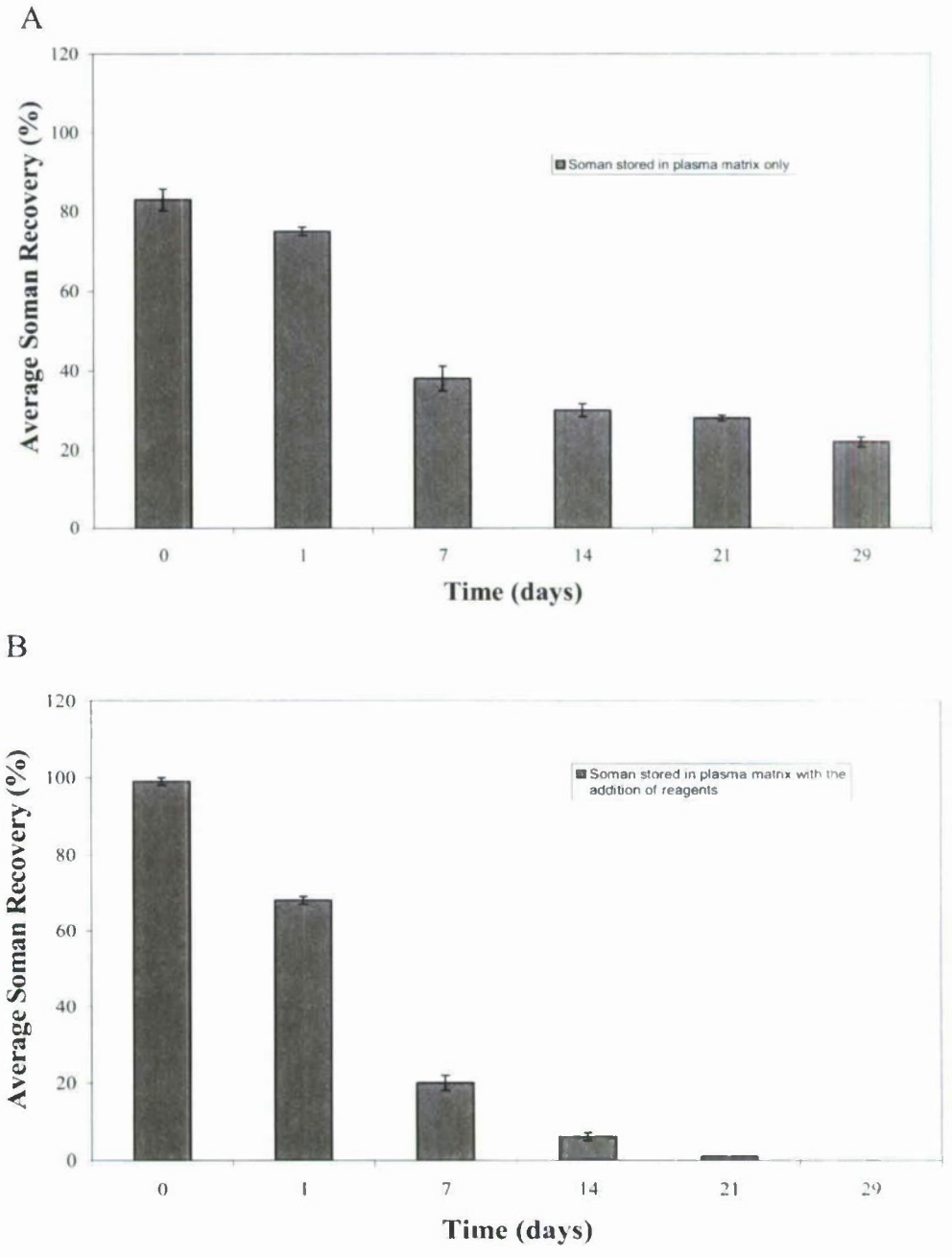

Figure 38. Average GD Recovery *versus* Time (days) Demonstrating Stability when Stored in Plasma Matrix Alone (A) and Plasma with the Addition of Assay Reagents (B)

The average recovery of GD spiked (99 ng/g) into control rat plasma (n = 5) decreased significantly over the course of four weeks when stored with and without acetate buffer and potassium fluoride in 4 °C indicating that GD is not stable at either step of the regeneration assay.

Agent Stability in Plasma Matrix with Assay Reagents

To determine how stable GD is in the plasma matrix with the addition of assay reagents, the experimental setup discussed above was followed with the addition of 1 mL acetate buffer (pH 3.5) and 200 µL potassium fluoride before spiking. Again, the initial set was prepared immediately after spiking while the others were stored in acetate buffer and potassium fluoride at 4 °C until the appropriate time, with d_4-GD added at the time of extraction. The results show a rapid decrease in the average percent recovery of GD as the time in storage increased (Figure 38B).

Both the GD-spiked plasma samples stored with and without the addition of buffer and potassium fluoride show a rapid decrease in recoveries over the course of four weeks. These results indicate that GD is not stable at either point during the regeneration assay when stored at 4 °C, demonstrating the need for rapid sample preparation immediately after GD exposure in order to obtain recoveries that accurately reflect the internal dose. Future studies will involve a stability test in plasma with the addition of buffer but without potassium fluoride as well as conducting the same series of tests in RBCs. In addition, analyzing the hydrolysate product pinacolyl methylphosphonic acid levels and the GD-AChE complex to compare the rate of hydrolysis and aging will better our understanding of the relationship of time and GD stability in biological matrices.

Agent Stability in Solvent

Using the samples from the matrix stability analysis above, a test was conducted to evaluate how stable GD is in ethyl acetate, the final matrix after extraction, in a freezer at -20 °C (normal storage conditions for our samples awaiting analysis). The five extracted samples at each of the six time points were injected four times under the same GC/MS conditions over the course of 29 days. Analysis of the four aliquots at each time point showed no significant decrease in the amount of GD recovered (Table 24), indicating that the ratio of GD to d_4-GD remained stable in ethyl acetate at -20 °C.

Table 24. Recovery of GD Stored in Ethyl Acetate at -20 °C over 29 Days*

Time Stored in Ethyl Acetate Post Sample Preparation	Time Stored in Acetate Buffer and KF Prior to Sample Preparation					
	Initial	24 h	1 week	2 week	3 week	4 week
Initial	984 ± 9	673 ± 12	197 ± 20	57 ± 12	13 ± 3	2 ± 2
24 h					13 ± 1	2 ± 1
7 days			200 ± 22			
8 days				58 ± 10	12 ± 1	
13 days		675 ± 15				
14 days	986 ± 5					2 ± 2
15 days			197 ± 21	60 ± 9		
21 days		672 ± 12			16 ± 3	2 ± 2
22 days	982 ± 7		195 ± 21			
28 days		650 ± 59		59 ± 10		
29 days	960 ± 70					

*Control rat plasma samples were spiked with GD (99 ng/g), stored in acetate buffer and potassium fluoride for the specified period of time (0-4 weeks), after which d_4-GD was added. Samples were then stored in ethyl acetate (the final matrix of the assay) at -20 °C and analyzed at four different times over the course of twenty-nine days and recoveries recorded as pg – on column ± standard deviation. Storing samples under these conditions post – sample preparation is typical for samples awaiting analysis. Results show that the ratio of GD to d_4-GD remains stable for up to twenty-nine days in ethyl acetate; n = 5 for each time point.

3.4.3.2 Application of Regenerated GD Detection in Rat Plasma

In applying this method, it should be noted that there are limitations. One limitation is that the bound and free agent fractions cannot be quantified separately without the use of an additional assay because the regenerated compound is identical to the parent compound. In addition, this method is not able to quantitate other biotransformation pathway products such as hydrolysis or "aging" products. Despite these limitations, this method has previously been successfully applied to the detection of regenerated GD in rat plasma and RBCs following 240 min whole-body inhalation exposures (14). In this study, levels of regenerated GD were measured in plasma samples immediately post-exposure and were correlated with the GD exposure concentration. Obtaining a closely correlated dose-response is typically difficult for GD due to its rapid "aging" (11). Additionally, the same study reported that regenerated GD was detected in the plasma at every concentration tested (0.033 to 0.280 mg/m^3). This is a significant finding because GD was detected at vapor levels that did not produce significant inhibition of AChE activity, a typical biomarker of nerve agent exposure, demonstrating that this method is approximately three-fold more sensitive than the AChE assay. In the present study, exposure of groups of rats to various concentrations of GD vapor for 60 min resulted in dose-dependent detection of GD in plasma (Figure 39). Linear regression analysis of regenerated GD data yielded an r^2 value of 0.754, demonstrating a solid relationship between GD exposure concentration and levels of regenerated GD. Using this method in conjunction with both free agent and hydrolysis product assays will provide a more complete picture of the pharmacokinetics and biotransformation of GD *in vivo*.

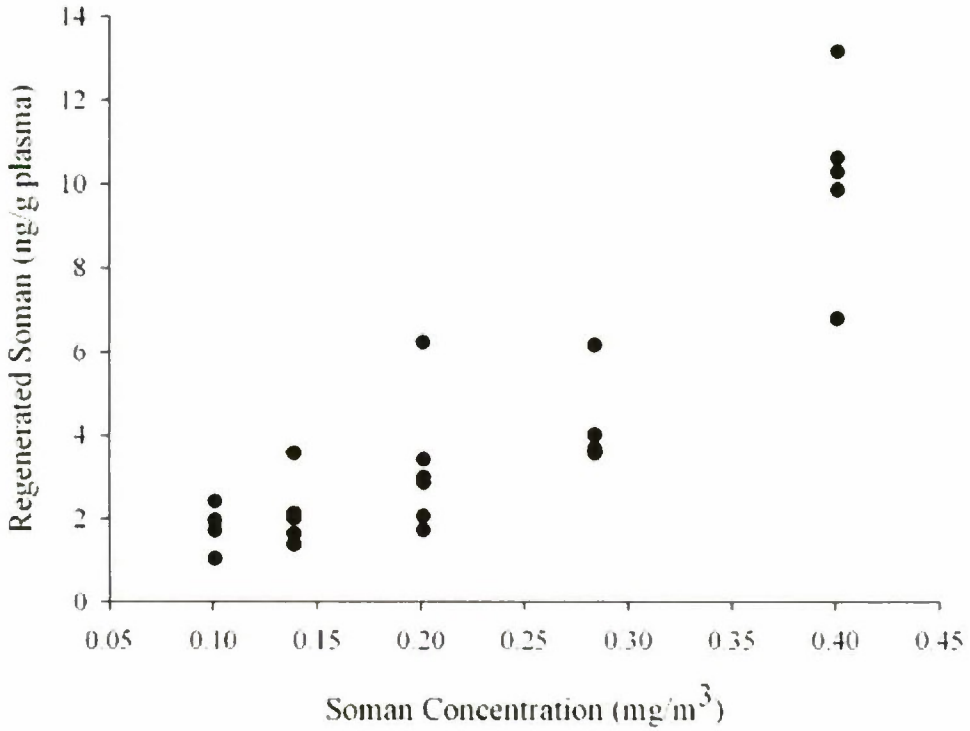

Figure 39. Regenerated GD in the Plasma Following Low-Level Vapor Exposure

Linear regression analysis yielded an r^2 value of 0.754 demonstrating a solid relationship between GD exposure concentration and levels of regenerated GD; each point represents a measurement from one animal.

3.4.4 Conclusions

A modified version of the fluoride regeneration assay and instrumental analysis method was validated and used immediately after GD vapor exposure to quantitate the concentration in rat plasma. This sensitive method was successfully applied to the analysis of GD in rat plasma, with the amount of GD regenerated from the blood correlating with the dose of GD to which the animals were exposed.

3.4.5 Acknowledgements

The authors greatly appreciate the hard work and technical assistance provided by: Emily A. Davis, James T. Taylor, William T. Muse, Dennis B. Miller, J. Steven Anthony, and Michael S. Horsmon; and Jacqueline A. Scotto, Megan L. Harris, and Ashley R. Fancher for veterinary care.

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3.5 Determination of VX-G Analog in Red Blood Cells via GC Tandem Mass Spectrometry Following an Accidental Exposure to VX

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3.5.1 Introduction

S-[2-(diisopropylamino)ethyl]-O-ethyl methylphosphonothioate (VX) is an extremely toxic organophosphate chemical warfare nerve agent (CWNA). Exposure to VX can result in a variety of toxicological effects with the most severe resulting in death. The toxicity of organophosphate nerve agents results from their effects on enzymes, particularly esterases. The most notable of these esterases is acetylcholinesterase (AChE), where the nerve agent phosphorylates a serine hydroxyl group in the active site of the enzyme, thus inhibiting the cholinesterase (ChE) from hydrolyzing the neurotransmitter, acetylcholine (1). Clinical effects manifested after exposure to these ChE inhibitors depend on the route, such as inhalation or percutaneous exposure and the amount of exposure. These signs or symptoms may include miosis due to contraction of pupillary sphincter muscles, tearing and salivation due to excess secretions of the exocrine glands, and twitching of large muscle groups due to hyperactivity in skeletal muscle (2).

Studies documenting human exposures to VX have been limited mainly to early military investigations (3,4,5,6), where volunteer subjects were administered sub-lethal doses of VX and monitored for signs and symptoms of anti ChE poisoning, including blood ChE activity. More recently, metabolites of VX were identified in the serum collected from a victim of VX poisoning in Osaka, Japan (7). In this latter study, ethyl methylphosphonic acid, a known hydrolysis product of VX, (8) was detected and quantitated along with 2-(diisopropylaminoethyl)methyl sulfide (DAEMS). The DAEMS was postulated to arise from enzymatic methylation of 2-(diisopropylamino)ethanethiol, an additional hydrolysis product of VX.

In addition to these traditional techniques for assessing exposure to VX, several researchers have demonstrated the utility of a fluoride ion regeneration method (9,10) for the production of the biomarker ethyl methylphosphonofluoridate (VX-G) from human serum exposed to VX. Jakubowski *et al.* (11) showed the method to be both selective and sensitive with a method detection limit of 10.5 pg of agent on column using GC with a flame photometric detector. Degenhardt *et al.* (12) analyzed extracts from the fluoride reactivation of human plasma samples that had been inhibited with VX by incubation via GC with high-resolution mass spectrometry (MS) and reported minimum measurable levels of VX-G as 3 pg/mL corresponding to 3 pg of agent on column for a 1 mL human plasma sample. Using gas chromatography with tandem mass spectrometry (GC-MS/MS), this fluoride ion regeneration technique was applied to the analysis of rat plasma exposed to VX and shown to be even more sensitive, with detection limits of <1 pg of VX-G on column (13).

Recently, we have applied the fluoride ion regeneration technique to the analysis of red blood cells (RBCs) collected from an individual accidentally exposed to VX vapor. The method employs GC-MS/MS on a triple quadrupole mass spectrometer using stable isotope dilution for quantitation. Results indicated that even 27 days after exposure measurable quantities of VX-G were detected.

3.5.2 Materials and Methods

3.5.2.1 Chemicals and Reagents

VX-G (Figure 40) of purity greater than 97% and $^{2}H_{5}$-ethyl methylphosphonofluoridate (deuterated VX-G, Figure 41) of isotopic purity greater than 95% were procured from ECBC. Potassium fluoride, 2-propanol, ethyl acetate, glacial acetic acid, and anhydrous sodium sulfate were obtained from Sigma-Aldrich (St. Louis, MO) at ≥99% purity. Sodium acetate was purchased from Fischer Chemicals (Fair Lawn, NJ) at > 99% purity. Ammonia and methane were obtained from Sigma-Aldrich, while helium and argon were obtained from Messer, Inc. (Malvern, PA). All the gases had purities >99.9%.

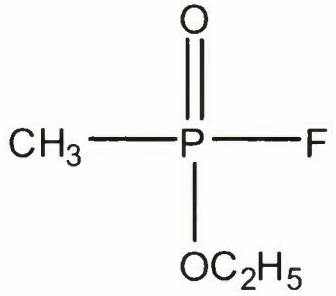

Figure 40. Structure of Ethyl Methylphosphonofluoridate (CAS Registry Number: 673-97-2)

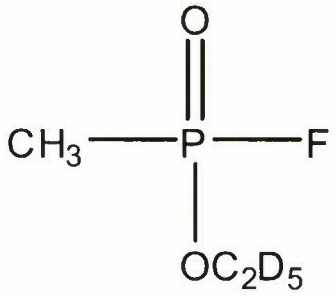

Figure 41. Structure of Deuterated Ethyl Methylphosphonofluoridate

3.5.2.2 Stock Solutions and Calibration Standards Preparation

Stock solutions of VX-G and deuterated VX-G were prepared in ethyl acetate at concentrations of 2.048 mg/mL and 1.203 mg/mL, respectively, and stored at -20 °C until used. Individual working solutions (nominally 50 µg/mL each) were prepared by diluting stock solutions in ethyl acetate for optimization of chromatographic and mass spectrometric conditions. Calibration standards of VX-G were prepared by diluting the working solution to obtain the following 10 concentration points: 4, 10, 25, 50, 100, 200, 400, 600, 800, and 1000 ng/mL. Each calibration standard also contained 1000 ng/mL deuterated VX-G diluted from the working solution. All of the calibration standards were also stored at -20 °C until analysis. Figure 42 shows the response of VX-G to the IS (deuterated VX-G), with the response being defined as Area $_{VX\text{-}G}$ X (Conc $_{IS}$/Area $_{IS}$) as a function of pg VX-G injected onto the column. These data are an average taken over the course of five weeks with n = 10, and show good correlation (r^2 = 0.9979) over two orders of magnitude.

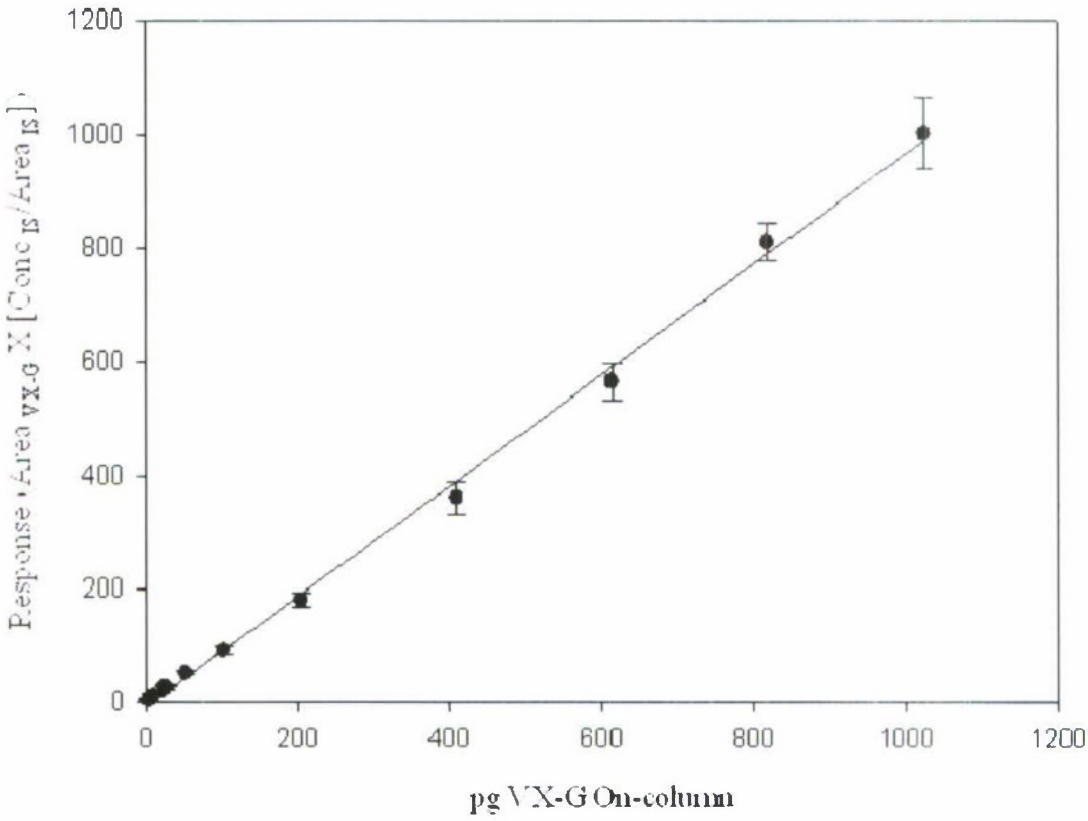

Figure 42. Response of VX-G to Deuterated VX-G *versus* pg VX-G Injected onto GC Column

3.5.2.3 Analytical Method

The assay was performed using an Agilent Technologies GC Model 6890 (Wilmington, DE) interfaced to a Waters Micromass Quattro micro GC™ tandem quadrupole mass spectrometer (Milford, MA). Gas chromatographic separations were achieved using a Restek Rtx®-5MS column (Bellefonte, PA) 30 m X 0.25 mm i.d. with a 0.25 µm film thickness. The carrier gas was helium with a flow rate of 1 mL/min. Injections of 1.0 to 3.0 µL were made by autoinjector (ALS Model 7683B, Agilent Technologies) into a splitless injector port at a temperature of 225 °C. The initial oven temperature of 35 °C was held for 2 min, then ramped at 15 °C/min to 125 °C, and then finally to 30 °C/min to 325 °C. Elution times for both VX-G and deuterated VX-G were typically 5 min as shown in Figure 43.

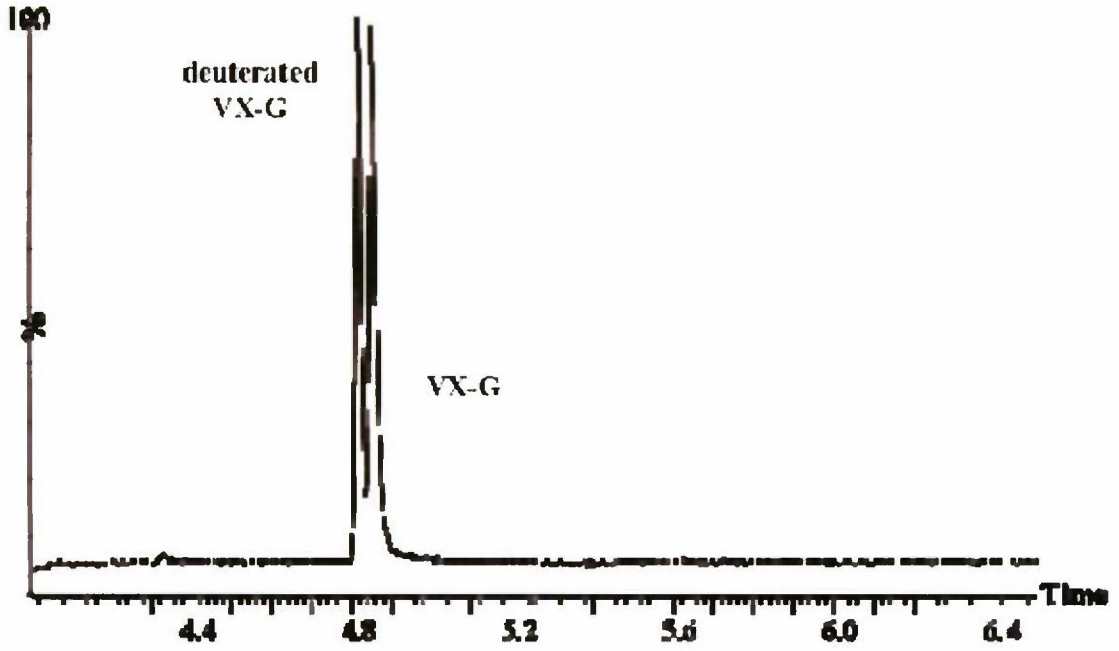

Figure 43. Total Ion Chromatogram Obtained from 1 µL Injection of VX-G Calibration Standard Containing 1000 ng/mL VX-G and 1000 ng/mL Deuterated VX-G

Samples were ionized by positive-ion CI with ammonia reagent gas. CI source conditions were optimized using Fluoroether E3 (CAS Registry Number: 3330-16-3, Agilent Technologies) tuning compound with methane reagent gas. Mass spectra were obtained at a dwell time of 0.05 sec for each transition in the MRM mode. Argon was used as the collision gas at a pressure of ~2 mTorr, with a CID energy of 10 eV. The CID energy was optimized for the m/z 144 > 99 transition for VX-G, and the m/z 149 > 100 transition for deuterated VX-G. Typical CID mass spectra obtained in our laboratory are shown in Figure 44. The MassLynx application software provided with the Quattro micro GC was used to process and analyze the data. The Quanlynx software provides automated peak detection, calibration, and quantification.

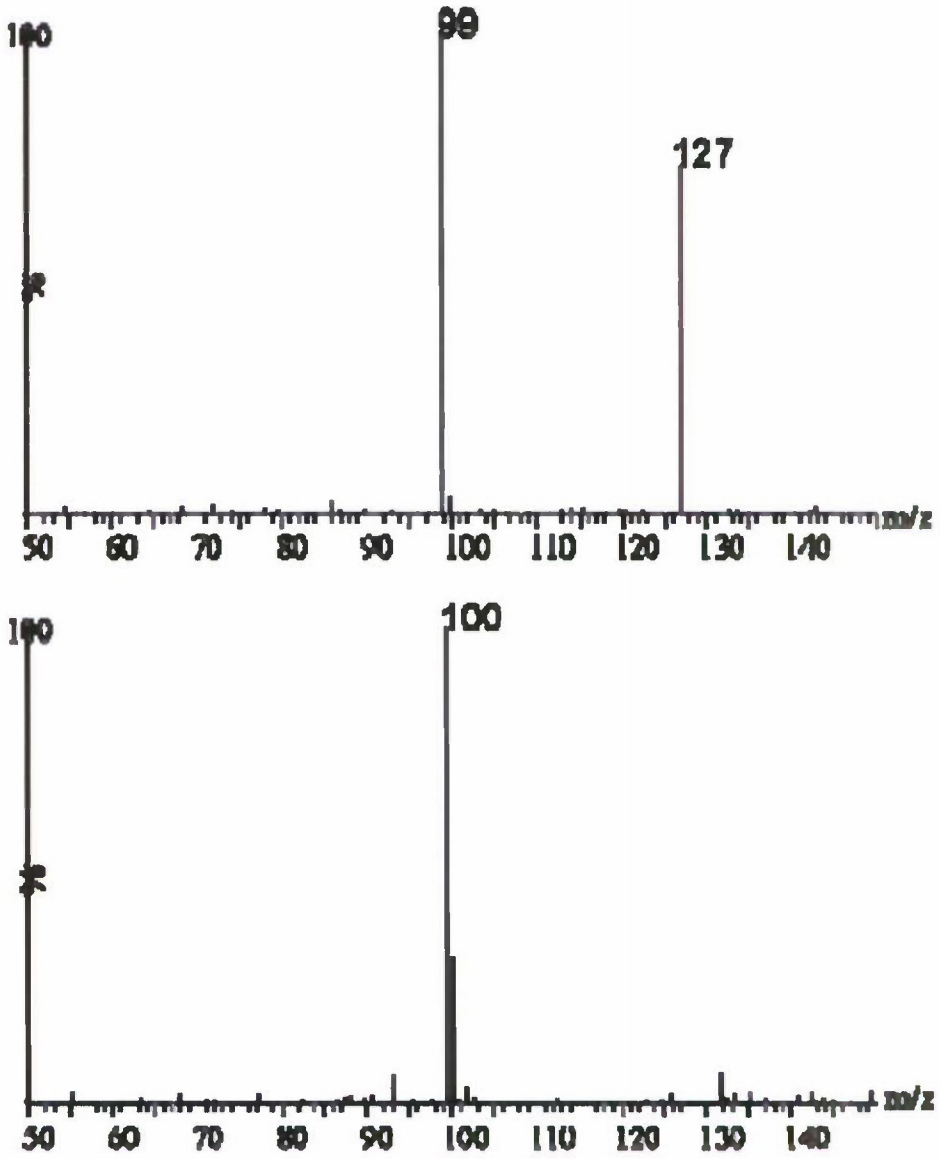

Figure 44. CID Mass Spectra of VX-G and Deuterated VX-G

Collision-induced dissociation mass spectrum (top) of m/z 144 from VX-G and (bottom) CID mass spectrum of m/z 149 from deuterated VX-G. Collision energy = 10 e.v. at 2.25 mTorr argon.

3.5.2.4 Sample Preparation

Sample preparations for this study were similar to those published by Jakubowski *et al.* (10) for fluoride ion released sarin. Whole blood samples obtained with informed consent from the patient were collected in glass evacuated blood collection tubes containing K_3EDTA. The blood samples were separated into RBC and plasma fractions by centrifugation at 4500 rpm for 10 min at 10 °C using an Allegra 25R centrifuge (Beckman Coulter, Inc., Palo Alto, CA). Following centrifugation, the plasma fraction was removed and immediately stored at -70 °C, while the RBC fraction was refrigerated until analyzed. RBC samples were extracted for VX-G using Sep-Pak® Vac 3cc C18 SPE cartridges (200 mg, Waters Corporation, Milford, MA), which were first conditioned with 1 mL each of ethyl acetate, followed

by 2-propanol, and then pH 3.5 acetate buffer (0.01 M sodium acetate, 0.2 M glacial acetic acid). To a weighed sample of RBCs (0.2-0.5 g) in a 2.0 mL microcentrifuge tube (Sigma-Aldrich, St. Louis, MO), 1 mL of acetate buffer, 200 µL of KF solution (6 M), and the IS, deuterated VX-G, were added. The mixture was vortexed for 10-20 sec and then centrifuged at 15,000 rpm for 5 min using a Micromax Microcentrifuge (Thermo IEC, Needham Heights, MA). Following transfer of the supernatant liquid to the SPE cartridge, the sediment at the bottom of the microcentrifuge tube was resuspended with 750 µL of acetate buffer and 200 µL of KF solution. This mixture was also vortexed and centrifuged and the resulting liquid added to the original solution. Fifteen minutes after the original addition of acetate buffer and KF, the combined reaction mixture was allowed to drain through the SPE cartridge under a gentle vacuum. The analytes were eluted with 1 mL of ethyl acetate that was collected and dried over anhydrous sodium sulfate. The ethyl acetate was withdrawn from the collection tube and filtered through a 0.2 µm nylon Acrodisc® syringe filter (Pall Gelman Laboratory, Ann Arbor, MI) into a GC autosampler vial (Agilent Technologies, Wilmington, DE) where it was concentrated to 50 µL for analysis.

3.5.2.5 Method Validation

Although the method described herein was not fully validated using human RBCs, the fluoride ion regeneration assay for VX-G in both plasma and RBC fractions from animal studies employing rats (14), guinea pigs, and minipigs (15) has been successfully implemented in our laboratory. In the case of minipigs (a typical mammalian animal model), typical RBC matrix spikes at nominal levels of 10, 200, and 1000 ng/mL yield RSDs of ±20.7, 4.6, and 3.8%, respectively. The limit of quantitation (LOQ) has been demonstrated at 5 pg on column.

3.5.3 Results and Discussion

3.5.3.1 Case History

On June 1, 2006, a laboratory worker at ECBC noticed dimness of vision and a feeling of tightness in the chest while performing routine laboratory work in an area where no known CWNA operations were to have occurred. Following standard operating procedures, the laboratory worker and fellow employees performed self decontamination and immediately contacted emergency response personnel. Because the lab worker exhibited early clinical signs of nerve agent intoxication, one MARK I nerve agent antidote kit (600 mg Pralidoxime chloride and 2 mg Atropine) was administered, and the worker transported to a local hospital for observation. The patient initially reported symptoms that included tightness in the chest and difficulty breathing, which was later followed by blurry vision, miosis, mild rhinorrhea, and eyelid muscle fasciculations. No significant depression in blood ChE activity was reported. All symptoms disappeared within 24 hr of the exposure.

After consulting other laboratory workers who were in the same area and who did not have signs or symptoms of exposure, it was determined that agent vapors could have originated in the room from a drying oven containing glassware that was cleaned after having been previously contaminated with VX. As compared to other workers who were in and out of the same area, the exposed worker was sitting closest to the oven for the longest period of time. Subsequent air monitoring of the laboratory at several locations in the room, including where the exposed worker was sitting, confirmed the presence of VX vapor with the highest concentration (0.000026 mg/m^3) reported in the room nearest the drying oven. This is significantly higher than the most recently recommended worker population limit expressed as an 8 hr time-weighted average of 0.000001 mg/m^3 (16). Furthermore, the actual vapor concentration during the exposure incident (approximately 90-120 min) was probably higher than 26 x WPL because the drying oven was turned off before the air samples were collected for safety reasons.

3.5.3.2 GC-MS/MS Analysis of RBC Samples

Because it has been shown that VX appears to inhibit RBC ChE preferentially over plasma ChE (5,17), efforts in our laboratory have focused on assessing exposure to VX via fluoride ion regeneration primarily by analyzing the RBC fraction of whole blood. RBC samples were obtained from the exposed laboratory worker on days 1, 6, 8, 20, and 27 following the inhalation exposure. Figure 45

shows the chromatographic trace obtained for the m/z 144 > 99 transition from the analysis of the RBC sample taken one day post-exposure. The on-column amount of VX-G was calculated to be 7.7 pg with a signal to noise RMS of 25.4:1. This calculates to a concentration of 242.1 pg/mL in the RBC sample. Typical recoveries from spiked quality control samples using minipig RBCs average between 110-120%. Table 25 summarizes the VX-G concentrations calculated for the five RBC samples obtained from the exposed laboratory worker. Average values and standard deviations are shown from a single injection of three separate sample preparations for each RBC sample. The relative standard deviations vary from a low of 13 to 62%.

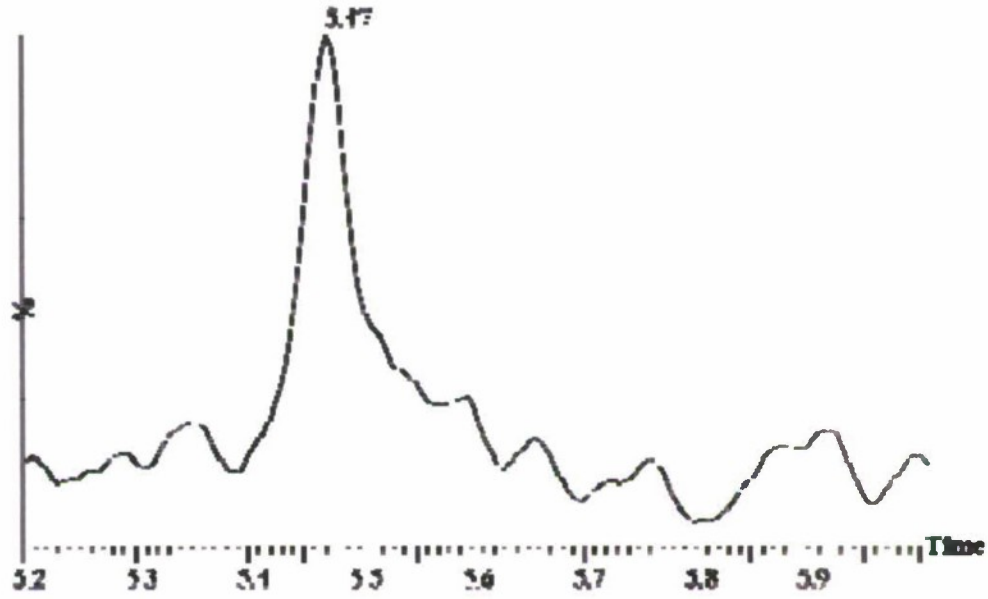

Figure 45. MRM of 144 > 99 Transition for VX-G from a 3 µL Injection of the Ethyl Acetate Extract from the Fluoride Ion Regeneration Preparation of RBC Taken 1 Day Post-Exposure*

*(S/N:RMS = 25.4)

Table 25. Observed Average (n = 3) Concentrations of VX-G in RBC Samples from Laboratory Worker Accidentally Exposed to VX

Collection Date	Days Post Exposure	VX-G Concentration (pg/mL)
2 June 2006	1	219.9 ± 97.1
7 June 2006	6	144.8 ± 57.8
9 June 2006	8	279.1 ± 170.5
21 June 2006	20	128.9 ± 17.5
28 June 2006	27	96.9 ± 60.4

Figure 46 shows the average VX-G concentrations as a function of time during which the samples were taken following the VX exposure. These data were fitted to a simple two parameter exponential decay equation (shown as a solid line) in order to approximate the half-life ($t_{1/2}$) of the fluoride ion regenerated biomarker VX-G. With a correlation coefficient of 0.6913, the $t_{½}$ was calculated to be

24.5 days, indicating that even several weeks after a low-level exposure to VX, detection and quantitation of VX-G should be possible.

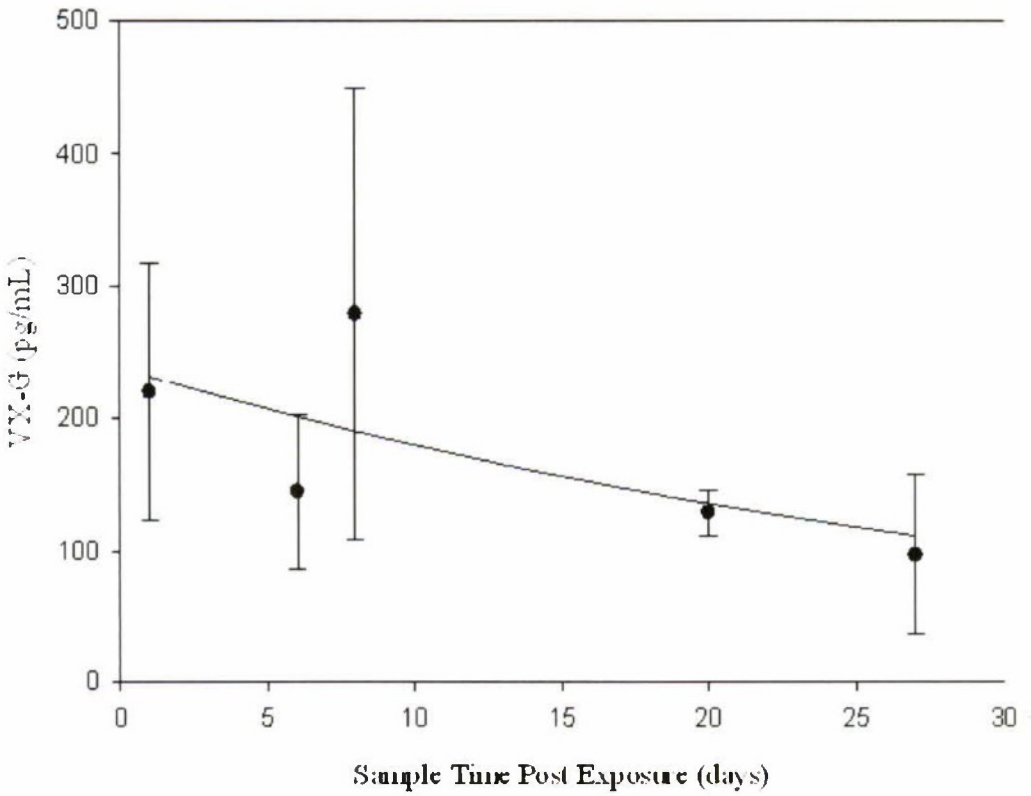

Figure 46. VX-G Concentration in RBCs *versus* Days Post-Exposure Sample was Taken

Solid line shows predicted curve for two parameter exponential decay, f = a*exp(-b*x)

3.5.4 Conclusions

The fluoride ion regeneration method has been shown to be an effective technique for producing the biomarker VX-G from human RBCs following exposure to VX. Using isotope-dilution GC-MS/MS the target analyte can be detected and quantitated in samples taken as late as three weeks post-exposure. For retrospective detection following a potential nerve agent exposure, this approach could be used even in the absence of gross clinical signs or when treatment has begun, such as oxime therapy.

3.5.5 Literature Cited

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3.6 Gas Chromatograph-Tandem Mass Spectrometry (GC-MS/MS) Analysis of Red Blood Cells from Göttingen Minipigs® Following Whole-Body Vapor Exposure to VX

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3.6.1 Introduction

Chemical warfare nerve agent (CWNA) S-[2-(diisopropylamino)ethyl]-O-ethyl methylphosphonothioate (VX) vapor is a highly toxic and potent cholinesterase (ChE) inhibitor. Older VX inhalation studies had assumed Ct = k (Haber's Law) (1) and had not elucidated the concentration-time profile. Haber's Law was viewed as a suitable approximation for offensive military applications (18),but this has since proved inadequate when extrapolating toxicity to longer exposure durations, which is required for hazard assessment involving defensive military applications. It has long been recognized in industry that for many acutely lethal gases the toxic effects cannot be correctly estimated by the Ct product (2.15). Many studies have indicated that an additional parameter, the toxic load exponent, is required to more accurately assess the toxic potential of an exposure. The resulting expression, $C^n t = k$, is known as the toxic load model, where n is the toxic load exponent and k is the toxic load constant (2,3,15). Only recently has the Department of Defense officially approved the use of the toxic load model for military applications (16). Both n and k are specific to the chemical agent and exposure scenario, and both are empirically derived. To elucidate this toxic load exponent, an experimental paradigm must be designed so that both exposure concentration and duration are varied within the same study (17). Although this limits the availability of repeated observations that are normally present in pharmacokinetic studies, indications of trends can still be observed.

The acute toxicity of VX is presumed to be caused by the inhibition of AChE, which reacts covalently with the organophosphorous CW agents at the serine residue active site to produce an inhibited *O*-alkyl methylphosphonylated enzyme. While blood ChE inhibition has shown to be a primary marker of CWNA vapor exposure, it is a poor indicator for the severity of exposure as well as the subsequent onset of clinical signs (*i.e.*, salivation, tremors, convulsions) (4,5). To better examine the degree of nerve agent exposures, a fluoride ion-induced nerve agent regeneration method has been utilized for the retrospective detection of CWAs in blood and tissues from exposed individuals that is both specific and quantitative (6,7).

The regeneration method is dependent upon incubation of nerve agent-exposed tissues with fluoride ions, after which the phosphonyl moiety of the nerve agent is released, resulting in regeneration of either the original CWA or a fluoro derivative (*O*-ethyl methylphosphonofluoridate; VX-G) in the case of VX. A variation of this fluoride reactivation assay for detecting VX-G has been previously applied as a biomarker for the presence of VX in spiked rat and human serum (8). This earlier work was conducted by injecting samples (40-400 µL) onto a Tenax®-TA sorbent tube followed by thermal desorption gas chromatograph-flame photometric detection (GC-FPD) analysis. The GC-FPD study established that VX can be both confirmed and quantified in blood following VX exposure.

The utility of this VX-G regeneration method has also been demonstrated in preliminary work clarifying the toxic potential of whole body VX vapor exposures in rats. This study was designed to establish values for lethal concentrations and times for half of the tested population, referred to as LCt_{50} (9). Analysis of blood from exposed rats relied on large volume injections (50 µL) into a GC/MS equipped for CI and utilizing ammonia as the reagent gas. With this method, it was found that even at 24 hr post exposure, relatively high concentrations of this fluoro derivative (or VX-G) were observed in both plasma and RBC fractions (0.5–1 ng/g), presumably due to the relatively high VX dosages administered (30 to 60 mg-min/m^3) (9). Additionally, the analysis of VX-G utilizing this regeneration method was also employed, examining low-level whole-body VX exposures (0.02-0.62 times 1.0 LC_{50}) in rats, where only the highest concentration produced clinical signs of toxicity, other than miosis. VX-G was detected in all samples at 30 min following a 60 min exposure (10).

VX can be difficult to analyze in biological systems, due in part, to the toxic effects of VX vapor at low doses, and the subsequent deposition of corresponding low levels of VX in exposed subjects. To better elucidate the effects of low-level vapor exposure scenarios where acute medical effects may not be obvious, a sensitive and selective analytical method for VX-G was required. In support of animal studies assessing low-level VX toxicity, by varying both VX vapor exposure concentration and duration in Göttingen Minipigs®, a GC quadrupole tandem mass spectrometry (GC-MS/MS) method was developed. This method employs the fluoride ion regeneration technique followed by ammonium chemical ionization (CI) GC-MS/MS using stable isotope dilution for quantitation. Preliminary studies employing this approach to monitor VX-G analog in rat blood (11) have shown significant increases in sensitivity over the earlier method, which utilized large-volume injections with single ion monitoring (9,10).

3.6.2 Materials and Methods

O-Ethyl-methylphosphonofluoridate and the internal standard (IS) $^{2}H_{5}$-O-Ethyl methylphosphofluoridate (d_5VX-G) were obtained from ECBC. All solvents and reagents used for the regeneration assay were of analytical grade or higher and obtained from Sigma-Aldrich Corporation (St. Louis, MO). Solid phase extraction (SPE) Sep-Pak cartridges (C_{18}; 200 mg) were obtained from Waters Corporation (Milford, MA).

3.6.2.1 Calibration Curve

Aliquots of neat VX-G and d_5VX-G were weighed and diluted with isopropyl alcohol (IPA) to create a calibration curve with concentrations of 5, 10, 25, 50, 100, 200, 400, 600, 800, and 1000 ng/mL, with 1000 ng/mL d_5VX-G IS in each. These calibration curve standards were stored frozen and remained stable for up to six months. Standards were analyzed to create a calibration curve each day minipig RBC samples were analyzed. Following analysis, a weighted (1/x) regression line was generated from the response factor (Area $_{VX\text{-}G}$ X (Concentration $_{IS}$/Area $_{IS}$) as a function of VX-G concentration. This regression line was used to quantitate minipig RBC samples.

3.6.2.2 Sample Collection

Samples of whole blood (approximately 1 mL) were collected from Göttingen Minipigs® (9-12 kg, Marshall Farms, North Rose, NY) via an indwelling jugular catheter, into BD Vacutainer® Blood Collection Tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) containing K_3EDTA. Catheters were implanted into anesthetized minipigs at least four days prior to nerve agent exposure. For whole-body exposure experiments, VX was administered via a 1000 L dynamic airflow inhalation chamber using concentrations ranging from 0.089-1.10 mg/m^3 and exposure times of 10, 60, and 180 min. Details of the inhalation chamber and methods for metering exposure levels are available from previously published research (4). Control blood samples were collected prior to exposure, with subsequent serial samples being collected at various time points during and following exposure. To help maintain homeostasis the blood volume withdrawn did not exceed 1% of the minipigs body weight over a one week span, and subsequent to each blood draw, an equivalent volume of Lactated Ringers was infused to replace sample volumes.

Whole blood samples were stored on ice immediately after collection, with a 100 µL aliquot from each used to assess ChE inhibition. After the terminal sample for that day's experiment was obtained, all samples were centrifuged at 15,000 rpm for 5 min. The plasma and RBC fractions were then separated, with the plasma stored frozen (-20 °C) and the RBC fraction refrigerated (5 °C) for 24 hr until analysis.

3.6.2.3 Regeneration Assay

VX-G was analyzed in RBC utilizing SPE C_{18} cartridges to extract the regenerated agent from the RBC fraction and then concentrated prior to analysis. Samples were first weighed (0.1-0.25 g) in a tared microfuge vial, then 1 mL of acetate buffer (pH 3.5), 200 µL of 6M KF, and 1 µL of d_5VX-G (50 µg/mL) IS was added. Included with each sample set was the control RBC fraction from that animal,

collected prior to nerve agent exposure. To this sample, 1 µL of d_5VX was added along with 1 µL of VX-G, resulting in a 100 ng/mL RBC sample utilized as a matrix spike. This mixture was vortexed for 20-30 sec and then centrifuged at 1500 rpm for 5 min. The resulting supernatant was then loaded onto a previously conditioned SPE cartridge. The SPE cartridge was conditioned by drawing through in order: 1 mL of ethyl acetate, 1 mL of IPA, and finally 1 mL of acetate buffer. The pellet from the centrifuged sample was re-suspended with an additional 750 µL of acetate buffer and 200 µL of KF, vortexed and centrifuged as before. The supernatant was then commixed with the previous addition and then allowed to drain through the SPE cartridge. A light vacuum was then applied for 5 min to remove any remaining aqueous component and ensure complete sample extraction. The extracted sample was then eluted from the SPE cartridge with an addition of 1 mL of ethyl acetate under a gentle vacuum. This fraction was then further dried with the addition of enough anhydrous sodium sulfate for it to freely flow in the vial. The dried sample was then filtered through a 0.2 µm nylon Acrodisc® syringe filter (Pall Gelman Laboratory, Ann Arbor, MI), pre-rinsed with 1 mL of ethyl acetate, then transferred to a GC autosampler vial. The sample was evaporated under a stream of nitrogen to a volume of approximately 50 µL, and then 1 to 3 µL was injected for analysis. If a 1 µL injection produced an analyte peak with a signal to noise ratio below 5:1, then up to 3 µL was then injected to increase analyte signal above this level.

3.6.2.4 Analysis

The 1 to 3 µL injection was introduced into an Agilent 6890 GC (Agilent Technologies, Wilmington, DE) equipped with a splitless injector port outfitted with a deactivated borosilicate single-taper inlet liner maintained at 250 °C. Separation was achieved with a Restek (Bellefonte, PA) Rtx®-5MS –30 m X 0.25 mm i.d. 5% diphenyl/95% dimethyl polysiloxane stationary phase capillary column with a constant helium flow rate of 1 mL/min. The oven program includes an initial oven temperature of 35 °C for 2 min, then ramped at 15 °C/min to 100 °C, held for 0.1 min, ramped again at 35 °C/min to 280 °C, and held for an additional 1.0 min.

The GC was coupled to a Waters (Milford, MA) Quattro micro GC tandem quadrupole mass spectrometer equipped with a CI source. The samples were ionized under positive-ion mode with ammonia utilized as the reagent gas at a pressure of 0.12 mTorr in the source. Argon was used as the collision gas at a pressure of approximately 2.25 mTorr, with a CID energy of 10eV. The detector was operated in MRM mode with a 0.05 sec dwell time allotted for the spectra for each transition. The transitions monitored were m/z 144>99 for VX-G and m/z 149>100 for d_5VX-G. The retention times were 4.87 min for VX-G and 4.83 min for d_5VX-G.

The method used for measuring whole blood AChE activity was a modification of the Ellman method (12). In brief, from each 100 µL whole blood sample, 10 µL was removed and added to a disposable borosilicate glass tube (Chase Scientific Glass, Rockwood, TN) containing 2 mL of distilled water, and 200 µL of 0.69 mM phosphate buffer at pH 7.4 (EQM Research, Cincinnati, OH). This mixture was then vortexed and incubated at room temperature for 15 min, at which time, 200 µL from each tube was transferred to a 96 well plate. Following the addition of 25 µL of 5,5-dithiobis-2-nitrobenzoic acid, the covered plate was incubated at 37 °C for 15 min. After incubation, 25 µL of a solution containing 10 mM acetylthiocholine and 200 µM of the BChE inhibitor 10-(α-diethylaminopropionyl)-phenothiazine was added to each well, shaken, then read at 450 nm at 37 °C using a SpectraMax Plus microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA). Data for AChE activity were expressed as units of activity per mL of whole blood, but reported herein as percent activity of control blood.

3.6.3 Results and Discussion

Analysis of the calibration standards proved to be linear (r = 0.9977) in the range of 5-1000 ng/mL of VX-G, as shown in the calibration curve depicted in Figure 47. This calibration curve was generated each day minipig samples were analyzed, with the weighted (1/x) equation of the line then used for quantitation of the regenerated samples.

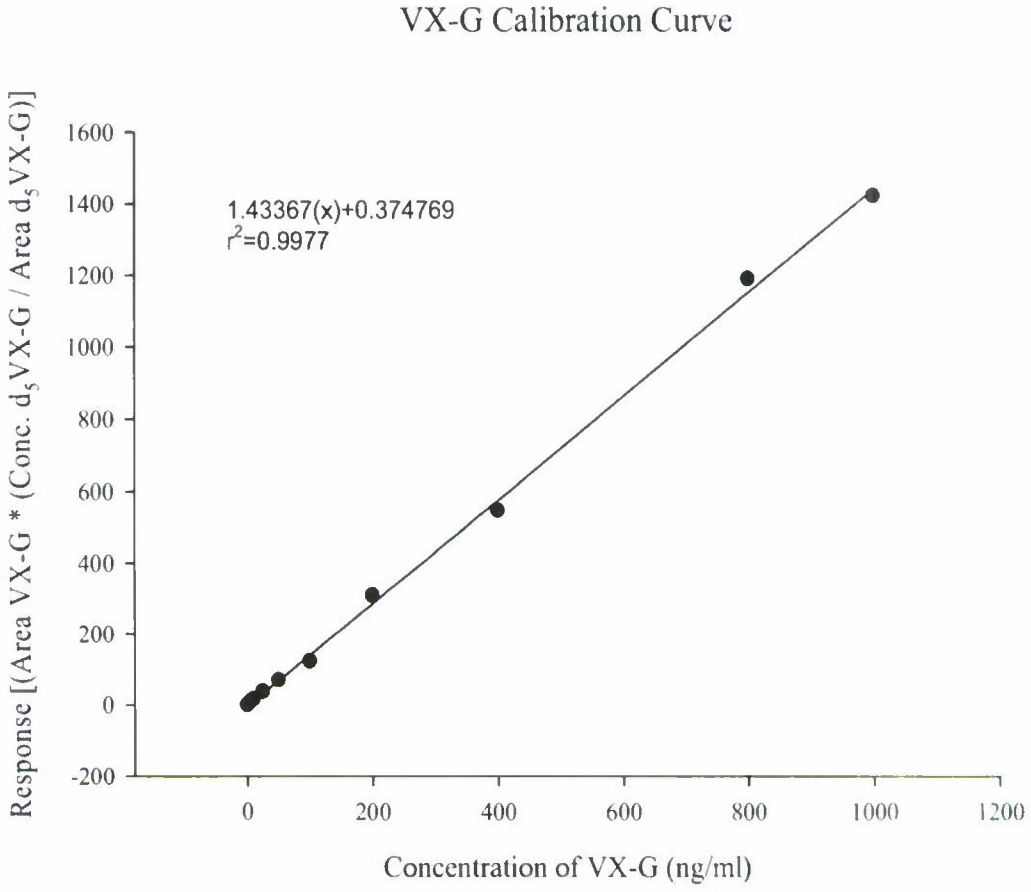

Figure 47. VX-G Calibration Curve

Calibration curve with weighted (1/x) least squares regression analysis, the resulting equation of the line and coefficient of determination. Data represent VX-G concentrations at 5, 10, 25, 50, 100, 200, 400, 600, 800, 1000 ng/ml versus response (Area $_{VX\text{-}G}$ X (Concentration $_{IS}$/Area $_{IS}$).

The linearity over two orders of magnitude for this detection method demonstrated a substantial increase from the original GC-FPD analysis that established VX-G as an accurate biomarker for VX exposure, where the standard curves were reported to be from 17.6-176.6 pg (5). The matrix spike samples, which were created from the control sample from each animal, had a mean of 108.6 ng/mL ± 8.7% RSD (n = 6). This finding is in accordance with previous analysis of matrix spikes at 10, 200, and 1000 ng/mL, where the RSD was ±20.7, 4.6, and 3.8%, respectively (n = 6). The LOQ with this method is 5 ng/mL (5 pg on column), which agrees with previous preliminary reports (10), whereas the GC-FPD method demonstrated a LOQ of 20 pg (8). In addition, the GC-FPD method lacks the specificity of the GC-MS/MS method.

Figure 48 illustrates a representative mass chromatogram of d_5VX-G (top trace) and VX-G (bottom trace), resulting from a 1 µL injection of extracted Göttingen Minipig® RBCs following whole body VX inhalation exposure and regeneration of VX-G. The whole blood sample was obtained at 60 min following the beginning of a 180 min exposure experiment. The top trace represents the m/z 149 > 100 transition for the IS d_5VX-G at 1000 ng/mL (1 ng on column), while the bottom trace represents the m/z 144 > 99 transition for VX-G at a calculated concentration of 6.0 ng/mL (6.0 pg on column). It should be noted there is an absence of any interfering peaks due to the MS/MS method that was utilized. The GC-MS/MS analysis provides the ability to minimize interfering peaks normally found in a complex biological matrix due to the selected and multiple reaction monitoring capabilities of the MS/MS instrument. Transition ions monitored result from the transmission of intact ionized precursor ions of a selected m/z

window through the first quadrupole, the fragmentation resulting from the applied collision energy encountered in the second quadrupole, and transmission of fragment product ions of an additional selected m/z window through the third quadrupole to the detector, which greatly enhances selectivity over previous GC-FPD and GC/MS analyses.

The VX-G concentrations found in minipig RBC, at two different vapor concentrations for each of three different exposure times (Figure 51: 10 min; Figure 52: 60 min; Figure 53: 180 min), versus sampling time are presented. Each data point represents the concentration of VX-G found in the RBC fraction of one whole blood sample at that particular time, while the line represents predicted data derived from the pharmacokinetic model. A control blood sample was taken just prior to exposure, with the exposure begun at time 0 and terminated at the end of the noted exposure time. Figure 51 depicts two experiments having a 10 min exposure time and doses of 1.1 mg/m^3 (●, solid line) and 0.63 mg/m^3 (▲, dashed line). Figure 52 shows an exposure level of 0.5 mg/m^3 (●, solid line) and 0.147 mg/m^3 (▲, dashed line), while Figure 53 shows 0.13 mg/m^3 (●, solid line) and 0.089 mg/m^3 (▲, dashed line). The data presented were fit to a pharmacokinetic model exhibiting constant IV input and first-order absorption and elimination {C(T) = (D/TI)*1/C*K10*[exp(-K10*TSTAR)-exp(-K10)*T]}; where D = dose(C*t), TI = length of infusion, STAR = T-TI for T > TI, and TSTAR = 0 for T ≤ TI (WinNonlin; Pharsight, Mountain View, CA). Owing to the primary objective of this study, to provide a greater understanding of the toxic potential of VX vapor exposures through varying exposure times and concentrations, repeated observations under identical conditions were not performed, therefore, limiting available sample size. While pharmacokinetic parameter estimates may be unreliable under these conditions, a dose-response relationship is evident (Figures 49-51).

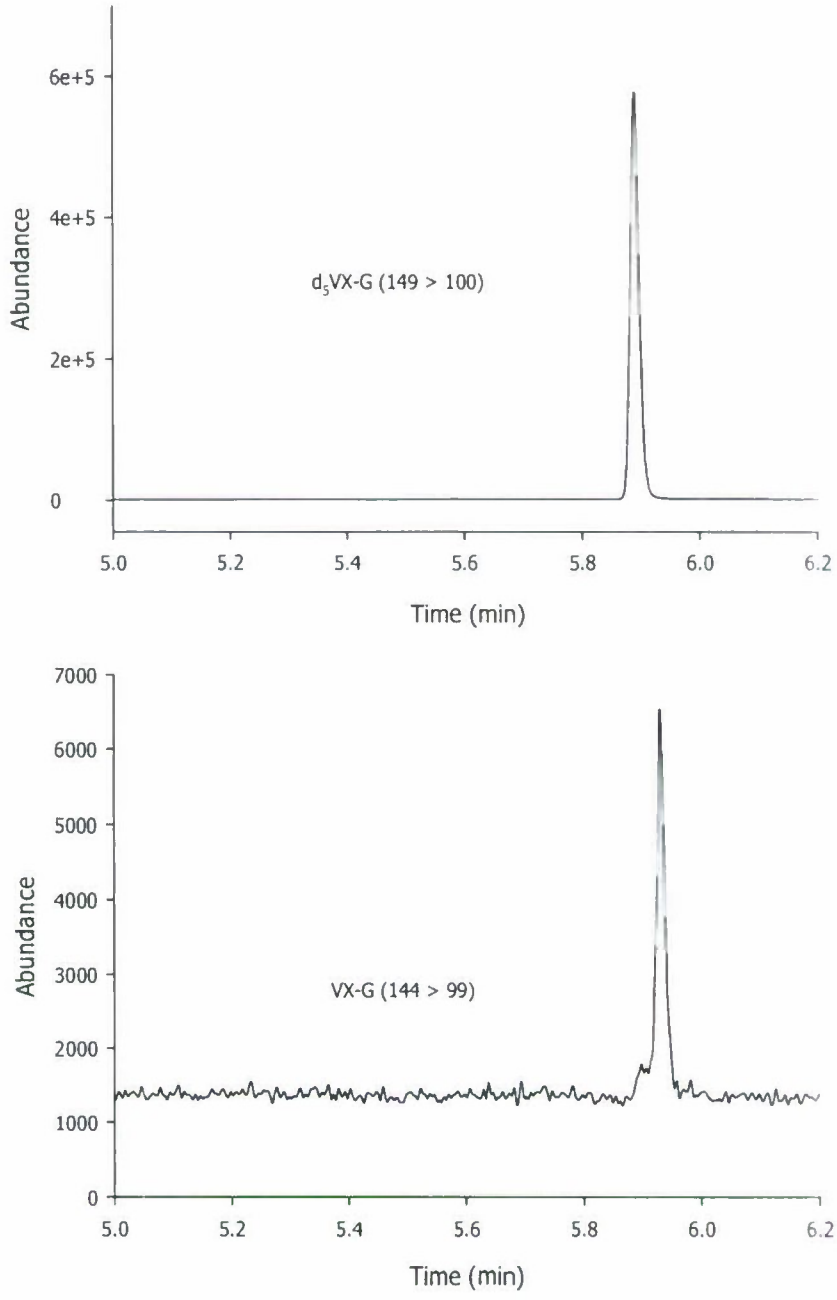

Figure 48. Representative Ion Chromatogram of VX-G and d_5VX-G Extracted from RBC

Representative mass chromatogram of VX-G (B) and d5VX-G (A) extracted from Gottingen minipig RBCs at 60 min of whole-body vapor inhalation exposure (0.089 mg/m3), and following regeneration as described in Regeneration Assay. The top trace represents the m/z 149 > 100 transition for the IS d5VX-G (5.90 min) at 1000 ng/ml, while the bottom trace represents the m/z 144 > 99 transition for VX-G (5.95 min) at a concentration resulting in 6.0 pg on column with a 1 µl injection.

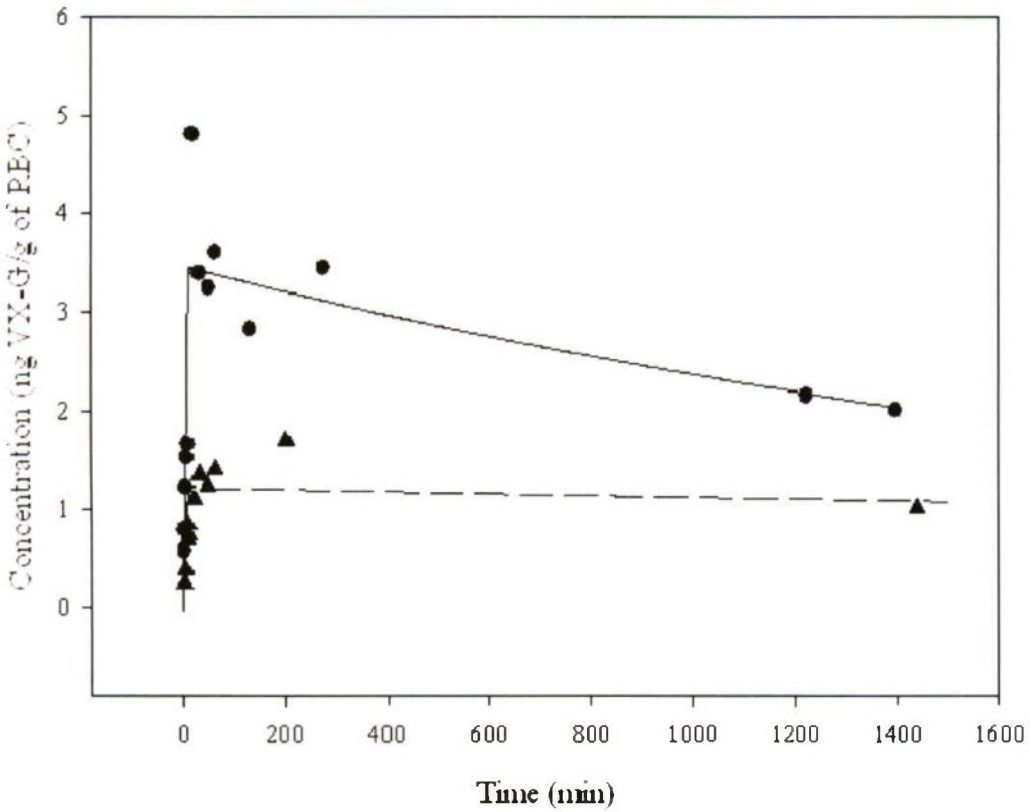

Figure 49. VX-G Concentration in the RBC Fraction as a Function of Sampling Time: 10 min

VX-G concentration in the RBC fraction as a function of blood sampling time for a 10 min whole-body vapor inhalation exposure at 1.1 mg/m^3 (•) and 0.63 mg/m^3 (▲).

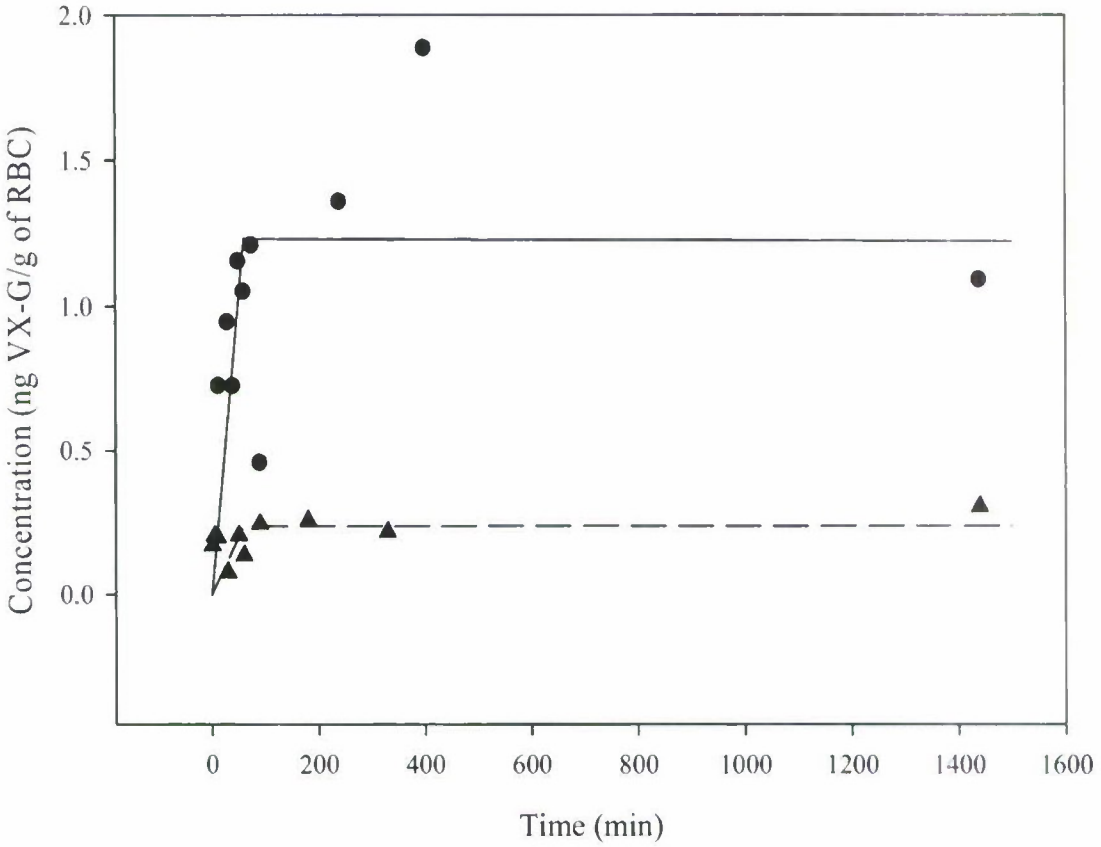

Figure 50. VX-G Concentration in the RBC Fraction as a Function of Sampling Time: 60 min

VX-G concentration in the RBC fraction as a function of blood sampling time for a 60 min whole-body vapor inhalation exposure at 0.5 mg/m^3 (•) and 0.147 mg/m^3 (▲).

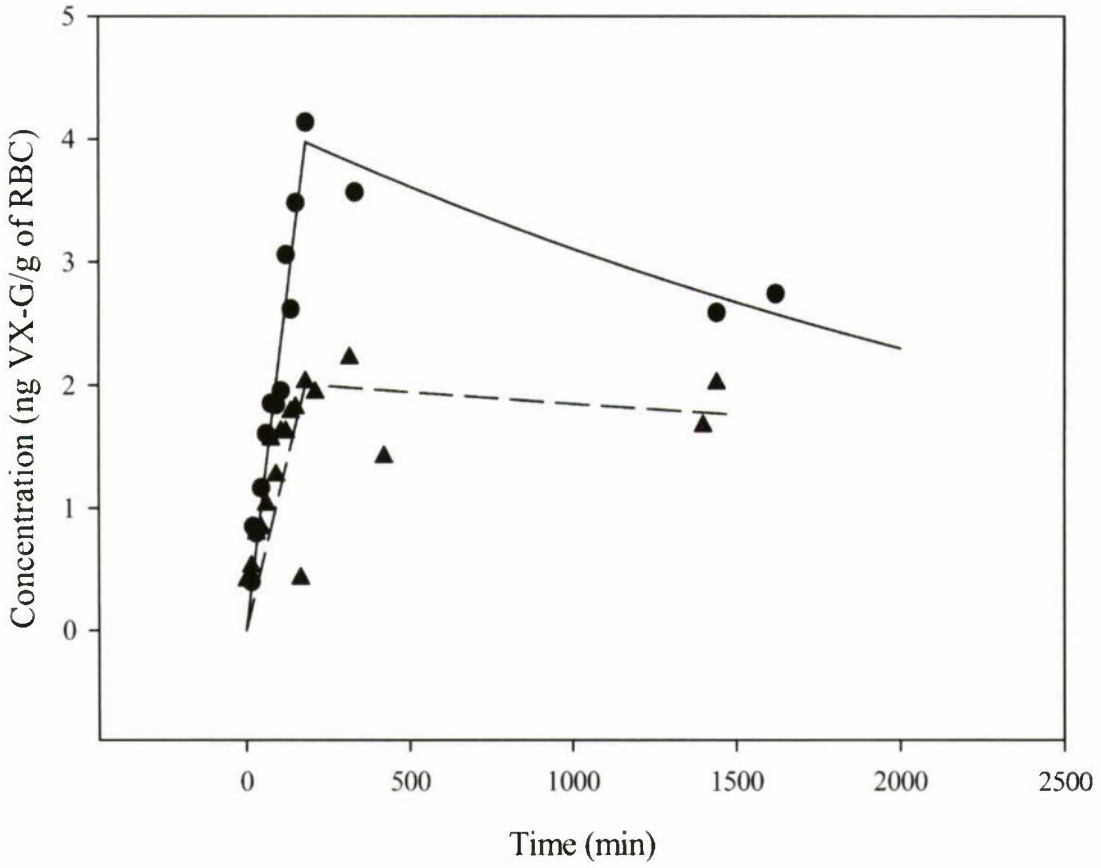

Figure 51. VX-G Concentration in the RBC Fraction as a Function of Sampling Time: 180 min

VX-G concentration in the RBC fraction as a function of blood sampling time for a 180 min whole-body vapor inhalation exposure at 0.13 mg/m^3 (•) and 0.089 mg/m^3 (▲).

The analysis of the ChE inhibition after a 180 min VX vapor exposure (0.13 mg/m^3 [•, solid line] and 0.089 mg/m^3 [▲, dashed line]) is presented in Figure 54. The inhibition of AChE activity is reported as percent of control, with the whole blood sample taken prior to exposure and identified at time 0 with a reported activity of 1.71 (0.13 mg/m^3) and 1.77 U/L (0.089 mg/m^3), serving as a control for each subsequent minipig blood sample. The data were best defined by an exponential linear combination equation (f = y0 + a * exp(-b * x) + c * x). The VX-G concentration in RBC and AChE inhibition have good correlation, in that, maximum inhibition is found at the 180 min sample, which is also the time for maximum concentration for VX-G. The maximum concentrations for VX-G following a 180 min exposure to 0.089 and 0.13 mg/m^3 are 4.14 and 2.03 ng/g, respectively, while the maximal inhibition of AChE was 7.06 and 10.59%, respectively. A 30% increase in dose administered correlated with a concomitant 33% increase in inhibition between doses, while the increase in recovered VX-G was 51% between doses. This small discrepancy may be an anomaly due to the limited sample size.

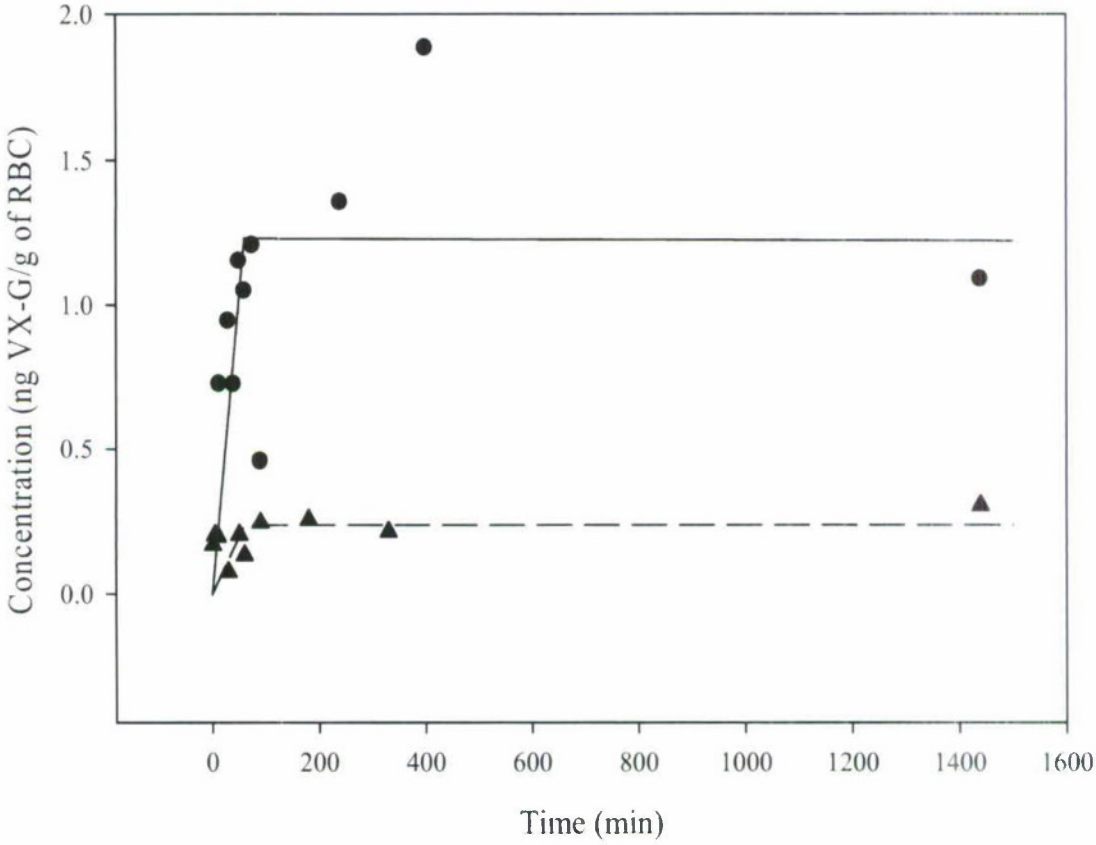

Figure 52. Curve Depicting the Exponential Decline of ChE Activity after 180 min Exposure

Following a 180 min whole-body vapor inhalation exposure at 0.13 mg/m^3 (•) and 0.089 mg/m^3 (▲). Data are presented as percent of control, with the control sample obtained prior to exposure and labeled as time 0.

The rate of AChE recovery seems to occur at a faster rate than the apparent elimination of VX when examining the slopes of the lines from 180 min to the 24 hr terminal sample. The enzyme activity recovers at a rate two orders of magnitude greater than the rate of decline in VX-G concentration at both doses analyzed (0.13 mg/m^3 and 0.089 mg/m^3). This much slower VX-G apparent elimination rate may be due to several factors. For example, VX-G regeneration may be occurring from locations other than the active site of the AChE. The fluoride ion-induced regeneration reaction will produce VX-G from unbound VX, as well as any of its breakdown products that retain the general *O*-ethyl methyl phosphonothioate (8). Unbound VX has previously been reported to be found in blood, tissues, and urine from exposed minipigs (13).

Factors contributing to the faster rebound of the inhibited AChE versus the slower apparent elimination of VX-G may be due, in part, to the spontaneous dephosphorylation and regeneration of active enzyme. In fact, the rate of spontaneous dephosphorylation is reported to be approximately three times faster than the rate of enzyme aging (14). Aging refers to the point that the enzyme activity can no longer be regenerated. This process of aging of the alkyl methylphosphonylated enzyme following VX exposure occurs at a slower rate when compared to many other organophosphorous CWAs. VX may also be bound to sites other than AChE, allowing the enzyme to rebound faster than VX-G is eliminated. Additional studies are suggested to establish an adequate sample size to substantiate these preliminary findings.

3.6.4 Conclusions

A method to detect VX-G in RBC from exposed Göttingen Minipigs® has been developed. The GC-MS/MS detection method demonstrates sensitivity and enhanced selectivity sufficient to analyze blood samples following whole body exposures to low levels of VX vapor. This method demonstrates significant advantages over analysis of AChE inhibition alone as an accurate measure of VX exposure.

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3.7 Determination of Partition Coefficients for VX in Biological Tissues and Fluids

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3.7.1 Introduction

Physiologically based pharmacokinetic (PBPK) models have become useful analytical tools to interpret pharmacokinetic data (1). These models are mathematical constructs that allow the coordination of species specific physiology, chemical specific characteristics, and the experimental protocol for the chemical and exposures of concern. The power of PBPK models lies in aiding the ability of scientists and decision makers to simulate the time-course concentration of chemicals in experimental animals and humans to better determine estimates of actual chemical doses delivered to the target tissue. Due to the physiologically based nature of these models, simulations of experimental data can be performed by one exposure route, to validate the PBPK model, and then this model can be used to simulate and predict the kinetics and pharmacodynamics in the human by one or multiple exposure routes. This provides decision makers with a fairly rapid method of comparing results from *in vitro* and *in vivo* laboratory studies to potentially real world exposure scenarios.

There are three basic critical components to PBPK models: 1) species-specific physiological parameters, 2) chemical-specific parameters, and 3) specific experimental details for the studies to be simulated. Species-specific physiological parameters are the organ weights and blood flow rates for the defined compartments in the PBPK model. These values are most often available in the published literature and when lacking, are derived from the closest like species. Chemical-specific parameters that are unique for each chemical include metabolism of the parent compound, plasma and tissue binding characteristics, and the tissue solubility or partition coefficient. In biological systems, this partition or distribution coefficient, which represents the ratio of chemical concentration in two interfacing phases when the chemical concentration is in equilibrium between the two phases is typically identified as the ratio of tissue chemical concentration to the concentration of chemical in the emergent venous blood of the tissue.

While many techniques, both *in vivo* and *in vitro* have been developed to quantify tissue:blood partition coefficients (2), there is no single best approach for their determination. For example, partition coefficients of low-molecular-weight compounds in tissues were measured experimentally by the vial equilibration method (3) due to the high volatility of the compounds studied. Conversely, for nonvolatile chemicals (chemicals with vapor pressures <1 mm Hg at 20 °C) an extraction procedure was developed for determining biological tissue partition coefficients (4). In support of recent efforts focused on integration studies to compare the dosemetric and response profiles for VX under inhalation and systemic exposure conditions, method development and implementation was undertaken for estimating the partition coefficient for VX in biological tissues and blood. Based on the earlier work by Jepson and colleagues for determining nonvolatile chemicals in biological tissues, a technique directed toward the determination of tissue:saline partition coefficients ($P_{T/S}$) for VX has been developed using hexane extraction from the saline solution followed by VX quantitation via gas chromatography-chemical ionization mass spectrometry with isotope dilution.

3.7.2 Materials and Methods

3.7.2.1 Chemicals

S-[2-(diisopropylamino)ethyl]-O-ethyl methylphosphonothioate (VX) of purity greater than 90% and $^{2}H_5$-*O*-ethyl-S-[2-(diisopropylamino)ethyl] methylphosphonothioate (d_5-VX) of isotopic purity greater than 90% were procured from ECBC. Paraoxon (Diethyl-p-nitrophenylphosphate, CAS No. 311-45-5) of purity greater than 98% was obtained from Chem Service, Inc. (West Chester, PA). Anhydrous sodium sulfate and 2-propanol ≥99% purity were purchased from Sigma-Aldrich (St. Louis, MO). Hexane (≥95% n-hexane, ≥99% hexanes) and buffer solution (pH 9.00) were obtained from Fischer Chemicals (Fair Lawn, NJ). Saline, 0.9% sterile and preservative free, was obtained from Phoenix Pharmaceutical,

Inc. (St. Joseph, MO). Ammonia and methane were obtained from Sigma-Aldrich, while helium was obtained from Messer, Inc. (Malvern, PA). All gases had purities >99.9%.

3.7.2.2 Chemical Stock Solutions and Calibration Standards Preparation

Paraoxon

A working solution of paraoxon was prepared in 2-propanol at a concentration of 126.83 µg/mL and stored at -20 °C until used for optimization of chromatographic and mass spectrometric conditions. Calibration standards of paraoxon were prepared by diluting the working solution to obtain the following nine concentration points: 10, 25, 50, 100, 200, 400, 600, 800, and 1000 ng/mL. All of the calibration standards were also stored at -20 °C until analyzed. Two chemical stock solutions of paraoxon were also prepared in saline at concentrations of 2.5 and 5 µg/mL, and stored at 4 °C until used.

VX

Stock solutions of VX and d_5-VX were prepared in 2-propanol at concentrations of 207.41 and 984.35 µg/mL, and stored at -20 °C until used. Individual working solutions (10.37 µg/mL for VX and 49.22 µg/mL for d_5-VX) were prepared by diluting stock solutions in 2-propanol for optimization of chromatographic and mass spectrometric conditions. Calibration standards of VX were prepared by diluting either the stock or working solution to obtain the following nine concentration points: 10, 25, 50, 100, 200, 400, 600, 800, and 1000 ng/mL. Each calibration standard also contained 1000 ng/mL d_5-VX diluted from the stock solution. All of the calibration standards were also stored at -20 °C until analyzed.

Two chemical stock solutions of VX were also prepared in saline at concentrations of 250 and 500 ng/mL and stored at 4 °C until used.

3.7.2.3 Blood and Tissue Sample Collection

Blood and tissue samples were collected from unexposed animals used as controls in various CWA inhalation exposure protocols. All studies were approved by the ECBC Institutional Animal Care and Use Committee and in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International. Blood draws were collected into BD Vacutainer® tubes containing K_3EDTA and stored at 4 °C until used. Following euthanasia, the tissues were immediately harvested and collected in polypropylene tubes, and stored at -80 °C. Prior to use, the tissue samples were thawed at room temperature for subsequent sectioning.

3.7.2.4 Analytical Methods

The assays were performed using an Agilent Technologies GC Model 6890 (Wilmington, DE) interfaced to a 5973 MSD. Gas chromatographic separations were achieved using a Restek Rtx®-5MS column 30 m x 0.25 mm i.d. with a 0.25 µm film thickness. The carrier gas was helium with an average linear velocity of 37 cm/sec at 70 °C. Injections of 3 µL were made by autoinjector into a splitless injector port at a temperature of 250 °C. The initial oven temperature of 70 °C was held for 2 min, then ramped at 25 °C/min to 300 °C and held for an additional 1 min. Elution times for both VX and d_5-VX were typically 8.8 min, while the elution time for paraoxon was 9.7 min. Mass spectrometric detection was by CI with ammonia reagent gas in the positive ion mode using the *m/z* 268/273 MH^+ ratio (VX/d_5-VX) for quantification and the *m/z* 269 and 274 ions (^{13}C isotopes) as qualifiers for VX determinations, and *m/z* 293 ($M+NH_4^+$) for quantification and *m/z* 294 (^{13}C isotope) as the qualifier for the paraoxon determinations. CI source conditions were optimized using Fluoroether E3 (CAS Registry Number: 3330-16-3, Agilent Technologies) tuning compound with methane reagent gas. Nine-level calibration curves using either the internal standard (IS) method for VX or the external standard method for paraoxon were created and updated periodically using the ChemStation software.

3.7.2.5 Sample Preparation

The appropriate weight of tissue (0.1 g) was placed in a tared 2 mL silanized clear crimp vial (Agilent Technologies, Wilmington, DE). A vial containing the chemical solution, but no tissue, was prepared to serve as a reference. The appropriate weight of the chemical solution (1.5 mL) was added to each vial and the crimp vials were sealed with 11 mm PTFE/S/PTFE (Teflon®) crimp caps (Agilent Technologies, Wilmington, DE). The crimp vials containing the tissue and chemical solution were placed in a shaker (The Jitterbug™, Model 130000, Boekel Scientific, Feasterville, PA) and incubated at 37 °C for 18 hr on mixer speed 6. Following the incubation period, the samples were centrifuged for 10 min at 1500 x g.

The supernatants resulting from the sample preparations were filtered with Ultracel YM-10 regenerated cellulose membrane centrifugal filter devices with a 10,000 nominal molecular weight cutoff (Microcon®, Millipore Corporation, Bedford, MA). Each filter device received 0.5 mL of sample supernatant and was centrifuged for 30 min at 8000 x g in order to collect approximately 0.3 mL of filtrate.

For the VX determinations, 50 µL of the pH 9.00 buffer, 500 µL of *n*-hexane/2-propanol (90:10), and d_5-VX IS was added to a weighed sample (0.1 g of filtrate) in a 1.5 mL microcentrifuge tube (Sigma-Aldrich, St. Louis, MO). For the paraoxon determinations, 500 µL of *n*-hexane was added. The hexane/filtrate samples were vigorously mixed for 30 sec, further shaken for 30 min using a mechanical mixer, and then centrifuged at 13,000 x g for 30 sec. The organic layer was transferred to a 2.0 mL microcentrifuge tube containing 1 g of anhydrous sodium sulfate and vigorously mixed. A second extraction with an additional 50 µL of buffer solution and 500 µL of *n*-hexane/2-propanol for VX or *n*-hexane only for paraoxon was done and also added to the 2.0 mL microcentrifuge vial and mixed. The organic layer was withdrawn and filtered through a 0.2 µm nylon Acrodisc® syringe filter (Pall Gelman Laboratory, Ann Arbor, MI), after pre-wetting the filter with either 1 mL of *n*-hexane/2-propanol for VX or *n*-hexane only for paraoxon, into a GC autosampler vial where it was concentrated/diluted for analysis.

3.7.2.6 Calculations

$$P_{T/S} = \frac{C_T}{C_S} = \frac{AMT_T / Wt_T}{C_S} = \frac{(C_R Wt_R - C_S Wt_S)/Wt_T}{C_S},$$

where

$$C_S = (C_{S,F})\left(\frac{C_{R,U}}{C_{R,F}}\right)$$

where

C_T is the chemical concentration (ng/g or ppb) in tissue, C_S is the chemical concentration (ng/g or ppb) in the saline fraction, AMT_T is the amount (ng) of chemical in the tissue, C_R is the chemical concentration (ng/g or ppb) in the reference solution, Wt_R is the weight (g) of the reference solution, Wt_S is the weight (g) of the sample solution, Wt_T is the weight (g) of tissue, $C_{S,F}$ is the chemical concentration (ng/g or ppb) in the saline filtrate, $C_{R,,U}$ is the chemical concentration (ng/g or ppb) in the unfiltered reference solution, and $C_{R,F}$ is the chemical concentration (ng/g or ppb) in the filtered reference solution.

3.7.3 Results and Discussion

Due to the low vapor pressure of VX (0.000495 mm Hg at 20 °C, (5), implementation of the vial-equilibration technique of Gargas did not appear feasible. Therefore, the Jepson method of incubating biological tissues with a saline solution containing the chemical of interest was investigated. In order to verify the validity of the method in our laboratory, an abbreviated study was initially done using paraoxon (vapor pressure = 0.0000378 mm Hg at 20 °C) to determine the partition coefficient between guinea pig blood and saline solutions at two different concentrations. Results indicated an average

tissue:saline partition coefficient ($P_{T/S}$) of 5.3 ± 2.7 for only two determinations. While this value is higher than those previously determined by Jepson for rat blood (1.8 ± 0.51 for a 50 µg/mL paraoxon concentration and 2.7 ± 0.5 for a 25 µg/mL concentration), it demonstrated the viability of this approach. Several factors may have influenced these discrepancies including different biological matrices (guinea pig versus rat blood) and the limited number of determinations. In Jepson's work, over 50 determinations were made, while in the present study only two determinations were done.

Based on these preliminary findings, tissue:saline partition coefficients were then determined for VX in various biological tissues. The $P_{T/S}$ values for whole blood and five different tissues from Göttingen minipigs at two different VX in saline concentrations are shown in Figures 53 and 54. Note that for those specimens where multiple determinations were done, error bars are quite large in some cases. This variability may be attributable to several factors inherent in the method itself, such as VX extraction efficiency and filtration recovery and is only exacerbated where partition coefficients are low. Also, the potential exists for metabolism of VX during the incubation phase of the method. However, all the tissues used in this study were perfused prior to harvesting. Therefore, any metabolic processes present should be compromised or negligible. Another factor is the possibility of binding between VX and proteins in the tissues. During this investigation, no precautions were taken to inhibit any protein or enzymatic interactions with VX. Finally, only a few measurements (n = 1-3) for each tissue have been done to date thus limiting the statistical significance.

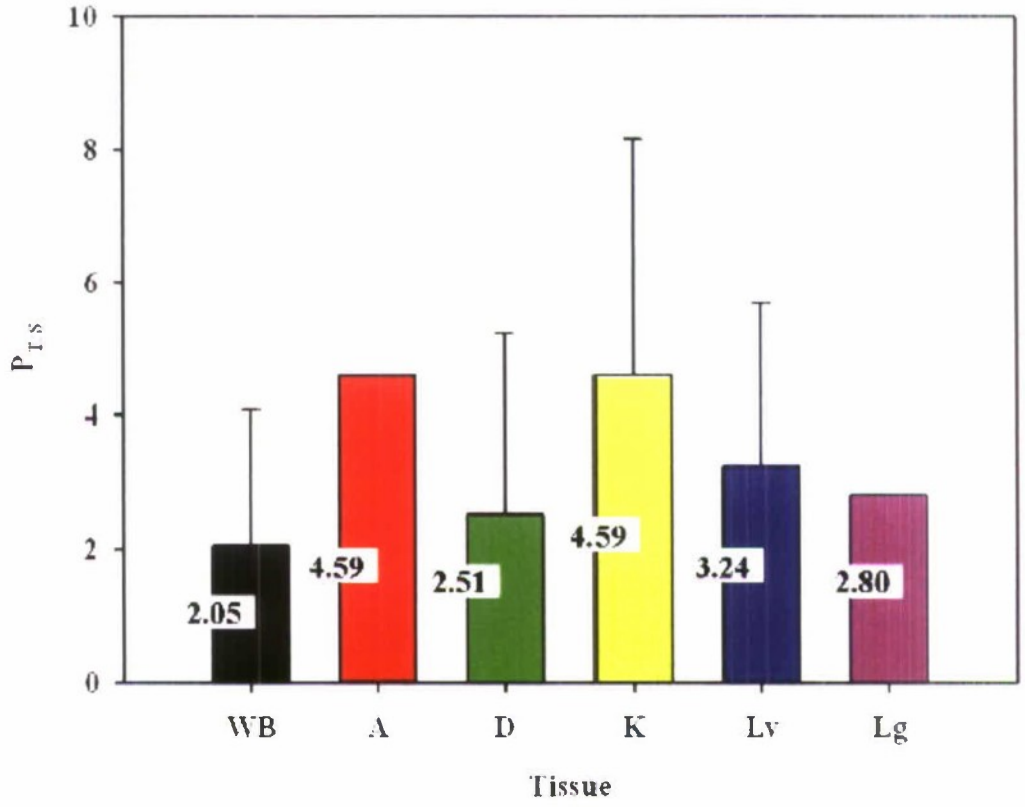

Figure 53. Tissue:Saline Partition Coefficient ($P_{T/S}$) for VX in Selected Göttingen Minipig Tissues

Saline Concentration of 250 ng/mL. Individual mean values are shown along with error bars for those specimens where multiple determinations were done. WB = Whole Blood, A = Adipose, D = Dermis, K = Kidney, Lv = Liver, Lg = Lung

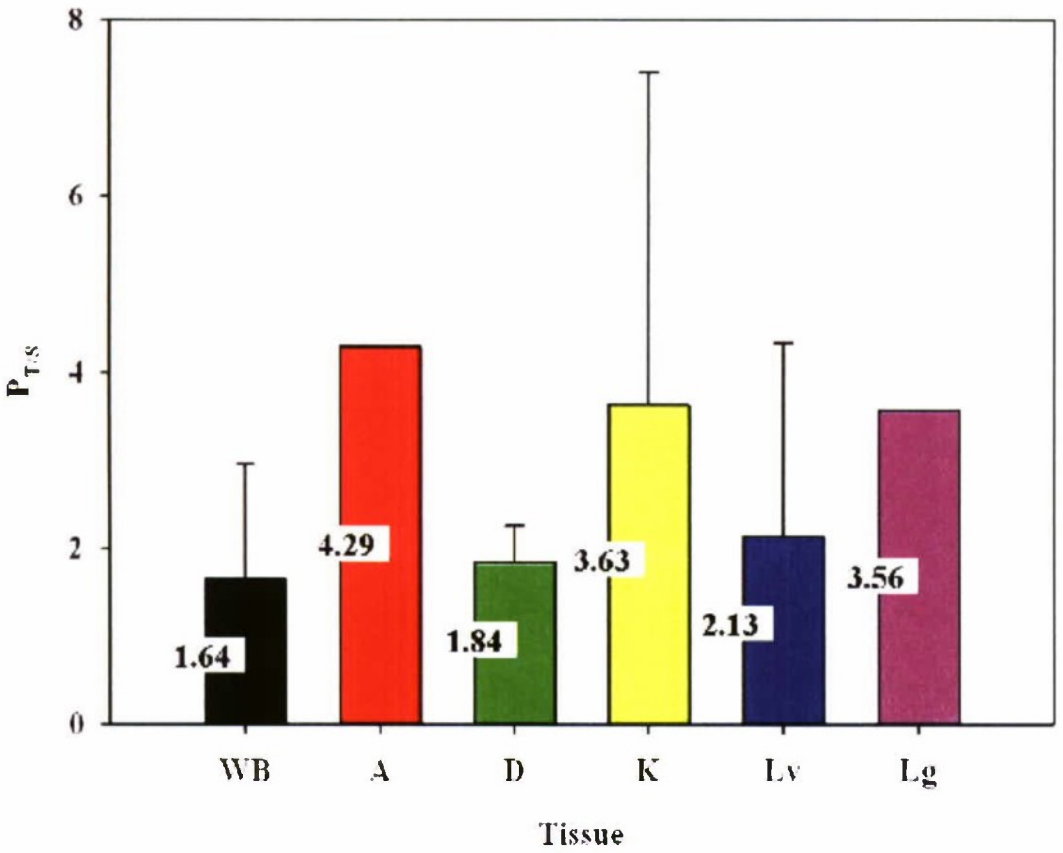

Figure 54. Tissue:Saline Partition Coefficient ($P_{T/S}$) for VX in Selected Göttingen Minipig Tissues

Saline Concentration of 500 ng/mL. Individual mean values are shown along with error bars for those specimens where multiple determinations were done. See Figure 53 for tissue key.

From the $P_{T/S}$ determinations, tissue:blood partition coefficients were calculated for the five tissues at the two different VX in saline concentrations and are shown in Table 26. In general, these values are reflective of the relatively low solubility of VX in these tissues. The results are consistent with recently published results for the octanol:water partition coefficient (log *P*) of VX as 0.675 ± 0.070 (6). Because VX is partly protonated at physiological pH (7.4) with a pKa of 9.4 (7), a low distribution into the lipophilic layer may not be too unexpected.

Table 26. Tissue:Blood Partition Coefficient for VX in Selected Göttingen Minipig Tissues at Two Different Saline Concentrations

Tissue	[VX in Saline] (ng/mL)	
	250	**500**
Adipose	2.2	2.6
Dermis	1.2	1.1
Kidney	2.2	2.2
Liver	1.6	1.3
Lung	1.4	2.2

3.7.4 Conclusions

A relatively simple method for the determination of biological tissue partition coefficients for nonvolatile chemicals has been adapted to calculate the tissue:blood partition coefficients for VX in the following Göttingen minipig tissues: adipose, dermis, kidney, liver, and lung. A determination of the tissue:saline partition coefficients is made for blood and the corresponding tissues, and the appropriate ratios calculated. Future work will focus on refining the method to reduce the variability and improve reliability, such as heating or chemically treating the tissues prior to analysis in order to minimize potential enzyme or protein interactions. Also, modifications to the extraction procedure will be investigated in order to permit the method to be applicable to the determination of partition coefficients for NTAs.

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4 CONCLUSIONS

For many years, issues unique to the skin have complicated a robust assessment of the hazard from "skin exposures" to chemical warfare agents (CWA) or to materials and equipment contaminated by CWA. With inhalation exposures, the very reasonable assumption that "what you breathe in is what you get" is largely borne out by experimental evidence. Lung internal anatomy is rather uniform even among different species, and it is not the controlling factor, necessarily, that determines how toxic a given inhalation exposure may be. The difference in potency observed from one animal model to another, or in an extrapolation to the human animal, is better explained by differences in upper respiratory configuration, metabolic capacities, systemic distribution, and similar systems level parameters.

In contrast, for dermal exposures the "receiving organ"—the skin, is not uniform at all. Skin is a very complicated structure and its architecture differs significantly from species to species and from various body regions—even within the same species. Unlike the lung, the external environment has a major influence on skin thereby affecting transfer and uptake of CWA. Conditions of humidity and temperature will substantially alter the risk calculation for a contact exposure. Furthermore, because it takes some time for a chemical to penetrate the skin and become available to the system there is often a delay period between contact and the onset of toxic signs. These issues have been "understood" on a qualitative level for years. The aim of this research program is to refine that understanding with scientific data that will allow us to predict more quantitatively the actual hazard posed to military operators from the potential contact to highly toxic chemical materials.

The studies, to date, that are captured in this report suggest the following conclusions that should guide the design and conduct of follow-on studies as well as the interpretation of historical data:

1. There is an unequivocal difference in the percutaneous toxicity between neat agent and diluted agent preparations. This is a critical issue as it is impossible to deliver increasingly smaller amounts of material to the skin surface when dosing into the less than lethal response range.

2. The onset of gross clinically-observable signs of intoxication from a contact exposure may be delayed until significant systemic absorption and distribution has occurred. Future studies will need to confirm the presence or absence of a "first noticeable effect" that would be a field expedient tool to trigger protective action. It may be discovered that unlike inhalation/direct ocular exposures where miosis is a key early first noticeable effect, contact exposures may not show an early warning sign. This is disconcerting, for if true, the window of opportunity to take protective action and thus prevent progression of signs in personnel would be vanishingly small—making contact exposures of any sort a mission compromising situation.

DEPARTMENT OF THE ARMY
US ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND
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1. Callahan, J.F. *The Relation between Skin Thickness and the Penetration Rate of VX through Skin.* In *Research Program of the Field Toxicology Branch;* CRDL TM 20-27; Callahan, JF, *et al.* Eds.; Directorate of Medical Research, U.S. Army Chemical Research and Development Laboratories, U.S. Army Chemical Center: Edgewood Arsenal, MD, 1962; UNCLASSIFIED Report. **CBRNIAC-CB-118810 Dist. E.**
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