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In vivo skin absorption and distribution of the nerve agent VX (*O*-ethyl-*S*-[2(diisopropylamino)ethyl] methylphosphonothioate) in the domestic white pig

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The purpose of this study was to characterize the skin absorption and distribution of VX (*O*-ethyl-*S*-[2 (diisopropylamino)ethyl] methylphosphonothioate) in the domestic pig in order to evaluate the animal as a potential model for assessing pretreatments against toxic anti-cholinesterase compounds. A liquid droplet (equivalent to a 2 $\times LD_{50}$ dose) of radiolabelled VX was applied to the inner ear-skin of each anaesthetized animal. Blood and tissue samples (liver, lung, kidney, heart and skin exposure sites) were obtained post-mortem. The amount of radioactivity in each sample was measured by liquid scintillation counting, from which the skin absorption rate and dose distribution of VX were calculated. A substantial proportion (22 $\pm$ 3%) of the applied dose remained within the skin at the site of application. It is conceivable that strategies to minimize or remove this reservoir may be of benefit in the early treatment of VX-exposed casualties. Image analysis of autoradiographs of exposed skin sites indicated that each milligram of radioactive VX covered an area of 1.2 $\pm$ 0.5 cm^2. The average skin absorption rate of ^{14}C-VX was 661 $\pm$ 126 µg/cm^2 per hour. Comparison of these data with previous studies suggests that human skin is less permeable to VX than pig skin, but VX spreads over a greater surface area when applied to human skin. Thus, paradoxically, while pig-ear skin is more permeable than human skin, the difference in skin surface spreading may lead to the absorption of an equivalent systemic dose. *Human & Experimental Toxicology* (2005) **24,** *347–352*

Key words: anti-cholinesterase; *in vivo*; pig

Introduction

The skin is a potential route of entry for a number of highly toxic anti-cholinesterase (nerve) agents, such as sarin (GB), soman (GD) and VX (*O*-ethyl-*S*-[2(diisopropylamino)ethyl] methylphosphonothioate). The development of effective medical countermeasures against such chemical warfare (CW) agents is primarily dependent on the use of appropriate animal models, as the toxicity of CW agents precludes the use of human subjects. Previous studies involving the skin absorption of CW agents have used rodent models, such as the rat, mouse and guinea pig,[1,2] as these animals have a well characterized response to anti-cholinesterase compounds. However, it is generally accepted that the most appropriate animal model for human skin absorption is the domestic pig.[3–5] Thus, the purpose of this study was to measure the skin absorption and distribution of VX in the domestic pig (*Sus scrofa*) in order to characterize it as a model for the development of effective pretreatments against nerve agents.

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Materials and methods

Chemicals

Radiolabelled ^{14}C-VX (2.5 MBq/mg) was synthesized at Dstl Porton Down and was of standard purity (89%). Soluene-350™ was purchased from Packard Biosciences (Canada). Liquid scintillation counting (LSC) cocktail, comprising toluene (90% v/v), isoamyl alcohol (10% v/v), 2,5-diphenyl oxazole (0.5 mg/mL) and bismethylstyrul-*o*-benzene (0.05 mg/mL) was prepared fresh for each batch of samples; Fisher ScintiSafe™ was used for the analysis of blood. Isofluorane was purchased from Abott Laboratories, Montreal, Quebec. Succinylcholine (SDC) was obtained from Burroughs Welcome Inc., Kirkland, Quebec. Hydrogen peroxide (30% solution, AnalaR grade) was purchased from Fisher Scientific (Canada). Reactive skin decontamination lotion (RSDL) was obtained from a current batch of Canadian Forces' stocks.

Animals

In conducting this research, the authors adhered to the *Guide to the Care and Use of Experimental Animals* and *The Ethics of Animal Experimentation* published by the Canadian Council on Animal Care (CCAC). The Defence Research and Development Canada (DRDC) is in full compliance with CCAC guidelines. Yorkshire-Landrace pigs ($n = 6$) were obtained from a local supplier and housed within a vivarium complex at CRDC. The animals were allowed access to water *ad libitum* and received 16% hog grower (Unifeed, Lethbridge, Alberta). Animal weights at the time of surgery were 20.5 ± 1.2 kg (average ± SD; range: 19.0–22.1 kg).

Anaesthesia was induced using isofluorane (5% in 8 L/min O_2) and each animal was placed into a dorsal recumbent position on a heated (38.5°C) operating table (Sage-London Industries Inc.). A transient muscle relaxant (20 mg SDC i.v.) was administered immediately prior to the introduction of a 6.5 mm internal diameter cuffed oral endotracheal tube (Ruschelit, Willy Rusch AG, 71394 Kernen, Germany), through which anaesthesia was maintained (2% isofluorane in 1 L/min O_2). All animals received normal saline (sodium chloride 0.9%, i.v., Abbott Laboratories Ltd.) at a rate of 9.50 mL/kg per hour ± 0.01 (mean ± SD, $n = 6$) via a volumetric infusion pump (Travenol FloGard 8000, Travenol Laboratories) for the duration of the study.

Percutaneous VX exposure

The dosing procedure was started at approximately the same time each day in order to limit diurnal variations in absorption, distribution, metabolism and elimination. A plastic octagonal weighing boat (diameter 3 cm across the flats) was inverted and attached via its rim to the inner ear-skin skin using an acrylic adhesive (Locktite 495, Locktite Corporation, Mississauga, Ontario). The base of each weighing boat contained a small (0.75 cm diameter) hole through which the VX could be applied to the skin surface using an Eppendorf™ micropipette. Each animal was administered a liquid droplet of VX (2.6–3.2 μL) equating to a 6-hour 2LD_{50} dose (120 μg/kg). After agent application, the boat was covered with a 3 × 3 cm^2 of charcoal cloth onto which surgical gauze pads (10 × 10 cm) containing approximately 2 mL of RSDL were placed. The whole ensemble was retained in place using surgical tape (Fisher Scientific, Nepean, Ontario).

Terminal procedures

Death was defined as having occurred 15 min after the onset of apnoea. At this time, apnoeic animals were euthanized by an i.v. injection of sodium pentobarbitol (6 mL, 540 mg/mL; Bimeda-MTC, Cambridge, Ontario, Canada). Animals that survived to 3 hours were euthanized by the same method. Following euthanasia, the gauze pads containing RSDL and charcoal cloth were removed, weighed and placed into glass vials which were stored at −70°C prior to sample analysis. An indelible marker pen was used to record the exact position of the weighing boat on the skin before the boat was removed, weighed, placed into glass vials and stored at −70°C. The whole exposed ear was excised, trimmed to leave a margin of up to 5 cm around the exposure chamber site and immediately autoradiographed (limit of detection 1 nCi/mm^2/100 h) at −70°C for 2 hours as previously described.[6] Autoradiographs were scanned using a Hewlett-Packard Scanjet 5550c scanner. A sheet of graph paper was placed behind the autoradiograph during scanning to superimpose a reference grid for calibration. The area over which staining of the autoradiograph had occurred was assumed to be representative of the area over which ^{14}C-VX had spread at the skin surface. The resulting digital images were analysed using Image Pro 4.1 software, which calculated the area of surface spreading. Each ear was then digitally photographed using a Kodak DC5000 digital zoom camera and the inner-ear skin (exposure site) was excised and stored at −70°C prior to sample analysis.

Following excision of the exposed ear, the animal was exsanguinated by cannulation and evacuation of the superior vena cava and/or carotid artery to eliminate any residual hazard from blood-borne ^{14}C-VX. The main thoracic and abdominal organs (heart, lungs, liver, kidneys and spleen) were removed and weighed prior to the removal of triplicate tissue samples which were individually stored in glass vials at $-70°C$ prior to sample analysis. Organ and skin samples were also obtained at post-mortem from animals that had been anaesthetized (but not exposed to ^{14}C-VX) in order to prepare 'control' tissue for sample analysis.

Sample analysis

All organ, weighing boat and ear-skin samples were placed into 15 mL Soluene-350™ and shaken at a temperature of 60°C until completely dissolved (<72 hours) after which aliquots (1 mL) were removed, placed into 18 mL LSC cocktail and analysed using a Beckman LS 6500 multipurpose liquid scintillation counter set to exclude single-photon (chemical or bioluminescent) events. Samples that did not dissolve (gauze and charcoal swatches) were subject to the same procedure but were centrifuged (Beckman J2-21M) at 20 000 rpm (10°C, 10 min) and samples (1 mL) of the supernatant were subject to LSC analysis. 'Standards' were prepared by the addition of 1 μL ^{14}C-VX to samples of organs and skin obtained post-mortem from animals that had not been exposed to ^{14}C-VX, or fresh gauze/RSDL and charcoal cloth swatches which were stored, prepared and counted in tandem with the exposed organ and skin samples. 'Blank' samples were prepared as above (using tissue obtained from animals that had not been exposed to ^{14}C-VX). Blood samples were prepared for LSC analysis using the method of Moore.[7] Briefly, 0.4 mL samples of whole blood were added to a 2 mL mixture of Soluene-350 and ethanol (50:50 v/v). Following a 2-hour incubation at 60°C, 0.6 mL aliquots were mixed with 1 mL hydrogen peroxide, incubated for a further 30 min (60°C), cooled to room temperature and mixed with 18 mL LSC cocktail prior to counting.

The quantity of radioactivity in each sample was converted to the amount of VX by reference to the blanks and standards. The total amount of VX per organ, skin sample, gauze or charcoal swatch was then calculated using the original weights of each sample. The total amount of VX in the systemic circulation was calculated using an estimate of total blood volume (see Equation 1), derived by regression analysis ($r^2 = 0.99$) from the data of Bush *et al.*[8]

$$\text{Total blood volume} = [161.48 \times \text{animal weight}^{-0.297}] \times \text{animal weight}/1000 \quad (1)$$

Limits of detection of the ^{14}C-radiometric analysis technique were set at twice the background decay rate. The coefficient of variation of background counts for each batch of (blank) samples did not exceed 0.12 and the coefficient of variation within triplicate tissue samples did not exceed 0.38, supporting this limit of detection.

Data analysis

The distribution of ^{14}C-radiolabelled material was defined as follows. The 'unabsorbed fraction' was equal to the sum of radioactive material recovered from the skin surface swabs and exposure chamber assembly (comprising charcoal cloth, plastic weighing boat and RSDL-soaked gauze). 'Skin absorption' was deemed to be the total radioactivity recovered from the skin exposure site. The difference between the applied dose and the total recovery of radioactivity (from the unabsorbed fraction, skin absorption and that recovered from the main organs) was assumed to be representative of material residing in unsampled tissues (such as muscle, brain and fat). 'Total systemic absorption' (A_{SYS}) was calculated as the sum of recovered and unrecovered fractions.

Two measurements of the rate of ^{14}C-VX absorption were calculated: 'skin' and 'systemic'. Skin absorption rates (μg/cm² per hour) were calculated from Equation (2):

$$J_{SKIN} = \frac{(\text{Total applied dose} - \text{unabsorbed fraction})}{\text{Area} \times \text{time}} \quad (2)$$

The surface area of contamination (Area) was measured directly from autoradiographs of each exposed skin site and time related to the duration over which absorption occurred (i.e., time to death).

Rates of systemic absorption (J_{SYS}) were calculated using Equation (3).

$$J_{SYS} = \frac{A_{SYS}}{\text{Area} \times \text{time}} \quad (3)$$

Unless otherwise quoted, all values represent average ± S.E.M. of $n = 6$ animals. Statistical analysis was performed using the Kruskal–Wallis ANOVA with Dunnett's multiple comparisons post-test.

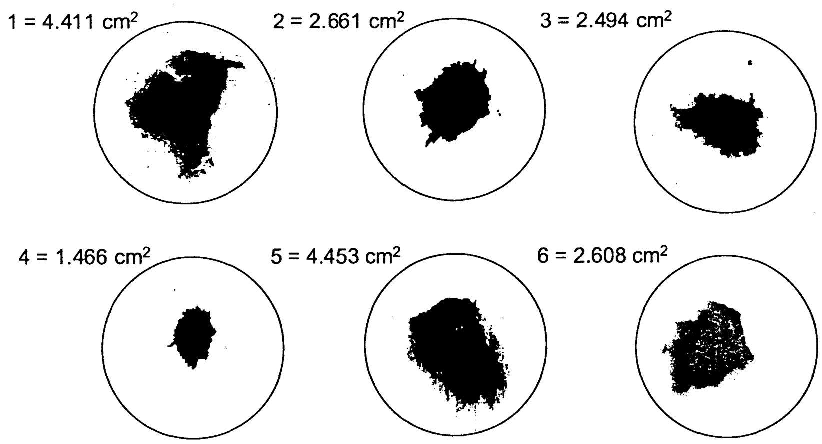

Figure 1 Digital image of autoradiographs taken immediately postmortem from the site of percutaneous exposure to ^{14}C-VX (liquid). Location of ^{14}C-radiolabelled material indicated by dark areas. The animal number (in order of exposure) and total surface area of the darkened regions are indicated in the top left corner. Circles indicate approximate position of the inner border of the dosing chamber. Time to death in each animal was 120, 62, 43, 180, 57 and 42 mins for animals 1–6, respectively.

Results

Absorption

The average area over which ^{14}C-VX was detected at each exposure site was 3.0 ± 0.5 cm^2 and was consistently retained within the exposure chamber (Figure 1). The corresponding rate of ^{14}C-VX skin absorption (J_{SKIN}) was calculated as 661 ± 126 μg/cm^2 per hour and the rate of systemic absorption (J_{SYS}) was 512 ± 96 μg/cm^2 per hour.

Distribution

Unabsorbed material accounted for 1.2 ± 0.2% of the applied dose (Table 1). Approximately one-fifth of the applied dose (21.7 ± 2.9%) remained within the skin exposure site and 53.3 ± 3.5% of the dose was recovered from the sampled tissues (Table 1). The remaining (unrecovered) fraction, assumed to be present in unsampled tissues, equated to 23.8 ± 3.6% of the applied dose and thus the total systemic absorption was approximately 77.1 ± 2.9%. The total, direct recovery of radioactive material was 76.2 ± 3.5% (Table 1).

Discussion

The results of this study have demonstrated that the *in vivo* percutaneous penetration of VX through pig-ear skin is both rapid ($\sim$660 μg/cm^2 per hour) and extensive ($>$98% applied dose absorbed). In contrast, the skin absorption rate of VX through human (breast) epidermal membranes is approximately 5.5-fold lower (120 μg/cm per hour; unpublished MoD report). This species difference is in agreement with previous studies using different chemicals.[9–11] However, it should be noted that the flux calculations in this study may underestimate the maximum absorption rate. This is because the method employed did not take into account the effects of lag-time or depletion of material at the skin surface, both of which would decrease the period over which steady-state penetration occurred.

The extent to which a chemical spreads out across the skin surface is of primary relevance to percutaneous toxicity studies, as the total absorbed dose will be proportional to the area of contaminated

Table 1 Radioactivity, expressed as a percentage of applied dose, recovered from exposure chamber assembly and skin surface ('unabsorbed material'), skin ('local recovery') and organs ('systemic recovery') and amount of VX (ng) per gram wet weight (organ) tissue in six pigs exposed to $2 \times LD_{50}$ ^{14}C-VX

Compartment	*Location*	*% Recovery*	*VX (ng)/g tissue*
Unabsorbed	Gauze	0.004 ± 0.003	–
	Charcoal	0.14 ± 0.02	–
	Plastic boat	0.63 ± 0.08	–
	Skin surface swab	0.56 ± 0.08	–
Local recovery	Skin exposure site	21.7 ± 2.9	–
Systemic recovery	Heart	0.33 ± 0.17	61 ± 32
	Lung	2.2 ± 0.4	201 ± 33
	Liver	11.6 ± 2.2	435 ± 85
	Kidney	1.3 ± 0.3	267 ± 62
	Blood	37.9 ± 3.5	546 ± 50.5
Total recovery	(Unabsorbed + local + systemic)	76.2 ± 3.5	–
Unaccounted	(Total recovered – applied dose)	23.6 ± 3.5	–

Total recovery of ^{14}C-radiolabelled material in the 'unabsorbed' and 'systemic' compartments were 1.2 ± 0.2% and 53.3 ± 3.5%, respectively. The 'unaccounted' dose was assumed to reside in unsampled tissues, such as the brain, muscle and fat. The calculated limits of detection for heart, lung, liver, kidney and blood were consistently lower than those measured in this study, being 45, 153, 148, 117 and 286 ng/g tissue, respectively. All values are mean $\pm$ SEM of $n = 6$ animals.

skin. Previous volunteer studies have demonstrated that the extent to which VX spreads over human (forearm) skin is $\sim$6 cm^2/mg of applied agent.[12] Such measurements were performed by adding a fluorescent 'tag' (1% w/w 4-methyl-7-dimethylcoumarin) to VX and illuminating the exposure site with UV light. In our study (based on image analysis of skin surface autoradiographs), each milligram of VX spread over an average area of 1.2 ± 0.5 cm^2. Clearly, such species differences merit further study in order to further characterize the pig as an *in vivo* model for human skin absorption.

In this study, it was assumed that unrecovered radioactivity was present in the unsampled tissues, viz., brain, muscle, fat, etc. This assumption was based on the fact that VX has a relatively low volatility, thus vapor loss from the dosing chamber or via expired air would be minimal. On average, $<0.005\%$ of the applied dose was detected in the gauze dressing of each exposure chamber, indicating that vapor loss from the exposure site was negligible. In addition, random swabs taken from the endotracheal tube contained only background quantities of ^{14}C-radioactivity. As a further precaution, the radiometric analysis was performed on skin that had been excised with a large (>2 cm) margin around the radioactive area (identified on the autoradiographs) to ensure that all the available radioactivity within the skin sample was recovered. Therefore, the unrecovered fraction of radioactivity can be deemed to be representative of ^{14}C-VX (or its radiolabelled metabolites) within unsampled body compartments. The relative quantities of radioactivity recovered from the liver, kidney and spleen were similar to those measured in mice exposed to ^{14}C-radiolabelled soman,[13] indicating that both anticholinesterase agents have a similar distribution in the two species. The substantial recovery of radioactivity from each exposure site may imply the presence of a VX skin reservoir. This is in agreement with a previous human volunteer study that inferred the presence of a 'dermal reservoir' from the observation that systemic absorption of VX continued for at least nine hours after the skin exposure site had been decontaminated.[12] If such a reservoir exists, it could be a target for decontaminants and 'catch-up' therapies; chemicals that can be delivered into the skin to preferentially neutralize the agent. Further work should be conducted to investigate this apparent reservoir in order to confirm the presence, intra-dermal location and availability of 'free' (unbound) VX. The build-up of a reservoir implies that diffusion of VX from the skin to the circulation is the rate limiting step for systemic absorption.

In conclusion, it is important that the fundamental differences in permeability and skin surface spreading characteristics between the two species should be considered when extrapolating the results of pig studies to man. Nonetheless, the pig should be considered the model of choice for future studies to investigate the effectiveness of topical pretreatments or therapies against percutaneous exposure to VX.

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