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13. Abstract

The objective of the project was to obtain knowledge on the biological and chemical fate and residual hazard of chemical warfare agents (CWA's) on contaminated human remains and personal effects. The study was anticipated to provide input for the development of a model to predict the residual hazard in time after death, as well as new decontamination technology and protocols for the safe handling of contaminated human remains and personal effects. Experiments have been conducted in which hairless guinea pigs have been contaminated with various agents (e.g., VX, sulfur mustard), through various routes of exposure. Subsequently, the fate of the agent after death of the animal has been followed by measuring levels of the intact agent and (potentially) toxic metabolites, both within the animal as well as on the skin. In addition, the fate of a number of agents on various personal effects has been determined. Finally, the effectiveness of skin decontamination has been determined for hairless guinea pigs which had been contaminated with various chemical and biological agents, including *B. anthracis* spores. Overviewing all results obtained in this study it can be concluded that scenarios can be envisaged in which vapor and or contact hazards are posed by human remains contaminated by VX or sulfur mustard. Decontamination of the remain shortly after death will reduce both the vapor and contact hazards. Chemically contaminated personal assets such as uniforms and boots also pose a considerable hazard, due to the high persistence of CWA on and in these materials, in particular the rubber and suede of the boots. For remains contaminated with *B. anthracis* a similar level of personal protection is required. Autopsy on such remains is preferably performed in a biosafety environment. Further research in this field could be aimed at similar 'postmortem' studies for NTA's in various physical states, with the emphasis on agent persistence, distribution and possibilities for decontamination. In addition, the biological fate of agents that cause contagious diseases under postmortem conditions and possibilities for decontamination, should be addressed.

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FOREWORD

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The objective of this study was to obtain knowledge - by literature search, experimental data and modeling - on the biological and chemical fate and residual hazard of chemical warfare agents (CWA's) on contaminated human remains and personal effects. The study was anticipated to provide input for the development of a model to predict the residual hazard in time after death, as well as new decontamination technology and protocols for the safe handling of contaminated human remains and personal effects.
So far, research efforts have mainly focused on the protection against CWA's and therapy to increase the survivability of victims after a chemical incident. However, after a severe intoxication, chances are profound that victims will not survive. Intoxicated, deceased victims will need to undergo recovery, decontamination, possibly autopsy, transportation into the home land, and finally interment. Several guidelines are available that describe these procedures, with a focus on the safety of personnel (Joint Publication 4-06; NATO STANAG 2070; London Mass Fatality Plan; Hanzlick *et al.*, 2006; Foust, 1997). During the handling of contaminated human remains and personal effects, the agent that is still present on (or inside) the contaminated body may pose a hazard to unprotected personnel. Therefore, the presence of (residual) agent is continuously monitored by detection equipment.
Limited attention has been paid to the scientific background of this secondary hazard. Agents may remain present on the skin or in body fluids, thus presenting both a contact and a vapor hazard. Agents may be present for a longer time in human remains compared to living human beings, due to postmortem changes of physiological processes that influence the biological and chemical fate of the agents.

Biological fate in healthy human beings

The distribution and metabolism of CWA's in living organisms have been studied extensively. After exposure, agents will be absorbed and distributed, depending on the route of administration. Some useful data was obtained from experiments on human volunteers in the 1950s, as well as from extensive toxicokinetic studies in laboratory animals (Van der Schans *et al.*, 2003 and 2008).
The biological fate of nerve agents is relatively simple (Noort *et al.*, 2002; Noort and Black, 2005; Black and Noort, 2005, 2007). The predominant process consists of hydrolysis (either spontaneous or enzymatic) to relatively non-toxic phosphonic acids that are readily excreted in urine. In addition to binding to its physiological target acetylcholinesterase, scavenging by butyrylcholinesterase (BuChE), carboxylesterase (CaE), and even albumin has been well documented (Williams *et al.*, 2007). The metabolism of sulfur mustard is complex, *inter alia* because of the agent's bifunctional alkylating character, resulting in a wide range of mixed hydrolysis/oxidation products and glutathione-derived conjugates in various oxidation stages. Also,

extensive binding to proteins occurs (Noort *et al.*, 2002, 2008; Noort and Black, 2005; Black and Noort, 2005, 2007).
Enzymatic activities and hydrolysis rates of CWA's in plasma and tissue homogenates have been well documented. These data were used to develop a PBPK model to predict the toxicokinetics of nerve agents in intoxicated (living) human beings (Langenberg *et al,* 1997; Sweeney *et al*., 2006).

Postmortem biological and chemical fate

After death, physiological processes start to change rapidly, many of which will have an effect on the biological and chemical fate of CWA's. When a victim dies slowly, some changes may start even before death (such as a reduced blood circulation and a changed percutaneous uptake). It is anticipated that CWA's will remain present for a longer period of time in human remains compared to healthy human beings, because of the slowed down or terminated processes of natural detoxification. On the other hand, certain biochemical mechanisms may be activated which may enhance destruction of CWA's.

The following postmortem physiological changes are important in this respect:

- The transportation of agent through the body by the blood circulation stops immediately after death. As a result, metabolism of the agent by the liver ceases.
- The body temperature gradually cools from 37 °C to ambient temperature (*algor mortis*).
- Postmortem redistribution of the agent may occur through the body.
- Gradual muscular stiffening (*rigor mortis*) will occur after about six hours after death, and is caused by a physico-chemical change in muscle protein.
- Postmortem decomposition of the soft tissues (putrefaction) by enzymes and bacteria starts between 36 and 72 hours after death. Putrefaction starts in the anterior abdominal wall and results in the gradual dissolution of all tissues into gasses, liquids and salts.
- Saponification is the hydrolysis of fatty tissues with the release of free fatty acids. This process starts within weeks after death and is therefore not considered relevant for this study.

The following mechanisms (and kinetics) of reaction between CWA's and physiologically active materials found in human remains are anticipated:

- *Postmortem Redistribution* Immediately after death, blood circulation is terminated. Still, agent concentrations in body fluids and organs may change, similar to drugs, due to postmortem redistribution (Pélissier-Alicot *et al.*, 2003). Agents may be redistributed from 'reservoir' organs such as the gastrointestinal tract, liver, and lungs to surrounding tissues. This may occur immediately after death by diffusion through blood vessels and transparietal diffusion towards the surrounding organs.

- *Postmortem Scavenging* As mentioned above, natural detoxification of nerve agents occurs by binding to scavenging enzymes. The total amount of available enzyme (BuChE and CaE) is normally limited to about 60 nM in human blood. Victims that suffered from a lethal dose of nerve agent will have enzyme activity levels that approach zero and detoxification by scavenging to these enzymes is no longer possible. Some enzyme activity may still be present, if there has been an alternative cause of death (e.g. the blast of an explosion) in addition to a non-lethal intoxication. Although enzyme activity will decrease in time due to the decrease in temperature, it has been shown that cholinesterase activity levels in postmortem blood of non-intoxicated patients were almost the same as those in serum samples from healthy controls (Klette *et al.*, 1993).

  In case of nerve agents, it is estimated that postmortem scavenging will play a limited role in their detoxification, when it is assumed that the received dosage will be far higher than the amount of available binding sites. Nonetheless, binding to sites with less affinity (e.g. the tyrosine-411 residue in albumin) might occur under these conditions. In case of sulfur mustard, alkylation of proteins (e.g. hemoglobin and albumin) and DNA will contribute to the post-mortem detoxification of this agent.

- *Postmortem Metabolism* The natural metabolism of CWA's is expected to continue postmortem, but at a much lower rate due to the decreasing body temperature and due to the fact that metabolism in the liver will stop. It has been shown that decomposition of organophosphate pesticides continues postmortem (Kupfermann *et al.*, 2004). Nerve agents will be detoxified by spontaneous and enzymatic hydrolysis, whereas sulfur mustard will undergo hydrolysis and oxidation. In addition, alternative, yet unknown degradation pathways may arise, e.g. by bacteria during putrefaction. In general, metabolism leads to agent detoxification, but it can not be excluded that under postmortem conditions the metabolism is incomplete or slightly altered, and consequently might give rise to the formation of toxic reaction products, such as desethyl-VX (EA2192) in the case of VX, and mustard sulfone in case of sulfur mustard. Many factors play a role during postmortem agent degradation. The process is complex and is expected to be slow, especially for the more persistent agents. This was shown, for example, by the reported presence of unmetabolized sulfur mustard in postmortem tissue samples from Iranian victims (Vycudilic, 1985; Drasch *et al.*, 1987). We estimate that complete postmortem metabolism of a high dosage of CWA may take weeks.

Hazard of contaminated human remains

The presence of toxic agents on or inside the body of deceased victims, as well as on their clothes, poses a threat to (unprotected) personnel handling the remains. Several (civil) cases are known from history, in which handling contaminated victims lead to intoxication of rescuers.

In 1943, German bombers attacked ships in the harbor of Bari, including one which was carrying sulfur mustard. Rescuers were unaware that they were dealing with sulfur mustard casualties, and many additional victims were caused among the rescuers by contact with the contaminated skin and clothing of victims.

Secondary exposure of ambulance personnel and medical personnel also took place after the terrorist attack in the Tokyo subway with sarin gas in 1995. Secondary exposure took place because victims were not decontaminated before transportation to hospitals, personnel did not wear adequate protection and ambulances were not ventilated. Thus, 135 ambulance workers (Okumura *et al.*, 1998a) and 110 members of the medical staff (Okumura *et al.*, 1998b) became intoxicated. It has been assumed that the secondary exposure resulted from off-gassing of sarin from the clothing of victims (Okumura *et al.*, 1998a; Tucker, 2006). No information was found on the off-gassing from the bodies of (deceased) victims.

The 1994 sarin attack in Matsumoto also lead to the contamination of rescuers, but this was probably due to direct contamination on-site, rather than secondary effects. The severity of intoxication was directly related to the time after the incident at which the rescuers arrived (Nakajima, 1997).

It has been estimated that 80 % of the contamination will be present on the clothing of victims after a chemical incident. Clothes will be removed from the body at some point, collected with other personal effects, stored and/or destroyed. These contaminated items will present both a contact and vapor hazard. Especially porous materials, such as rubber, are known to absorb CWA's, leading to a prolonged hazard. Obviously, the hazard of contaminated clothing is also relevant in case of surviving victims.

The secondary hazard from (decontaminated) human remains may seem less important than the hazard from contaminated clothing. However, after removal of clothing and decontamination of skin and hair, the hazard may not be completely removed as deceased victims may continue to emit fluids or gases after decontamination. A vapor concentration may build up when a body has been temporarily stored in a body bag or a (refrigerated) coffin at a morgue. After opening the coffin at room temperature, e.g. for autopsy examination, the vapor may be released and pose an unexpected hazard.

An essential tool for the determination of the actual hazard of contaminated human remains and personal effects, is the use of chemical agent monitors. This handheld detection equipment responds to the presence of vaporized agents, and is required for ongoing detection, identification and monitoring of human remains in order to detect the presence of hazards (Joint Publication 4-06).

The expected hazard will depend on the type of CWA. For sulfur mustard, the contact and evaporation hazard are equally important. VX is known to be a slowly evaporating CWA. It was recently confirmed that the agent poses a contact hazard, rather than an evaporation hazard (Boone *et al.*, 2008). On the other hand, sarin is known to evaporate rapidly. The agent poses an evaporation hazard, but only for a short period of time since the agent is rapidly fully evaporated. It is anticipated that the hazard of sarin will be more significant on contaminated clothing materials than on human remains.

Similar problems may occur with respect to deceased persons externally and/or internally contaminated with *Bacillus anthracis*. Performing an autopsy on such a victim is risky for the persons who perform the autopsy, and can only be done when using adequate personal protective equipment. *B. anthracis* is a sporeforming bacterium, and can survive for decades as a spore. ln the literature, accounts can be found that people became sick by digging at a site where 70 years earlier an animal was buried that had died of Anthrax. The data available from such literature accounts are, however, mainly qualitative. Numbers on the degree of transmission of *B. anthracis* from external and internal infected dead animals are not available. Furthermore, it is unknown to what extent decontamination with hypochlorite solution or other decontaminants will reduce such a transmission. These are the questions that have been addressed in this study, using laboratory animals.

Due to the current lack of scientific knowledge on the subject, certain choices had to be made concerning the experimental set-up in this study. The here presented work will be a first step to fill this knowledge gap.

Within the current project, the biological and chemical fate of CWA's in human remains will be studied by literature search and animal experiments on hairless guinea pigs. The biological and chemical fate of CWA's (concentration and metabolism) will be determined in intoxicated and euthanized animals in time after death. Obtained experimental data will be used in a Physiologically-Based PharmacoKinetic (PBPK) model to predict the kinetics and detoxification of CWA in human remains in time after death. The residual vapor and contact hazard will be measured from intoxicated, deceased animals, as well as from contaminated clothing material and rubber boots. It will be attempted to extrapolate the obtained experimental data towards the situation of contaminated human remains and personal effects. Finally, the results will be integrated and evaluated against available toxicological data. It will be shown whether secondary exposure may lead to negligible or significant toxicological effects. Advice shall be provided concerning future research and the way ahead.

The following tasks have been defined:

Task 1: Literature study on postmortem processes that might occur with highly reactive chemicals, such as chemical warfare agents.

Task 2: Animal study – contact hazard resulting from liquid VX on skin

Task 3: Animal study – contact and vapor hazard resulting from liquid sulfur mustard on skin

Task 4: Animal study – contact and vapor hazard resulting from sulfur mustard vapor exposure

Task 5: Animal study at low temperature – contact and vapor hazard resulting from liquid sulfur mustard on skin

Task 6: Physiologically-based pharmacokinetic (PBPK) modeling of VX in the deceased hairless guinea pig.

Task 7: Contact and vapor hazard resulting from clothing contaminated with sulfur mustard or sarin

Task 8: Extrapolation of the measured hazards towards human remains and personal effects

Task 9: Integration: toxicological evaluation and way ahead

Task 10: Effect of decontamination on the residual contact hazard of liquid VX

Task 11: See part II of this report (classified)

Task 12: Pilot on feasibility of determination of contact hazard through the use of pig ear skin

Task 13: Hazards from remains externally or internally contaminated with *Bacillus anthracis*, effect of decontamination

II LIST OF ABBREVIATIONS

AChE	AcetylCholinesterase
B. anthracis	*Bacillus anthracis*
B.a.	*Bacillus anthracis*
BuChE	ButyrylCholinesterase
BSL-3	Bio Safety Level-3
CaE	Carboxylesterase
CAM	Chemical Agent Monitor
CD_3-VX	VX, deuterated at the P-methyl
CFU	Colony Forming Units
ChE	Cholinesterase
CWA	chemical warfare agent
d7-GB	Sarin with a deuterated isopropyl function
d8-HD	deuterated sulfur mustard
DEMP	Diethyl methylphosphonate
DES	Diethyl succinate
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid
ECt_{50}	Concentration that causes an effect in 50% of the group studied
EMPA	Ethyl methylphosphonic acid
ES	Electro Spray
FRET	Fluorescence Resonance Energy Transfer
GB	Sarin
GC	Gas Chromatography
HD	sulfur mustard
i.v.	intravenous
ICAM	Improved Chemical Agent Monitor
IPA	isopropyl alcohol
LC	Liquid Chromatography
LCt_{50}	Concentration that causes lethality in 50% of the group studied
MS	Mass Spectrometry
MS/MS	Tandem MS
NFI	Netherlands Forensic Institute
NPD	Nitrogen Phosphorus Detector
NTA's	Non Traditional Agents
OP	organophosphate
p.a.	pro analyse
p.c.	percutaneous
PBPK	Physiologically-Based PharmacoKinetic
PES solid	Polyether sulfone solid

pm	post mortem
Q-TOF	Quantum-Time of Flight
RF	Relative fluorescence
RSDL	Reactive skin decontamination lotion
SIM-EI	Selected ion monitoring, electron impact

III. EXPERIMENTAL PROCEDURES

Materials

Sulfur mustard, d_8-sulfur mustard, VX, CD_3-VX and (S-[2-(diisopropylamino)ethyl]-O-n-propyl methylphosphonothioate (the O-n-propyl analogue of VX; V33) were used from stocks within our laboratory. Solvents (p.a. purity or higher) were used from various commercial sources.

Instrumentation/devices

LC/electrospray tandem mass spectrometric analyses were conducted on a Q-TOF hybrid instrument equipped with a standard Z-spray electrospray interface (Micromass, Altrincham, UK) and an Alliance, type 2690 liquid chromatograph (Waters, Milford, MA, USA). The chromatographic hardware consisted of a pre-column splitter (type Acurate; LC Packings, Amsterdam, The Netherlands), a sixport valve (Valco, Schenkon, Switzerland) with a 10 or 50 µL injection loop mounted and a PepMap C_{18} (LC Packings) or Vydac C18 column (both 15 cm x 300 µm I.D., 3 µm particles). A gradient of eluents A (H_2O with 0.2% (v/v) formic acid) and B (acetonitrile with 0.2% (v/v) formic acid) was used to achieve separation. The flow delivered by the liquid chromatograph was split pre-column to allow a flow of approximately 6 µl/min through the column and into the electrospray MS interface. MS/MS product ion spectra were recorded using a cone voltage between 25 and 40 V and a collision energy between 30 and 35 eV, with argon as the collision gas (at an indicated pressure of 10^{-4} mBar).

GC-MS analyses were conducted using a HP6890 gas chromatograph with a 5973N MSD (Agilent). For specific columns used, applied conditions etc, see the various sections.

Protocols for Animal Ethics Committee

The various protocols for the animal experiments were drafted and subsequently submitted to the Animal Ethics Committee within TNO. After modification (if required) and approval, they were subsequently submitted to ACURO (proposal numbers CB-2010-130.01, CB-2010-130.02 and CB-2010-130.03). After approval, the animal work was initiated. Any modification of the protocol was reported to ACURO for approval.

Task 2: Contact hazard of VX

Pilot experiments for exploring tape stripping procedure

Contamination

Pieces of pig ear skin or glass plates (both approximately 1 x 1 cm) were contaminated with VX in IPA solution. The following range of VX concentrations in IPA was used: 10 mg/mL, 1 mg/mL, 100 µg/mL, 10 µg/mL and 1 µg/mL. From each solution, 10 µL was applied to the pig ear skin or a glass plate. The samples were dried at room temperature during 5 minutes. The experiments were carried out in duplicate or triplicate (glass plate and pig ear, respectively) for each concentration.

Tape stripping

Tape stripping was performed directly after a drying time of 5 min subsequent to the contamination with VX. The tape stripping procedure is rather straightforward:
A piece of adhesive tape (FixoMull from Beiersdorf AG, Germany), cut to the desired size (about 12x12mm) is applied onto the surface and firmly pressed. After 2 min, the tape was carefully removed with a clean forceps under a 45 ° angle. This procedure was repeated once.

Extraction of VX

Each tape sample was transferred to a glass vial of 4 mL with screw cap. To each vial n-heptane/acetone (9/1, 1 ml) and 10 µl of an internal standard solution (S-[2-(diisopropylamino)ethyl]-O-n-propyl methylphosphonothioate (V33) in IPA) was added. The concentration of the internal standard solution depended on the applied amount of VX. All samples were extracted by gently shaking of the vials during 2 hours.

Contamination of animals with agent (VX or sulfur mustard)

Adult male hairless guinea pigs (Crl: IAF(HA)-hrBR); 500 and 600 g; Laboratories Wilmington, MA, USA) were used. The animals were anesthetized with fentanyl/fluanisone (hypnorm 1ml/kg) and midazolam (5 mg/kg) in water (1:1:2) by i.p. injection. The animals were placed on their back at a special homemade fixation holder. When fully under sedation, control blood samples were taken from the ear of the animal. The animal in the fixation holder was placed under a homemade laser pointer device, pointing out the 8 spots that are to be contaminated with liquid agent on the skin of the belly of the animal. The same device also points out 4 additional spots which were used as a reference point. The latter 4 spots were marked with a marker pen in order to assure that the animal will always stay in the correct position when collecting samples. Neat agent (VX or sulfur mustard) was applied at the belly of the animal at each of the 8 spots. The animal was placed in a glass chamber with lid (150x200x350 mm) and 30 min after contamination the animal was euthanized with a mixture of oxygen and carbon dioxide which flowed through the chamber (20 l/min) for 3 minutes.

At time t=0, 2.5, 5, 23, 29, 48, 72 and 96 hours, skin tape stripping was carried out and blood-, liver- and lung samples were taken. Each time a skin sample was taken the animal was moved out of the glass chamber and placed under the laser pointer device.
Blood samples were taken from the heart as long as possible, depending on the degree of coagulation. The first blood- liver- and lung samples were taken by a small incision in the thorax and a small part of the belly, away from the contaminated spots. After sampling, the incision was firmly closed by 9 mm auto clips and the animal was moved back into the glass chamber. Work-up of the blood-. liver-, and lung samples was carried out as described in the procedures. At t = 96 hours, skin samples were taken from the 8 contamination points (+ 2 control skin samples) with a 8 mm disposable punch and stored in the freezer at –70 °C.

Tape stripping for determination contact hazard by VX; extraction of tapes

Prior to starting the tape stripping procedure, 3 strips of tape (type Fixomull transparent tape) with a size of 12x12 mm where precut. On specified time points a single piece of tape was applied to a specific spot on the skin of the animal. The tape was firmly pressed to the skin with a cotton applicator and the top layer was removed with a pair of tweezers. After 2 min the tape was removed with a sharp tipped pair of tweezers and placed in a 4 mL glass vial. Immediately after removing the first piece of tape from the skin, a second piece of tape was applied on the same spot and manipulated as described above. Internal standard (V33; 10-100 µg/mL in n-heptane/acetone, 9/1, v/v; concentration depending on the expected VX concentration in the sample), and 2 mL of extraction solvent (n-heptane/acetone, ratio 9/1) were added to the vial. The amount of internal standard used was adapted to the expected concentration of VX. The vials were placed in a rotation mixer for 2 hours to promote extraction under gentle mixing. 1 mL of extract was placed in a 1.5 mL vial for GC/MS analysis.

GC-MS analysis of VX in skin tape extracts

Extracts of tapes (in 1.5 mL GC vials) were analyzed for the presence of VX by GC-MS, using an Agilent GC-MS system equipped with a Combipal auto sampler (CTC, Zwingen, Switzeland) and a JAS PTV injector (JAS, Eindhoven, The Netherlands). Injection volume: 1 µL. The injector was set to splitless mode and the temperature was 275 ºC. To the injector, a VF5ms (30 m x 0.32 mm id. x 1.0 µm df) column (Varian, Middelburg, The Netherlands) was connected. The column was placed in an Agilent 6890n GC (Agilent technologies, Santa Clara CA, USA) and the flow was 2 mL/min (helium). Temperature program: 45 to 325 ºC (15 ºC/min). For detection, the 5973n MSD (Agilent technologies, Santa Clara CA, USA) was operated in the most sensitive SIM EI mode (m/z 114). The concentration of VX was calculated from the area ratio between VX and V33 (internal standard).

Sample preparation for LC-MS analysis of VX in blood samples

To an aliquot of blood (100 µl) in an Eppendorf tube was added internal standard (CD_3-VX; 10 µL of a 0.2 – 20 µg/mL solution in isopropanol) and water (890 µl). The sample was mixed on a Vortex and put on ice for 15 minutes. Subsequently, a part of the solution (500 µl) was put on a 3 kD molecular weight cut-off filter (Millipore/Amicon Ultra, 0.5 mL 3 K), followed by centrifugation at 3000 rpm in an Eppendorf centrifuge, at 4 °C. The filtrates were stored at – 30 °C and analyzed by means of LC tandem MS.

Sample preparation for LC-MS analysis of VX in liver samples

After euthanasia an incision in the thorax was made and at defined time points a aliquot of liver tissue (at least 0.35 g) was collected with a biopsy punch (8 mm). The liver sample was transferred to a glass tube (on ice) and weighed. A solution of internal standard (CD_3-VX; 10 µL of a 0.2-20 µg/mL solution in isopropanol) was added and the sample was diluted with water (liver/water, 1/3, v/v). After mixing with a Vortex the sample was put on ice. Subsequently, the sample was homogenized with a Polytron (PT100). The homogenate was centrifuged at 3000 rpm in an Eppendorf centrifuge at 4 C. An aliquot of the supernatant (500 µL) was transferred to a 3 kD molecular weight cut-off filter. Further sample preparation as described above for the blood samples.

LC-MS analysis of VX in processed blood and liver samples

LC conditions:

Eluent A: H_2O (0.2 % formic acid). Eluent B: CH_3CN (0.2 % formic acid). A gradient was applied from 100 % A to 70% B in 45 minutes.

Flow: approx. 40 µL/min.

Column: PepMap C_{18}; 3 µm; 150 x 1 mm. Loop volume: 10 µL.

MS conditions:

Positive ion electrospray mass spectra were recorded and a collision energy of 18 eV. Product ion spectra (MS/MS) of protonated molecular ions MH^+ 268 (VX) and MH^+ 271 (CD_3-VX; internal standard) were recorded at a cone voltage of 20 V and a collision energy of 18 eV. The argon gas pressure was approximately 10^{-4} mBar.

Cholinesterase determination

For determination of cholinesterase activity, an aliquot of blood (20 µL) was mixed with 1% saponine (180 µL) on a Vortex and stored at -70 °C. Total cholinesterase was determined with the Ellman method, by using acetylthiocholine and DTNB.

Task 3: Contact and vapor hazard of sulfur mustard

Tape stripping for determination contact hazard by sulfur mustard; extraction of tapes

Tape stripping was performed in a similar way as described for VX, with the exception that d_8-sulfur mustard was used as internal standard and that ethyl acetate was used for extraction.

GC-MS analysis of sulfur mustard in tape extracts

Extracts of tapes (in 1.5 mL GC vials) were analyzed for the presence of sulfur mustard by GC-MS, using an Agilent GC-MS system equipped with a Combipal auto sampler (CTC, Zwingen, Switzeland) and a JAS PTV injector (JAS, Eindhoven, The Netherlands). Injection volume: 1 µL. The injector was set to splitless mode and the temperature was 250 ºC. To the injector, a VF5ms (30m x 0.32 mm id. x 1.0 µm df) column (Varian, Middelburg, The Netherlands) was connected. The column was placed in an Agilent 6890n GC (Agilent technologies, Santa Clara CA, USA) and the flow was 2 mL/min (helium). Applied temperature program: 50 to 225 ºC (15 ºC/min). For detection, a 5973n MSD (Agilent technologies, Santa Clara CA, USA) was operated in the most sensitive SIM EI mode (m/*z* 109, 115, 158, 166). The concentration of sulfur mustard was calculated from the ratio of sulfur mustard and d_8-sulfur mustard (internal standard) peak areas.

Sample preparation for GC-MS analysis of sulfur mustard in blood samples

In view of the expected low levels of sulfur mustard in the samples and the fact that sulfur mustard tends to stick to plastic materials, glass tubes were used. To the collected blood sample (100 µL), d_8-sulfur mustard was added (10 µL of a solution of 2 – 2000 µg/mL in ethyl acetate; concentration depending on expected concentration of sulfur mustard in sample) together with 1 mL of ethyl acetate. The sample was mixed with a Vortex for 10 seconds. To promote further extraction, the tubes were placed in an ultrasonic bath for 10 minutes. The blood and ethyl acetate layers were separated by centrifugation. The ethyl acetate layer was collected in a 1.5 mL vial with an insert for small sample volumes. The entire procedure was repeated once. The two extracts were pooled and analyzed with GC/MS.

Sample preparation for GC/MS analysis of sulfur mustard in liver samples

To a piece of liver (approximately 0.5 g) in a 10 mL glass tube on ice, 10 µL of internal standard (d_8-sulfur mustard; 2 µg/mL in ethyl acetate) was added. Water was added in a ratio of 3/1 (v/v, water/liver). The sample was mixed with a Vortex and stored on ice. Subsequently, the sample was homogenized with a polytron (PT100). The homogenate was transferred to a 2 mL test tube and centrifuged for 3 minutes at 4000 rpm. An aliquot (500 µL) of the top layer was transferred to a glass reaction tube for extraction with ethyl acetate (1 mL). After mixing with a Vortex for 10 s the tube was placed in an ultrasonic bath for 10 min to allow maximum extraction. Each sample was centrifuged and the organic (top) layer was collected in a 1.5 mL vial for analysis with GC/MS.

GC-MS analysis of sulfur mustard in processed blood and liver samples

A set of glass tubes (Gerstel, Müllheim ad Ruhr, Germany) filled with Tenax^{TM} TA (a specific type of absorbent material) where preconditioned by rinsing the tubes with methanol and ethyl acetate. After chemical cleaning, the tubes where placed in an oven (225 ºC) under helium atmosphere for a bake-out procedure (overnight). An aliquot (100 µL) of blood or liver extract (see above) was loaded on a tube with a 100 µL syringe. Solvent was evaporated by flushing the tubes with nitrogen (225 mL/min) for 10 minutes. A second portion of 100 µL of the same extract was loaded on the same tube. Again solvent was evaporated with nitrogen (g) for 15 minutes to remove any remaining solvent. Tubes were placed in a TDS autosampler (Gerstel, Müllheim ad Ruhr, Germany) at room temperature.
Desorption of the Tenax^{TM} tubes was performed by heating the TDSa autosampler to 200 ºC (60 ºC*min^{-1}). All components were transferred to a cryogenically cooled CIS (cooled injection system) injector (-75 ºC) with a split flow of 30 mL/min and a column flow of 1.5 mL/min. After desorption was complete, the injector was heated to 260 ºC (12ºC/sec) and components were transferred to the gas chromatograph 6890n (Agilent tech., Santa Clara CA, USA). The GC was equipped with a VF5ms (30 m x 0.32 mm; id. X 1.0 µm df) column (Varian, Middelburg, The Netherlands). Temperature program: 40 to 300 ºC (10 ºC/min). For detection, a 5973n MSD (Agilent technologies, Santa Clara CA, USA) was operated in the most sensitive SIM EI mode (m/*z* 109, 115, 158, 166). The concentration of sulfur mustard was calculated from the ratio between sulfur mustard and d_8-sulfur mustard (internal standard) peak areas.

Determination of vapor hazard

In this task, the evaporation hazard is studied after contamination of animals by liquid sulfur mustard on skin. Animal experiments are conducted on adult, anesthetized hairless guinea pigs, that were contaminated with a sublethal dose of liquid sulfur mustard on the skin applied on multiple spots.

The used dose is 8 droplets of 1 µL (< 1 LD_{50}). The contaminated animals were placed in a glass chamber, in which a controlled air flow of 50 ml/min is sweeping over the animal. Animals were euthanized 30 min after contamination. The outlet air was lead through two bubble cell in series containing 1 mL solvent (diethyl succinate) each, in order to sample the agent in intervals. The experimental set-up is given in Figure 1.

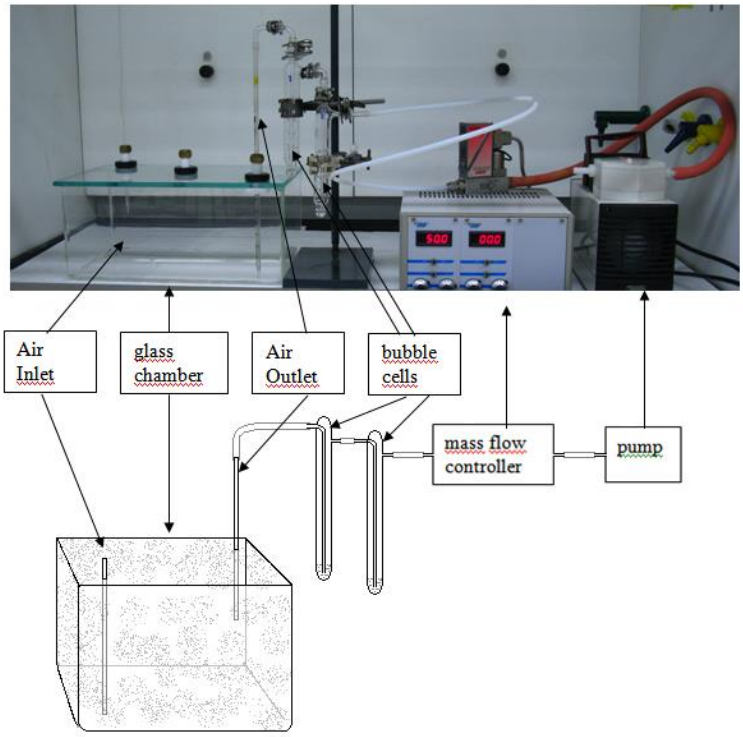

Figure 1 Experimental set-up for determination of off-gassing of contaminated animal.

The bubble cells were replaced 1, 2, 3, 4, 6, 24, 48, 72 and 96 hours after euthanization of the animal. A known amount of deuterated sulfur mustard was added to the diethyl succinate as an internal standard. The solvents were quantitatively analysed by gas chromatography mass spectrometry (GC-MS). The GC-MS used was a HP6890 gas chromatograph with 5973N MSD (Agilent). The gas chromatograph was equipped with a Factorfour VF-5MS column (Varian, 30 m x 0.32 mm ID, film thickness 1 µm). Helium was used as carrier gas with a flow-rate of 1.5 ml/min. The sample volume was 1 µL and was injected at 250 ºC with a split ratio 25:1. The temperature program used was 100 °C (1 min), 10 °C/min to 260 °C (5 min). The mass spectrometer was used in SIM-mode (m/z 158 for the trapped sulfur mustard and m/z 166 for the internal standard deuterated sulfur mustard).

In addition, the response of a CAM (chemical agent monitor; used by the Dutch Armed Forces) and later an ICAM (provided by US Army) was used to measure the released vapor above the animal. For the measurements the (I)CAM was operated in the H-mode (mustardgas-mode). The nozzle of the (I)CAM was pressed against the air outlet of the glass chamber with the deceased and contaminated animal.

Task 4: Contact and vapor hazard of sulfur mustard after vapor exposure

Whole body exposure, followed by determination of contact risk

The anesthetized animal was exposed to sulfur mustard vapor within the equipment depicted in Figures 2 and 3.

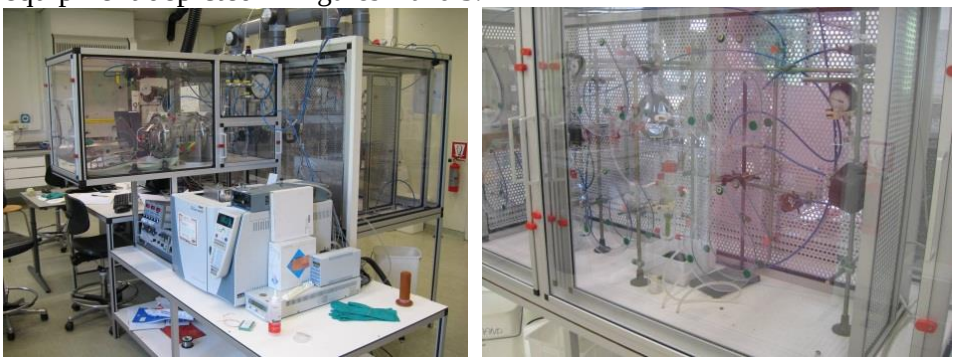

Figure 2 Vapor exposure equipment for whole body exposure of guinea pigs to highly toxic chemicals. The left picture shows the entire set-up, with the on-line GC in the front, the exposure chamber on the left and the vapor generation unit on the right (in the back). The right picture shows the vapor generation unit.

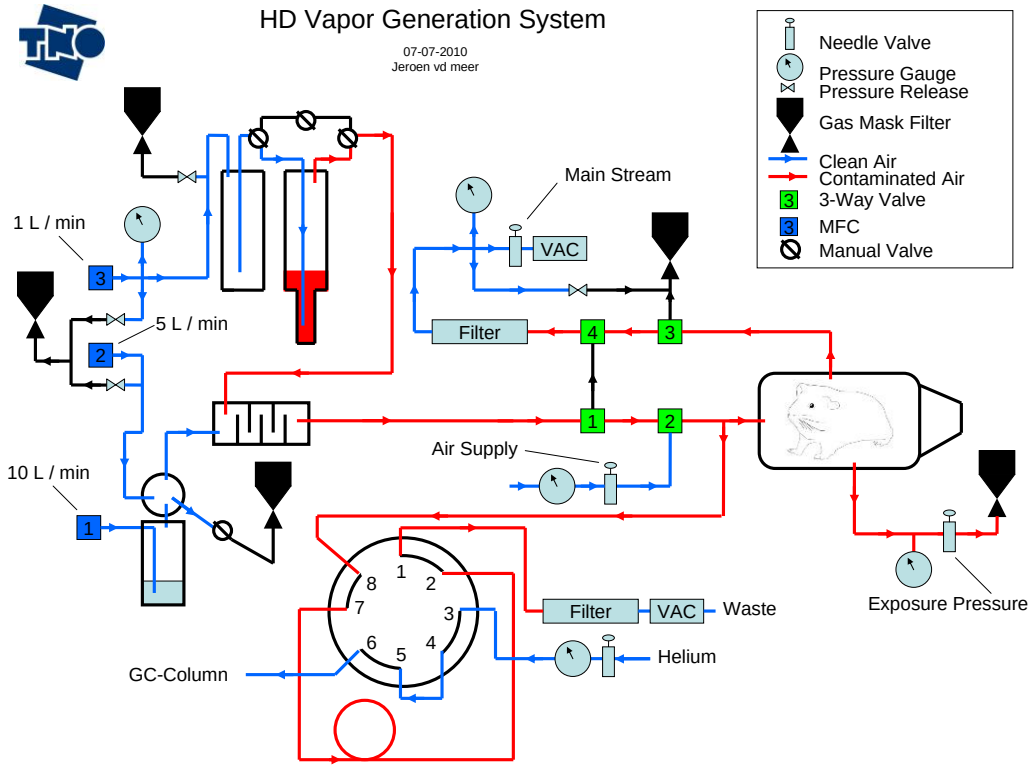

Figure 3 Schematic representation of vapor exposure equipment for whole body exposure of guinea pigs to highly toxic chemicals.

After the exposure (10.000 mg.min.m^{-3}, i.e., 250 mg.m^{-3} during 40 minutes, equivalent with 1 LCt_{50} (percutaneous) or 12 LCt_{50} (inhalation)), the animal was transferred to a glass chamber. The animal was left for 30 minutes and subsequently euthanized (carbogen for 1 min, followed by CO_2 for 3-4 minutes). Tape stripping was applied for determination of contact risk, or the animal was transferred to a glass chamber for determination of the evaporation hazard, in a similar way as described for Task 3. Further analyses (sulfur mustard in blood, liver etc) was carried out as described for Task 3.

Task 5: Animal Study at Low Temperature - Liquid Sulfur Mustard on Skin

For these experiments the same procedures as described above were followed, except that after euthanasia, the animal was kept in a refrigerator at 5-7 °C in a closed glass chamber. For collecting samples at the specified times, the animal was temporarily removed out of the glass chamber and put back in the refrigerator at 5-7 °C when finished. Collection of vapor samples for determination of 'off-gassing' was carried out as described above. For this purpose the refrigerator containing the remains of the animal was equipped with gas in/outlet tubes (see Figure 4 for experimental set-up).

Figure 4 Experimental set-up used for performing experiments at low-temperature; the picture on the right shows the gas in- and outlet tubes, necessary for vapor sampling.

Task 7: Hazard of contaminated clothing

Vapor risk experiments

The evaporation of sulfur mustard and sarin in time was measured using a similar evaporation chamber as described for the animal studies (Task 3) followed by GC analyses of collected vapor. In addition, the response of a chemical agent monitor, - ICAM - provided by the US Army, to the released vapor above the animal was assessed. Small disks (with a diameter of 12 mm; n=3) of the requisite material were contaminated with the neat agent (1 µL; sarin or sulfur mustard), within a glass container. A flow of air was led over the contaminated disks; the air was led through bubble cells (2, in series) filled with DES. Bubble cells were replaced at specific times (1, 2, 3, 4, 6, 24, 48, 72 and 96 hours). Finally, the collected DES fractions from the bubble cells was analyzed for the presence of intact agent, by using d_8-HD or d7-GB as internal standard.

Contact risk experiments

The contact risk of VX and sulfur mustard was studied by quantification of CWA's on absorbent material (aluminum sheets covered with silica) after contact with contaminated materials under a fixed pressure. One contact time was used (15 minutes). The contact risk was determined in time up to 96 hours after contamination.

Small disks (with a diameter of 12 mm; n=3) of the requisite material were contaminated with the neat agent (1 µL; VX or sulfur mustard), within a glass container. On specific time points (0, 2.5, 5, 23, 48, 72 and 96 hours) three disks were removed from the glass container. Subsequently, these disks were covered with a disk of aluminum foils covered with silica gel (DC-Alufolien, Kieselgel 60 W; Merck) of equal size. A weight of 20 g was placed on top of the silica gel foil for 15 minutes. Subsequently, the silica gel foil was transferred into a vial, ethyl acetate (2 mL) was added, and the sample extracted for 2 hours. The piece of contaminated material (suede leather/rubber sole/trousers) was transferred to a different vial, followed by extraction with ethyl acetate (2 mL) for 2 hours. Finally, extracts were analyzed by means of GC-MS, using d_8-HD or V33 as internal standard.

Task 10: Effect of decontamination on residual chemical hazard of VX

Contamination

The animals were contaminated by applying 8 spots with 1 µL droplets of neat VX, as has been described previously for Task 2. The droplets were not visible anymore after 30 minutes following application, indicating they had been fully absorbed by the skin.

Decontamination

From a commercially available RSDL pouch the lotion was removed by wringing the pouch. Subsequently the pouch was rinsed with water and dried. With a punch small pouches (0.8 mm in diameter) were manufactured from the original RSDL pouch. These small pouches were soaked with RSDL by immersing them in the earlier removed lotion.

Figure 5 Pouches with RSDL (left) and application of RSDL on contaminated spot (right).

Decontamination of the spots contaminated with VX was carried out by rubbing the spot with a RSDL saturated pouch during 30 seconds (see Figure 5). The RSDL lotion is removed with a cotton wool swab 10 minutes after application.

Tape stripping

At the designated sampling time points the spots were stripped four times. After addition of ethyl acetate and a known amount of the internal standard (V33, the O-n-propyl analogue of VX), the strips were extracted for 2 hours on a rotator. Subsequently, the extracts were analyzed with GC-MS for VX and internal standard.

Skin samples

After stripping the last spot the skin of all eight spots contaminated, decontaminated and stripped were removed with a punch (diameter 8 mm). The pieces of skin were separately extracted with ethyl acetate after addition of a known amount of the internal standard V33 for 2 hours on a rotator. The extracts were analyzed with GC-MS for VX and V33.
In the experiments HR031, HR033, HR035 and HR037 decontamination was directly after death (30 minutes after contamination). In the experiments HR032, HR034, HR036 and HR038 decontamination was carried out 24 hours after death. Experiment HR039 was a positive control without decontamination.

Schedule for experiments with direct decontamination with RSDL (animals HR031, HR033, HR035, HR037).

Time (hr)	Action
	Narcosis
-0,5	Contamination 8 spots with 1 µL VX
0	Euthanasia
0	Decontamination of 8 spots, 1 pouch per spot, 30 sec. rubbing
0:10	Removal of RSDL with swab
3	Stripping of spots 1 and 2 (4x)
24	Stripping of spots 3 and 4 (4x)
48	Stripping of spots 5 and 6 (4x)
96	Stripping of spots 7 and 8 (4x)
	Skin sampling of the 8 spots

Schedule for experiments with delayed decontamination with RSDL (animals HR032, HR034, HR036, HR038).

Time (hr)	Action
	Narcosis
-0,5	Contamination 8 spots with 1 µL VX
0	Euthanasia
24	Decontamination of 8 spots, 1 pouch per spot, 30 sec. rubbing
24:10	Removal of RSDL with swab
27	Stripping of spots 1 and 2 (4x)
48	Stripping of spots 3 and 4 (4x)
72	Stripping of spots 5 and 6 (4x)
96	Stripping of spots 7 and 8 (4x)
	Skin sampling of the 8 spots

In experiment HR039 (positive control) the schedule for experiments with direct decon is used, with the exception that the decontamination step is skipped.

For the quantification of VX peak area's, the m/z 114 ion chromatograms of VX and the internal standard (V33) are used.

Task 12: Pilot on feasibility of determination of contact hazard through the use of pig ear skin

Schedule for experiments without decontamination (HR052, HR054, HR056).

Time (hr)	Action
	Narcosis
-0,5	Contamination 8 spots with 1 µL VX
0	Euthanasia
3	Spot 1: sampling with pig ear skin Spot 2: sampling by tape stripping (4x)
24	Spot 3: sampling with pig ear skin Spot 4: sampling by tape-stripping (4x)
48	Spot 5: sampling with pig ear skin Spot 6: sampling by tape stripping (4x)
96	Spot 7: sampling with pig ear skin Spot 8: sampling by tape stripping (4x)
96	Skin sampling of the 8 spots

Schedule for experiments with decontamination (HR053, HR055, HR057).

Time (hr)	Action
	Narcosis
-0,5	Contamination 8 spots with 1 µL VX
0	Euthanasia
0	Decontamination of 8 spots, 1 pouch per spot, 30 sec. rubbing
0:10	Per spot removing of RSDL with swab
3	Spot 1: sampling with pig ear skin Spot 2: sampling by tape stripping (4x)
24	Spot 3: sampling with pig ear skin Spot 4: sampling by tape stripping (4x)
48	Spot 5: sampling with pig ear skin Spot 6: sampling by tape stripping (4x)
96	Spot 7: sampling with pig ear skin Spot 8: sampling by tape stripping (4x)
96	Skin sampling of the 8 spots

The animals were contaminated on 8 spots with 1 µL droplets of VX. The procedure for decontamination is similar to the procedure used in Task 10. From a commercially available RSDL pouch the lotion is removed by wringing the pouch. Hereafter the pouch was rinsed with water and dried. With a punch small pouches (0.8 mm in diameter) were manufactured from the original RSDL pouch. These small pouches were soaked with RSDL by immersing them in the earlier removed lotion. Decontamination of the spots contaminated with VX was carried out by rubbing the spot with a RSDL saturated pouch during 30 seconds. The RSDL lotion is removed with cotton wool swab 10 minutes after application. At the time points of sampling spots were stripped four times. After addition of ethyl acetate and a known amount of the internal standard V33 the strips were separately extracted for 2 hours

on a rotator. The extracts were analysed with GC-MS for VX. Pieces of pig ear skin are placed on (de)contaminated skin sites of the hairless guinea pig under a pressure of 20 $g.cm^{-2}$ for 15 minutes. An impression of the preparation of the pieces of pig ear skin used for sampling is given in Figure 6.

Figure 6 Removal of skin from pig ears and preparation of pieces of approximately 1 x 1 cm.

After preparation the pieces of skin were separately placed in vials and stored in a freezer at -20 °C. About 1 hour before sampling a piece of pig ear skin was defrosted. It is placed on a (de)contaminated spot with on top a piece of non-absorbing paper (Figure 7). On top of the paper, a weight of 20 gram was placed for a period of 15 minutes. The piece of paper is used to avoid contamination of the weight.

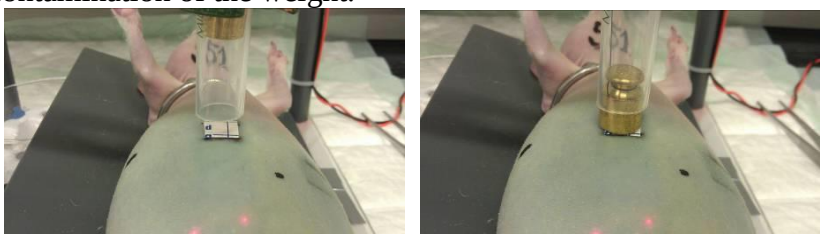

Figure 7 Experimental set-up for determination of contact hazard with pig ear skin.

After the sampling period of 15 minutes the weight is lifted and the pig ear skin is put into a vial. Ethyl acetate and a known amount of the internal standard V33 were added and the pig ear skin was extracted for 2 hours on a rotator. The extracts were analysed with GC-MS for VX.

Skin samples

After sampling of the last spots (at t= 96 hours) the skin of all eight spots of the hairless guinea pig (after the various contamination/decontamination and sampling cycles) were removed with a punch (diameter 8 mm). These pieces of skin were extracted with ethyl acetate after addition of a known amount of the internal standard V33 for 2 hours on a rotator. The extracts were analysed with GC-MS for VX.

Task 13: Hazards from remains contaminated with *Bacillus anthracis*

Application of spores/counting experiments

On the dead guinea pigs at 8 places on the abdominal skin small quantities of *B. anthracis* spores were applied (Delta Sterne strain BM1221, 10 µL of a 5.10^9 suspension and Vollum strain BM 233, 10 µL of a 6.10^9 suspension). The spores had a purity of 99%. At various time points (0, 4, 22, 26, 46, 50, 70 and 92 hours after infection) swab samples were taken to investigate whether the number of spores on the abdominal skin changes over time. After putting the swab sample in culture medium, the bacteria colonies were counted, according to standard protocols.

Intranasal infection with B. anthracis spores

Hairless guinea pigs (n=4) were placed in the isolator in our BSL-3 facility. *B. anthracis* (Vollum strain) spores were administered intranasally (50 µL of a 6.10^4 CFU suspension). After 72 hours the animals were euthanized, which should be sufficient time for the infection to spread within the body, although the animals may not show clear clinical signs. At 0, 3, 6, 22, 29, 48, 72 and 96 hours after euthanasia swabs were taken from premarked spots from the belly. Also samples were taken from blood, liver and lung. Presence of *B. anthracis* was assessed by using colony counting and by using the FRET-technology.

Fluorescence Resonance Energy Transfer (FRET) assay

The FRET assay was performed after preculturing the collected spores, using the substrate peptide BikKam1, i.e., FITC-Leu-D-Leu-Lys-Dbc (see Kaman *et al.*, 2011 for full experimental details). Assays were performed in Blackwell, clear bottom 96-well plates (Corning, Lowell, U.S.A.). Enzyme activity in bacterial supernatants was determined by incubating 16 µM substrate with 100 µL of filtered culture supernatant at 37 °C. Filtered BHI medium was used as a negative control. Plates were read with 10 minutes intervals on a CytoFluor 4000 (Applied Biosystems, Foster City, U.S.A.) with excitation at 485 nm and emission at 530 nm. Relative fluorescence (RF) is the value obtained after correction with the negative control, BHI medium. The measured enzyme activity is defined in RF per minute (RF/min).

IV. RESULTS AND DISCUSSION

Task 1: Literature study on postmortem processes

A literature study has been performed by dr M. Verschraagen and dr C.M. Boone of the Netherlands Forensic Institute (NFI), department of Toxicology, and by dr. M. Polhuijs and dr. D. Noort of TNO Defense, Security and Safety. The entire report can be found in Appendix A. The following is the abstract of the report.

The aim of the literature study was to investigate the postmortem (PM) detoxification of chemical warfare agents (CWA's) in contaminated human remains. The study focused on PM redistribution and metabolism of CWA's and related compounds.

PM redistribution involves the transfer of agents through the body after death by passive release from 'reservoir' organs such as the gastrointestinal tract, liver and lungs to surrounding tissues and organs. Thus, agent concentrations in body fluids and organs can change. In the report, factors influencing PM redistribution is outlined, such as the chemical properties of the involved compounds. PM redistribution can be studied by comparing agent (or drug) concentrations in multiple specimens i.e. body fluids and tissues. Data was collected from literature on PM tissue distribution of CWA's and organophosphorus (OP) pesticides. OP pesticides are less toxic but chemically similar to nerve agents and are frequently used for suicidal and homicidal intoxications. An overview of pesticide concentrations in body fluids and tissues after death is presented, with data of about 20 OP pesticides, such as parathion, malathion, omethoate, and dichlorvos. In the majority of cases in which various organs were examined, there was a tendency to cumulate OP pesticides in adipose (fat) tissue.

The distribution of CWA's before death was investigated to get some information on the possibility of contact or evaporation hazard during autopsy. A similar phenomenon as for the OP pesticides was observed for an Iranian soldier who died after mustard gas exposure. Based on concentrations sulfur mustard in various organs that were found at autopsy, it can be concluded that sulfur mustard seems to accumulate in the lipid compartments of the human body. Since these concentrations were measured in samples which were taken seven days after exposure, redistribution of sulfur mustard from the lipid depots before death occurs at a rather slow rate. Since all processes are slowed down postmortem, it is likely that the same will apply to postmortem redistribution. The concentrations found in the lipid tissues, including skin with subcutaneous fat, suggest ante mortem and postmortem stability of mustard gas. Therefore, any lipid rich tissue poses a hazard for secondary contamination.

Some data on *in vivo* skin absorption and distribution of VX in the domestic white pig which were exposed percutaneously to a 6-hour 2 LD_{50} is

presented in the report. These data suggest that approximately one-fifth of the applied dose of VX remained in the skin exposure site, suggesting postmortem risk on contact hazard. Decontamination procedures before autopsy might lower the risk of contact hazard. High concentrations were also found in blood and other tissues.
The metabolism of CWA's is expected to continue after death and by different mechanisms than prior to death. The following processes are being discussed: scavenging, metabolism, chemical degradation and putrefaction.
After a fatal dose of OP (nerve agent or pesticide), scavenging will play a limited role in PM detoxification. This was confirmed by data of cholinesterase (ChE) activity levels of victims of fatal OP pesticide intoxications. Although it is clear that many processes contribute to (slow) degradation of agents after death, the compounds are not completely detoxified. However, PM formation of additional toxic compounds has not been described and seems to be unlikely. So far, literature did not provide any information on other CWA metabolites than the ones that are commonly found.

Task 2. Contact hazard by VX

Nerve agents of the so-called V-type are several orders of magnitude less volatile than G-type agents and act primarily as a liquid via the percutaneous route. The most important representative of V-agents, VX, is several orders of magnitude more lethal percutaneously than sarin. VX is more persistent *in vivo* than G-agents (Van der Schans *et al.*, 2003). Significant VX levels have been reported in the skin exposure site; up to 20% in domestic pigs (Chilcott *et al.*, 2005). The metabolism of VX is relatively simple (see Figure 8). Hydrolysis to ethyl methylphosphonic acid is the major metabolic route. Hydrolysis to the toxic metabolite desethyl-VX has also been reported, but not under *in vivo* conditions (Van der Schans *et al.*, 2003b). The other hydrolysis product, i.e., diisopropylaminoethanethiol (RSH), can undergo a number of conversions, including conjugation to proteins or oxidation (see Van der Schans *et al.*, 2000 for a good overview).

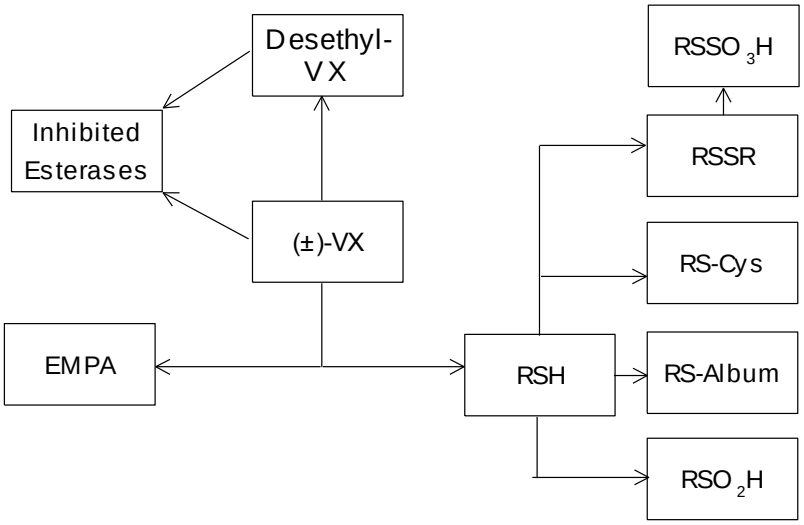

Figure 8 Metabolic pathways for VX.

Exploration of tape stripping for determination of contact hazard by VX

Tape stripping is a frequently used method to quantify residual contamination on the skin (including liquid droplets and agent penetrated in the first layer of the skin). In the current project, the tape stripping procedure is used in a quantitative way. The most critical question is: what is the recovery of agent, present on the skin, by tape stripping? Usually, recovery issues can be dealt with through the addition of a well-defined amount of an internal standard. However, in this case we have to be careful with this. Addition of a solution of an internal standard solution might seriously interfere with the sampling spot. On the one hand it might dissolve some of the agent from lower layers in the skin, which by itself would no longer pose a real contact hazard. On the other hand it might 'flush' remaining agent from the sampling spot, resulting in lower quantities that are being analyzed. We have therefore determined the recovery of agent by tape stripping, directly after application of agent on the skin, for a wide concentration range of the respective agent. Subsequently, external standard (the O-n-propyl analogue of VX) was added to the piece of tape, followed by extraction, GC-MS analysis and quantification.

After the extraction, the extracts were transferred to autosampler vials and analysed by GC-NPD. From the ratio VX and V33, the amount of VX that could be removed from the surface by tape stripping was calculated and expressed as a percentage from the originally applied quantities of VX.

The following results were obtained with glass plates (see Table 1).

Table 1 Recovery of VX from glass plates.

Contamination VX (µg)	% recovery strip 1	% recovery strip 2	% recovery total
100	83	1	84
100	*	*	*
10	96	3	99
10	82	9	91
1	60	1	61
1	51	1	52
0.1	71	2	73
0.1	85	2	87
0.01	43	9	52
0.01	81	6	87

* experiment carried out with wrong dilution.

Table 2 Recovery of VX from pig ear skin.

Contamination VX (µg)**	% recovery strip 1	% recovery strip 2	% recovery skin	% recovery total
100	58	14	51	123*
100	59	11	49	119*
100	77	11	25	113*
10	54	12	42	108
10	50	11	41	102
10	53	10	42	105
1	44	4	28	76
1	43	8	24	75
1	47	7	27	81
0.1	39	3	39	81
0.1	45	3	29	77
0.1	35	3	34	72

* at higher contamination levels the total recovery exceeded 100%, probably because of non-linear response of the detector at these very high concentrations.

** in this case 0.1 µg was taken as the lowest because it was envisaged that the 0.01 µg level was around the detection level (when using GC-NPD).

The following results were obtained with pig ear skin samples (see Table 2). It can be concluded that the described tape stripping procedure seems to work satisfactory for VX. Even at low contamination levels (< 1 µg), the recovery after one round of stripping is still approximately 40%. The components released from the tape during extraction did not interfere with the actual GC analysis. Also, the use of an internal standard in the extraction and analytical procedure, i.e. by addition just after the actual tape stripping, is quite advantageous. If desired, a second round of tape stripping can be applied, but in case of low contamination levels (0.1 µg) with pig ear skin this hardly results in a significant increase in recovery. We envisage that a similar procedure will work for sulfur mustard contaminated skin (Task 3).

Sampling of postmortem blood

We envisaged that we might encounter problems during blood sampling from the deceased guinea pigs. In contrast to man, blood of a guinea pig coagulates very rapidly and we have no experience with sampling postmortem blood from animals for a prolonged period of time. It was discussed whether the antemortem administration of heparin to the guinea pig would make it possible to sample postmortem blood over a longer period of time. An alternative would be to sample tissue that is rich in blood. In case of humans, liver is often used for postmortem sampling. Therefore, the sampling of postmortem blood, and sampling liver with and without the heparin procedure was tested in a pilot experiment with blank rats which were available from another experiment. Without heparin, it was only possible to sample blood for up to three hours after death. Heparin only worsened the situation. It is anticipated that results with guinea pigs will be even worse. Without heparin, it was possible to sample the liver during a period of up to 72 hours. So, probably only the liver sampling procedure will be applicable for the guinea pigs tests.

Literature data on autopsy data from an Iranian soldier that died seven days after a mustard gas exposure show that mustard gas can be found in blood (1.1 mg/L) as well as in liver (2.4 mg/kg). In an experiment in which the domestic white pig was exposed percutaneously to a 6-hour 2 LD_{50} VX, intact agent could be measured (at a maximum time of 3 hours after exposure) both in blood (546 ± 51 ng/g) and liver (435 ± 85 ng/g).

Pilot experiment with VX

A pilot experiment was carried out for the percutaneous VX contamination experiments (Task 2) in order to assess whether the developed sampling and analytical methodologies could readily be applied, whether blood and liver sampling proceeded satisfactorily, and whether the additional time points (48 and 96 hours) for sampling were feasible.

First, a small device was developed in order to easily locate the contaminated skin during tape stripping. It contains a number of small laser beams (see Figure 9) that point to the 8 spots on the belly where the animal has been contaminated; the black dots are used to keep the animal in a fixed position relative to the laser beams. We did not want to use a marker pen because this might negatively interfere with the tape stripping procedure.

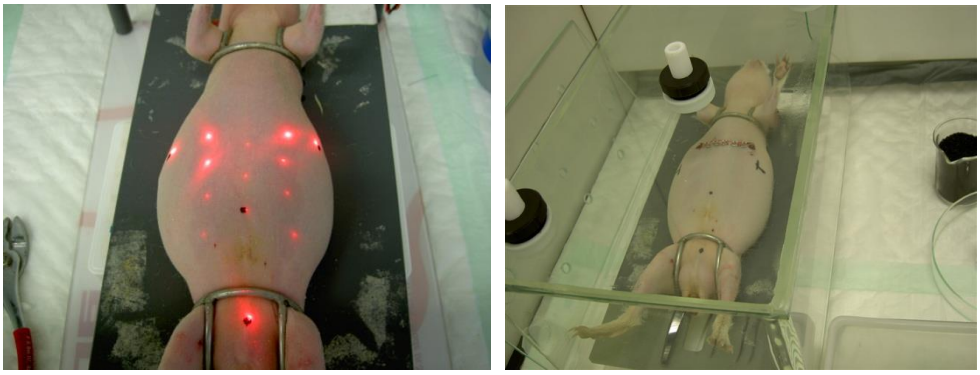

Figure 9 Experimental set up with laser beams that mark exposure sites on hairless guinea pig.

The hairless guinea pig (approximately 800 grams) was anesthetized before contamination. Before the start of the experiment, a blank blood sample was taken from the ear. Subsequently, 8 drops of 1 µL neat VX were applied onto the belly of the guinea pig. The VX drops vanished after 20 to 30 seconds. 30 Minutes after application of VX the guinea pig was euthanized with Nembutal®. This euthanasia procedure was not very effective and the animal died only after 60 minutes (from beginning of the experiment). However, the time between application and sampling should be well-defined and identical in each experiment, because it can be envisaged that it will have large effect on the various toxicokinetic processes. Therefore, in subsequent experiments euthanasia was performed using a lethal mixture of CO_2 and medicinal oxygen; this normally leads to death within a few minutes.
The tape stripping procedure resulted in bruises on the skin (appearing at later time points). Also, some skin stuck onto the tape at the later time points and the skin was severely damaged after the skin stripping procedure (see Figure 10).

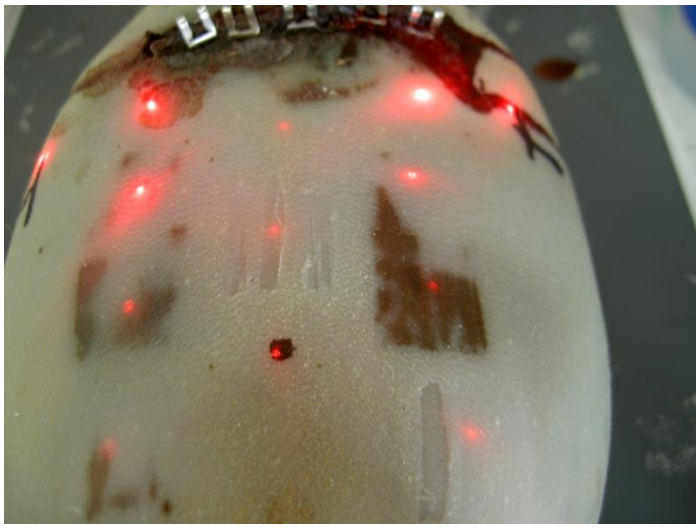

Figure 10 Guinea pig skin after tape stripping at time point 96 h, showing bruises and damaged skin. The site where liver and lung samples were taken throughout the experiment is visible at the top of the picture.

Tape stripping was performed using two tape strips for each time point, and for each time point a new VX spot was used. As was to be expected, the amount of VX on skin gradually decreased with time (see Figure 11). At the later time points (see, e.g. the 29 hours point), the amount of VX that was sampled with the second tape strip clearly increased; this is probably related to the fact that the upper part of the skin is removed by the first tape stripping, making more VX available for the second tape strip.

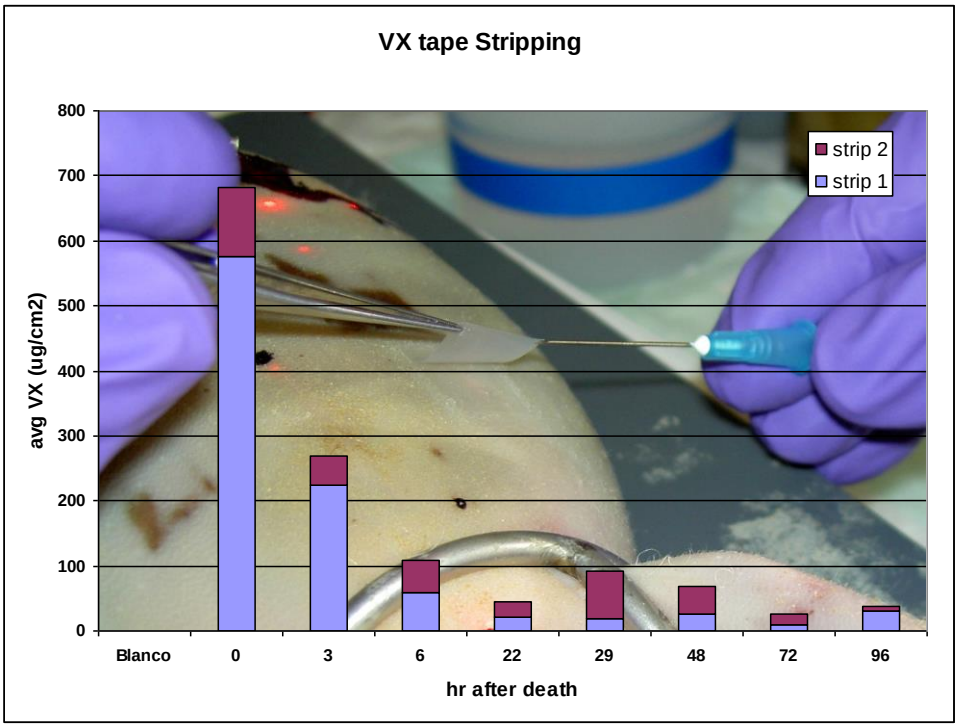

Figure 11 VX levels (expressed as µg/cm^2) on skin at various sampling points as determined by tape stripping.

During the experiment blood and liver samples were taken at predetermined time points. It was possible to take blood samples (100 to 200 µL at each time point) up to 22 hours. Sampling of the lung and liver was difficult because of the presence of body fluid. Analysis of the blood samples was performed in a quantitative way, by using an internal standard. The concentration of VX in blood was quite low (approximately 20 ng/ml) and remained rather constant over time (see Figure 12). Surprisingly, the concentration of VX in the liver appeared to be much higher than the VX concentration in blood, i.e, up to 700 ng/g liver. This finding might indicate that VX readily distributes to the liver and that it is not metabolized extensively. It is clear that this finding might have important implications in view of the aim of the project, i.e., assessment of the contact hazards of contaminated human remains: the liver might pose hazards during autopsy manipulations. Self-evidently, care has to be taken with interpretation of this set of data, because they represent only a single experiment.
Liquid chromatography – mass spectrometry (LC-MS) was used advantageously for (qualitative) analysis of VX and peculiar (toxic) metabolites, both in blood and in liver samples (see Figure 13). In general,

the same findings were obtained, i.e., higher concentrations of VX in liver. Also, it appeared that LC-MS analysis of VX was more sensitive than GC-MS analysis, so for subsequent experiments we decided to use LC-MS analysis for quantitative analysis of VX in aqueous samples.

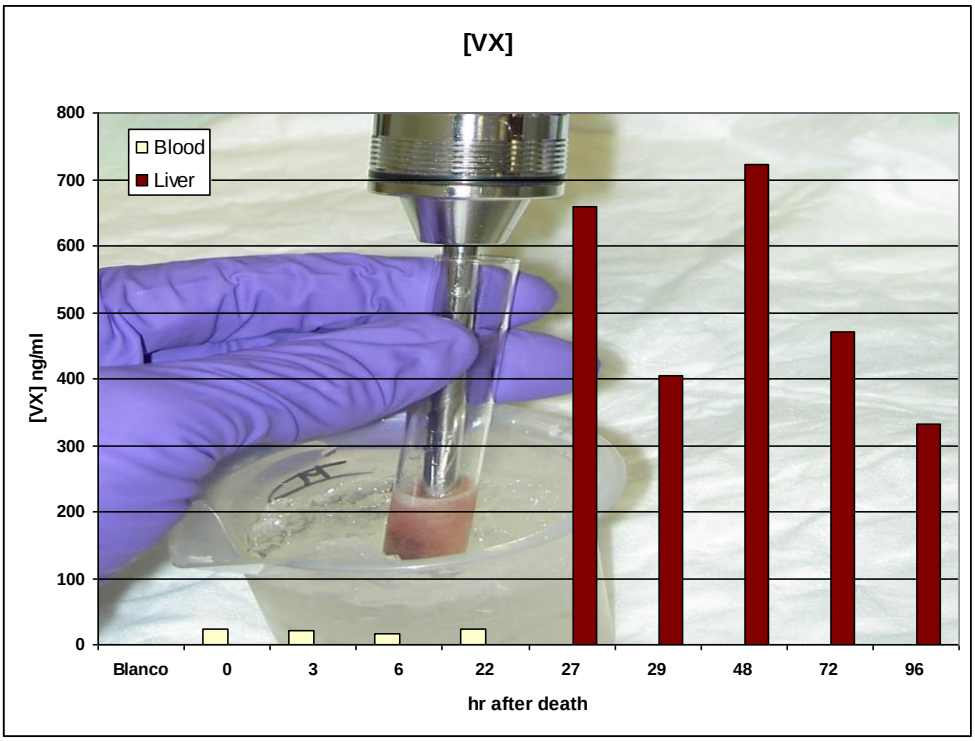

Figure 12 Measured concentration VX in blood (expressed in ng VX/mL blood) and liver (expressed in ng VX/g liver) at various sampling points.

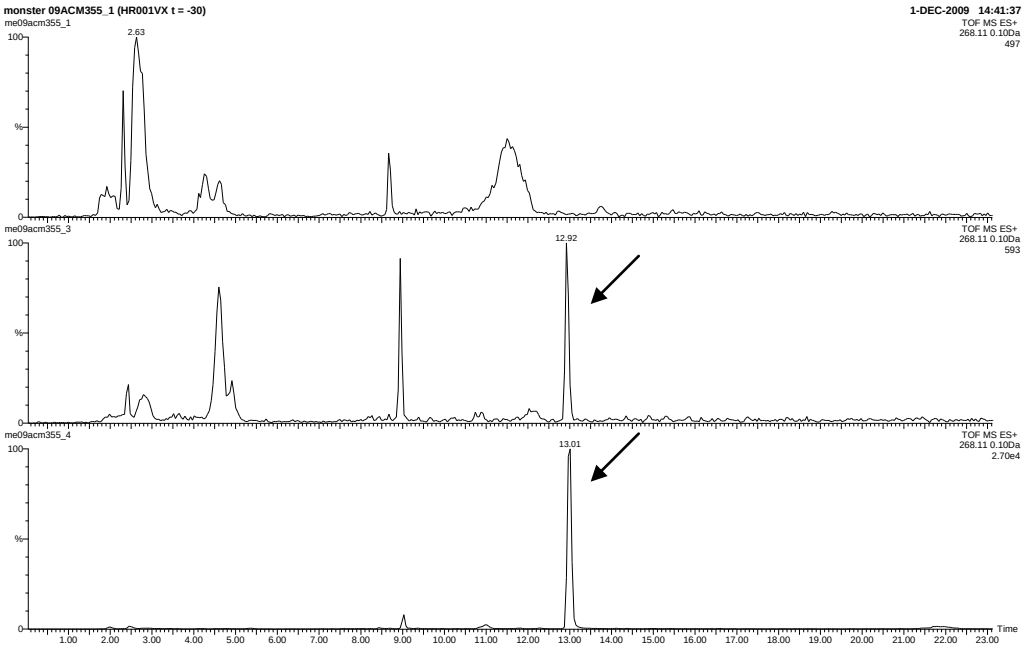

Figure 13 Liquid chromatography – mass spectrometry (LC-MS) analysis of blank blood sample at t = - 0.5 h (HR001VX, upper trace), blood sample at t = 3 h (HR001VX; middle trace) and liver sample at t= 27 h (HR001VX; lower trace). The peak of VX is indicated by an arrow.

At the end of the experiment a sample of the body fluid was taken out of the belly of the animal. This sample will be analyzed with LC-MS for the presence of VX and possible hydrolysis products.

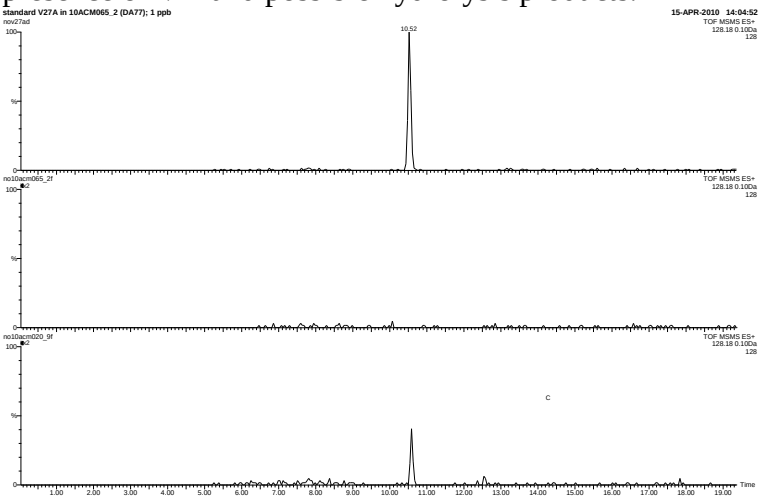

Figure 14 LC ES-MS/MS analysis of desethyl VX (V27A). Extracted ion chromatograms of m/z 128. A. standard addition experiment; V27A in sample DA77 (1 ppb), B. liver sample DA77, C. liver sample DA33.

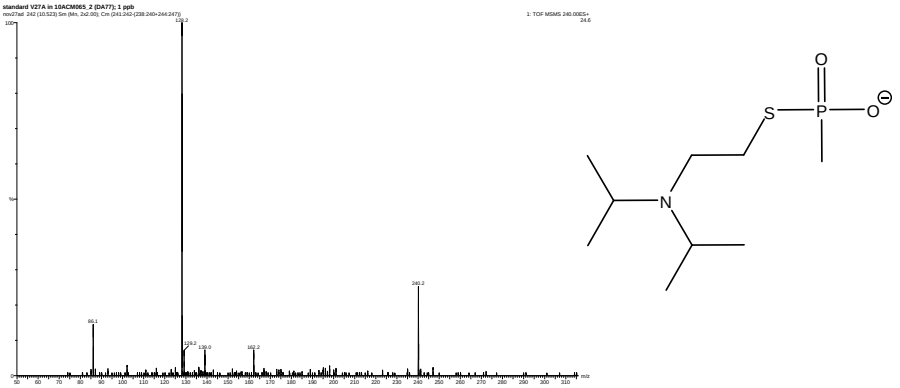

Figure 15 Mass spectrum of desethyl VX, as detected in blood and liver samples of VX-contaminated animal remains.

Earlier in the study it was found that the toxic metabolite V27A (desethyl VX) could be detected in one of the blood- and in one of the liver samples, at a level of 0.2 ng/ml, which is very low in comparison to the VX levels. This finding has been further corroborated by standard addition experiments with synthetic reference standards (see Figures 14 and 15). The non-toxic hydrolysis product EMPA could only be analyzed in liver samples at variable levels (2-200 ng/ml), which is in agreement with the high and variable concentrations of VX in liver.

Based on these pilot results, several preliminary conclusions could be drawn:

- experimental set-up works nicely, and animal can be sampled up to 22 hours for blood samples and up to 96 hours for liver and lung samples;
- the amount of VX on skin decreases gradually, but VX is still present after 96 hours;
- VX is present in blood for up to 22 hours (last possible sampling time point) and liver for up to 96 hours, and levels seem to be rather constant over time;
- VX is present at much higher levels in liver than in blood.

It might be possible to sample even after 96 hours, but 96 hours was agreed upon as the last sampling point within the SOW.

Determination of contact hazard by VX, with n=4

Subsequently the entire series (n=4) was carried out. Tape stripping was performed using two tape strips for each time point, and for each time point a new VX spot was used. After addition of internal standard to the tapes followed by liquid extraction, the extracts were quantitatively analyzed by means of GC-MS. For the tape stripping results, see Figure 16. As was observed during the pilot experiment, the amount of VX on skin gradually decreased in time.

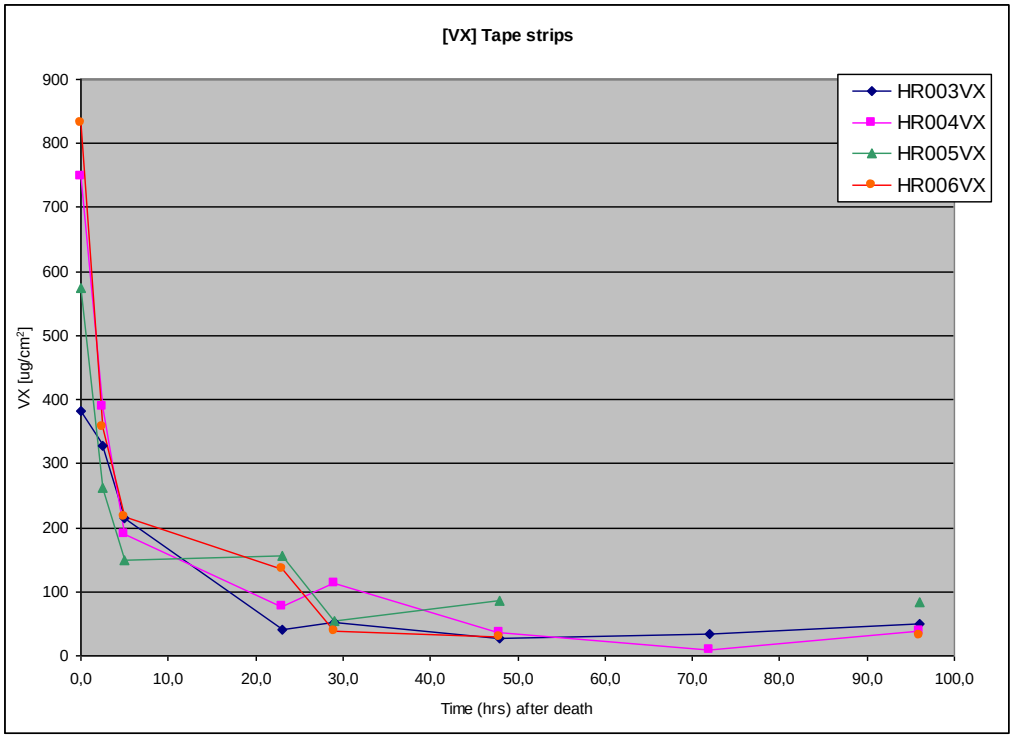

Figure 16 VX levels (expressed as µg/cm^2) on skin at various sampling points as determined by tape stripping, after percutaneous exposure of hairless guinea pigs (n=4) to VX. For 2 animals, samples could not be taken at t= 72 h.

During the experiment blood, liver and lung samples were taken at predetermined time points. In this series, it was possible to take blood samples (100 to 200 µL at each time point) up to 5 hours after death of the animal (in the pilot experiment blood samples could be taken up to 22 hours). Liquid chromatography – tandem mass spectrometry (LC tandem MS) was used for quantitative analysis of VX in blood. At t=5 hours, still significant levels of VX could be analyzed in blood, at slightly lower levels than in case of the pilot experiment (see Figure 17).
Cholinesterase activity determination showed that already at t=0 the activity had already dropped to < 10%, as was to be expected for these high exposure levels.

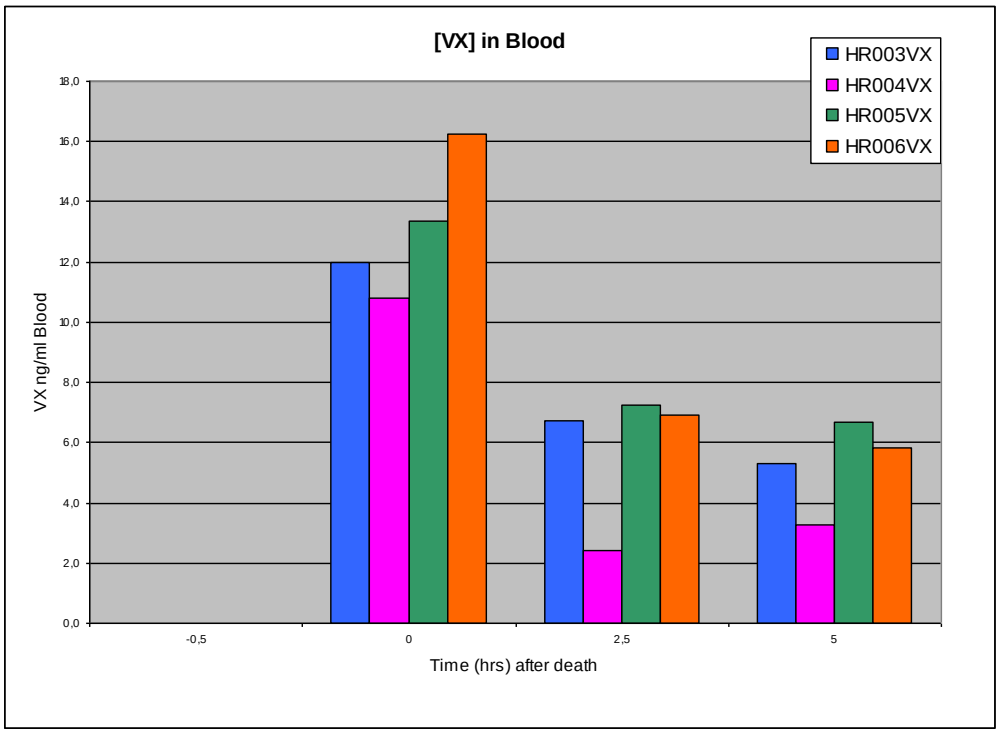

Figure 17 Measured concentrations of VX in blood (expressed in ng VX/mL blood) at various sampling points, as determined by means of LC-tandem MS, after percutaneous exposure of hairless guinea pigs (n=4) to VX.

A high variability in the levels of VX in liver was observed, which might indicate that the site of sampling is important (see Figure 18). Remarkably, during the first hours after death, levels in the liver are much lower (lowest level 11 ng/ml; highest level 77 ng/ml) than at later time points. During the experiment, extreme attention was paid to tissue manipulations in order to circumvent contamination of the internal parts of the animal by the exposed skin site. It is proposed to determine the total agent in the liver, after homogenization, in future experiments.

The toxic metabolite V27A (desethyl VX) was detected in one of the blood- and in one of the liver samples, at a level of 0.2 ng/mL (which is very low in comparison with the VX levels)
The hydrolysis product EMPA could only be analyzed in liver samples at variable levels (2-200 ng/ml), which is in agreement with the high and variable concentrations of VX in liver.

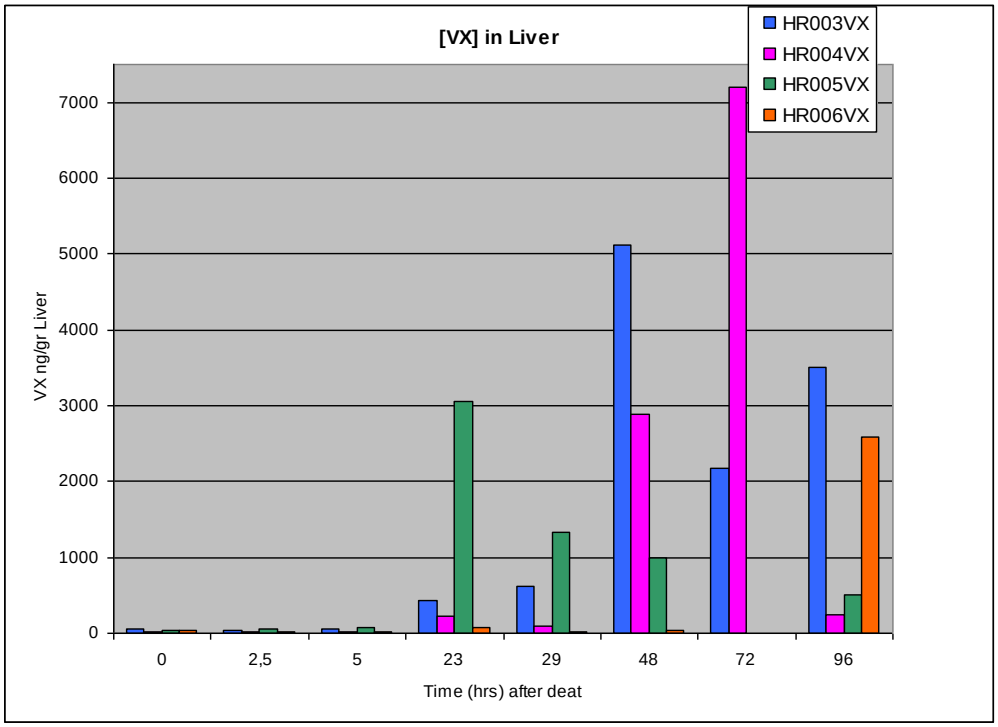

Figure 18 Measured concentrations of VX in liver (expressed in ng VX/g liver) at various sampling points, as determined by means of LC-tandem MS, after percutaneous exposure of hairless guinea pigs (n=4) to VX.

Task 3: Animal study – liquid sulfur mustard on skin

Sulfur mustard is a vesicant that has been used in World War I and more recently by Iraq during the Iran-Iraq conflict. It is a bifunctional alkylating agent that reacts rapidly with various nucleophilic sites in proteins and DNA under physiological conditions, via the intermediate episulfonium ion. Its metabolism is much more complex than that of nerve agents (see Figure 19; Noort *et al*., 2002; Black and Noort, 2005). Sulfur mustard distributes strongly to various organs (e.g. lung, spleen, liver), in contrast to nerve agents; accumulation in fat tissue has also been reported. Despite its high reactivity, intact sulfur mustard could be detected up to 6 hours after exposure, at ng/mL levels in toxicokinetic experiments in hairless guinea pigs (Langenberg *et al*., 1998).

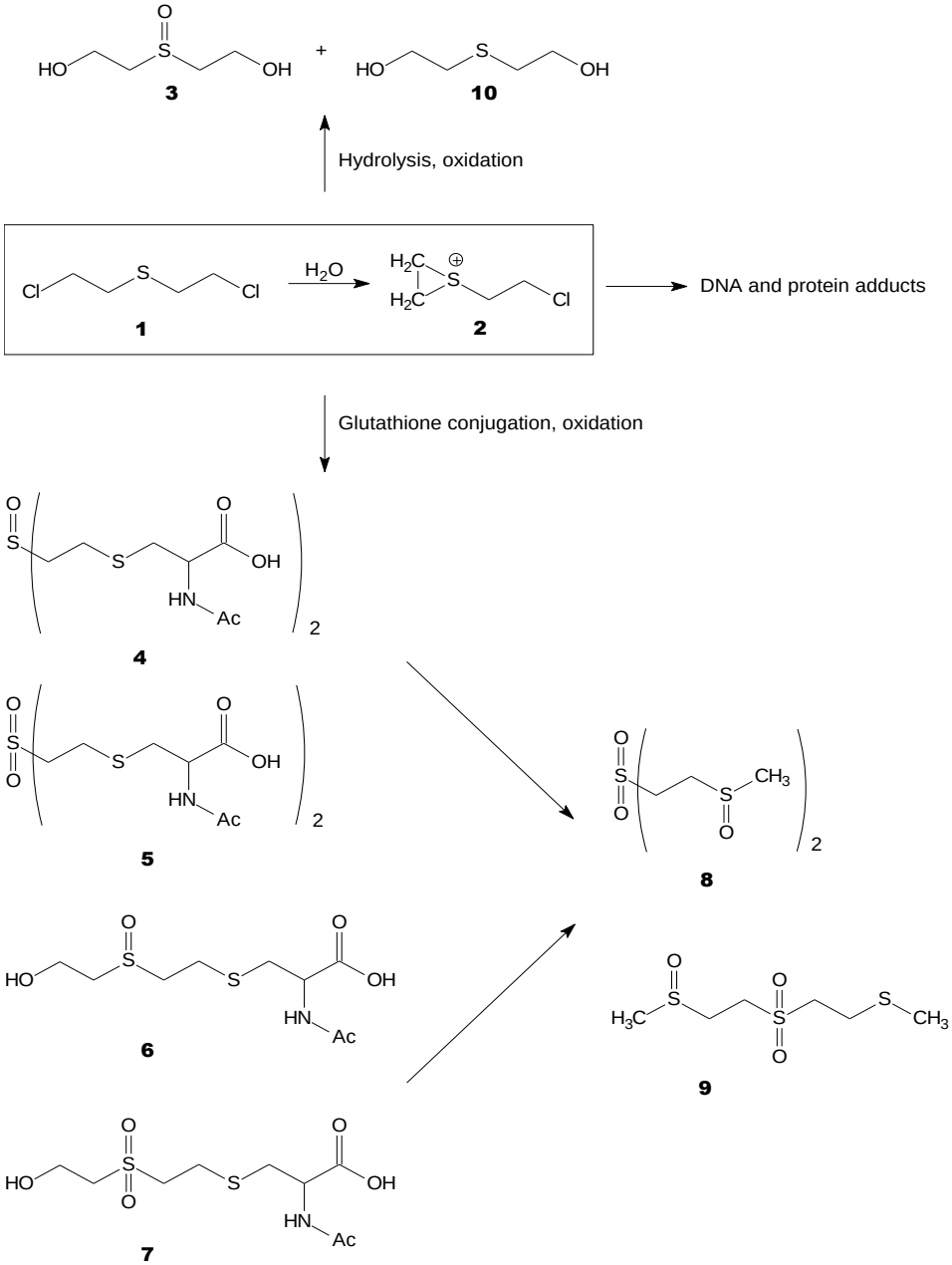

Figure 19 Metabolic pathways of sulfur mustard.

Exploration of tape stripping for determination of contact hazard by sulfur mustard

In order to carry out the animal experiments with liquid sulfur mustard, we explored the required analytical methodology. Tape stripping was carried out in an analogous way as reported for VX, *i.e.*, by using Fixomull transparent tape. Both glass slides and pig ear skin were used; experiments were carried out in triplicate. The tapes were extracted with n-heptane/acetone, 9/1, v/v (2 mL). Deuterated sulfur mustard (d_8-sulfur mustard) was used as an internal standard and was added prior to extraction of the strip. The results for glass slides are shown in Table 3. No sulfur mustard could be detected in the second tape that was used for tape stripping.

Table 3 Tape stripping experiments with sulfur mustard on glass slides.

Contamination (µg)	Recovery strip 1 (%)	Recovery strip 2 (%)	Recovery total (%)
130	95	0	96
130	92	0	92
130	94	0	94
8	52	0	52
8	56	0	56
8	59	0	59
1	17	0	17
1	56	0	56
1	38	0	38
0.1	0	0	0
0.1	0	0	0
0.1	31	0	31
0.01	0	0	0
0.01	0	0	0
0.01	0	0	0

In the second series of experiments the tape stripping procedure was used for recovery of sulfur mustard from pig ear skin; see Table 4 for results. For contamination levels <0.1 µg, no sulfur mustard could be detected after tape stripping. Analogous to the contaminated glass slides, nearly all sulfur mustard could be removed by using one strip. Hardly any sulfur mustard could be analyzed in the skin after the experiment.

Table 4 Tape stripping experiments with sulfur mustard on pig ear skin.

Contamination (µg)	Recovery strip 1 (%)	Recovery strip 2 (%)	Recovery skin afterwards (%)	Recovery total (%)
130	83	0.4	0.4	84
130	95	1	0.2	96
130	106	1	1	107
8	75	0	0	75
8	93	2	0	95
8	110	0	0	110
1	85	4	2	88
1	97	2	2.24	101
1	68	0	0	68
0.1	48	0	0	48
0.1	56	0	0	56
0.1	55	0	0	55
0.01	0.01	0	0	0
0.01	0.01	0	0	0
0.01	0.01	0	0	0

In conclusion, the tape stripping procedure, including extraction of the tape, seems to work satisfactorily for sulfur mustard.

Determination of contact hazard by sulfur mustard

Subsequently, hairless guinea pigs were exposed percutaneously to sulfur mustard (8 * 1 µL), analogous to the experiments with VX. In case of skin samples, sulfur mustard could only be analyzed at t=0 in case of 2 animals (see Figure 20), which is in sharp contrast with the results obtained for VX. This might be due to: 1. a higher penetration rate of sulfur mustard. 2. a higher chemical reactivity of sulfur mustard (hydrolysis, adduct formation with skin proteins) and 3. a higher volatility of the agent.

In blood, intact sulfur mustard could still be observed after 23 hours, which is the last sampling point for blood, for each animal that could be sampled (see Figure 21). In general, results are quite scattered: in a number of animals, no agent could be detected anymore in the blood, while in other animals the blood levels were significant. Sulfur mustard could also be observed in liver samples, with initial concentrations significantly higher than concentrations observed in blood. Sulfur mustard could still be detected in the liver for up to 48 hours (see Figure 22) in a number of animals. In contrast to the results obtained for VX, the pronounced accumulation of agent within the liver was not observed. This might be caused by different distribution characteristics, or might be due to the higher inherent reactivity of sulfur mustard compared to VX, or due to a higher (post-mortem) metabolism in liver compared to VX.

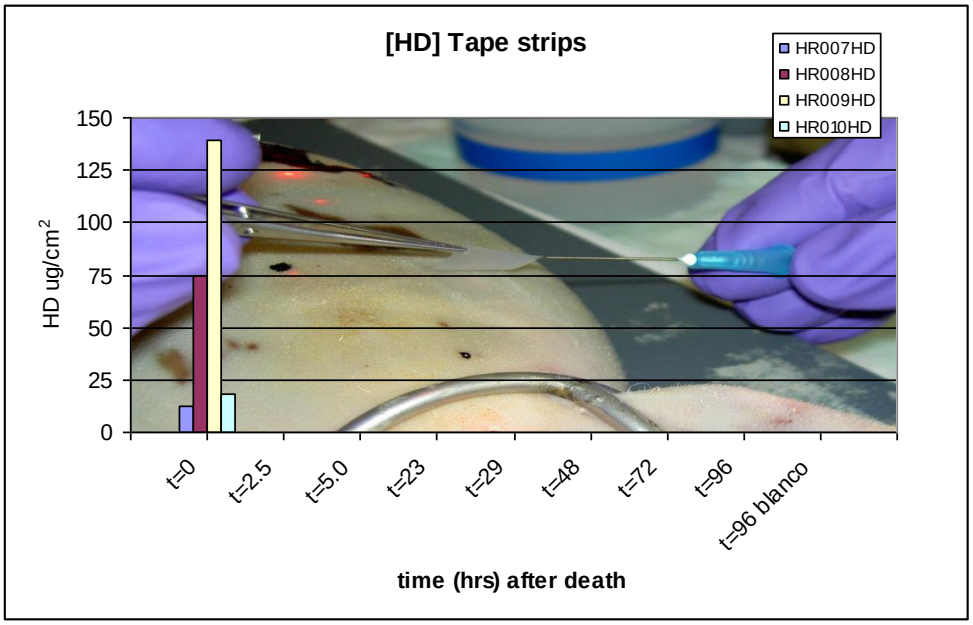

Figure 20 Sulfur mustard levels (expressed as µg sulfur mustard/cm^2) on skin at various sampling points as determined by tape stripping, after percutaneous exposure of hairless guinea pigs (n=4) to liquid sulfur mustard.

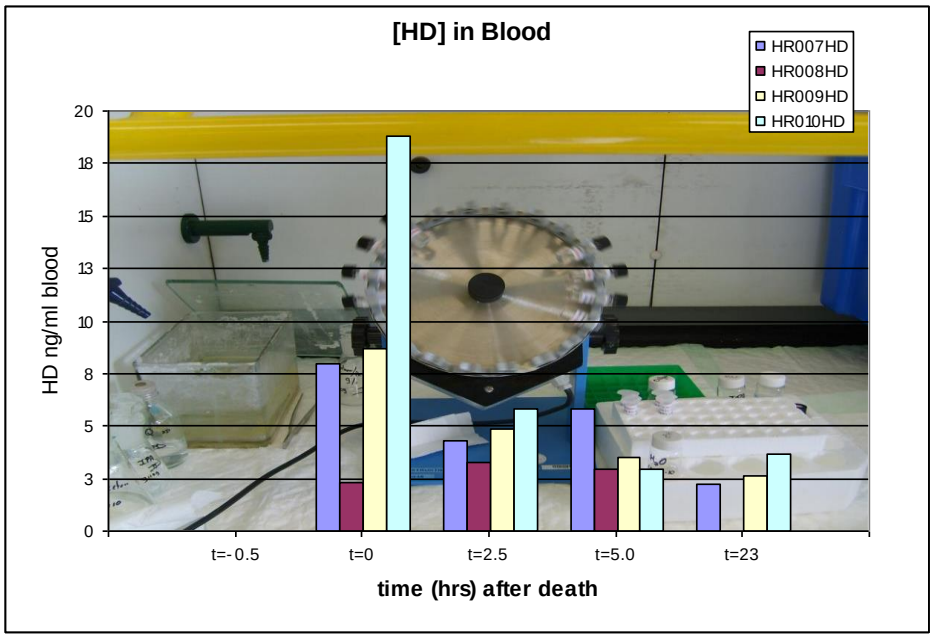

Figure 21 Measured concentrations of sulfur mustard in blood (expressed in ng sulfur mustard/mL blood) at various sampling points, as determined by means of GC-MS, after percutaneous exposure of hairless guinea pigs (n=4) to liquid sulfur mustard.

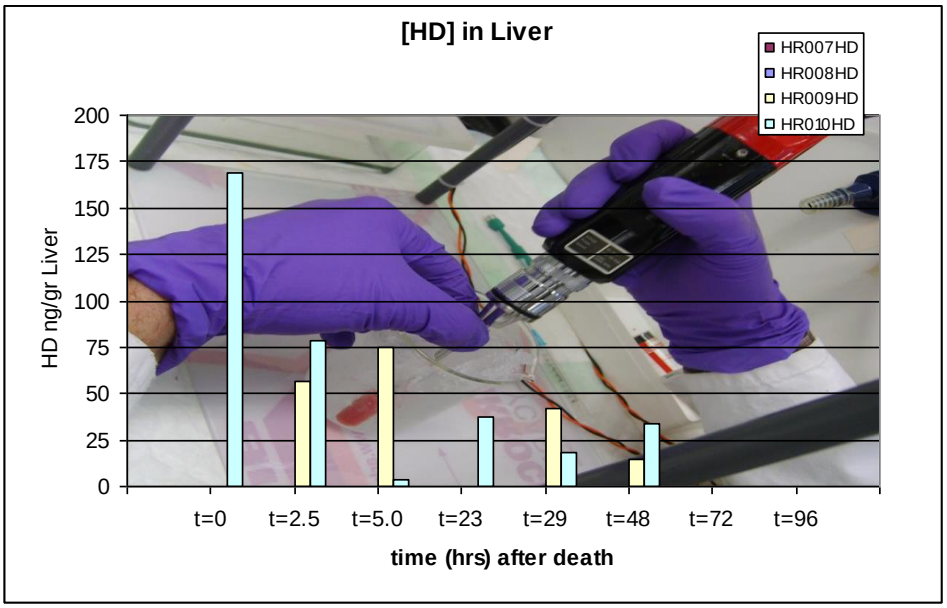

Figure 22 Measured concentrations of sulfur mustard in liver (expressed in ng sulfur mustard/g liver) at various sampling points, as determined by means of GC-MS, after percutaneous exposure of hairless guinea pigs (n=4) to liquid sulfur mustard.

Determination of evaporation hazard after liquid contamination with sulfur mustard

In a similar experimental set-up, the evaporation hazard after contamination with 8 x 1 µL droplets of sulfur mustard was studied. Animals were contaminated and euthanized 30 minutes after contamination in a glass chamber, in which a controlled air flow was sweeping over the animal; see experimental part for a full description of the equipment. The outlet air was lead through two bubble cells in series containing 1 mL solvent (diethyl succinate) each, in order to sample the agent in well-defined time intervals. After addition of a specific amount of internal standard (deuterated sulfur mustard), the collected solvent fractions were analyzed by means of GC-MS. See Figure 23 for the results.

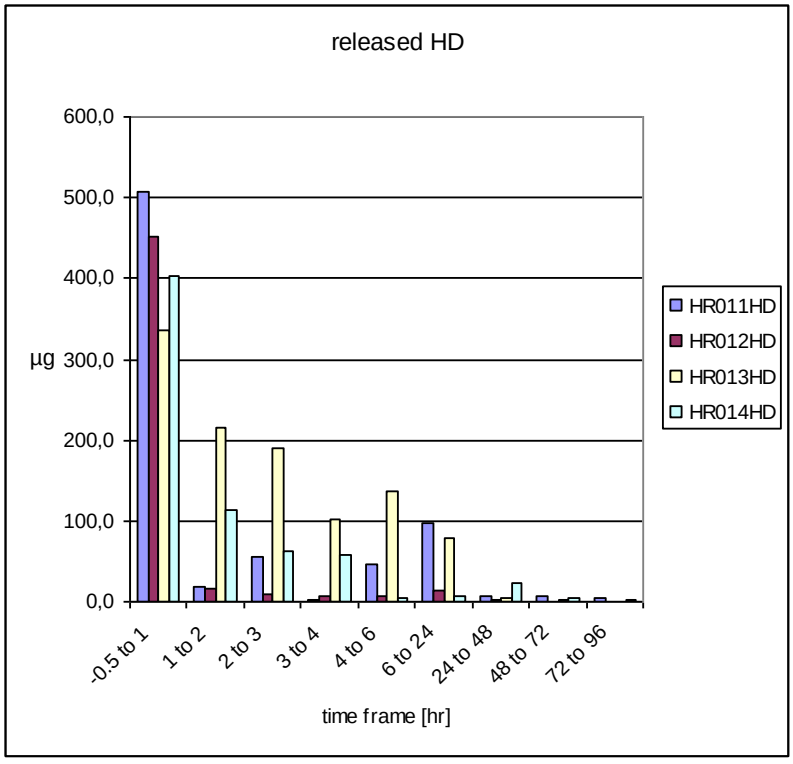

Figure 23 Released amount of sulfur mustard vapor, as determined by means of GC-MS, after percutaneous exposure of hairless guinea pigs (n=4) to liquid sulfur mustard (8 x 1 µl).

Although the measured amounts of sulfur mustard seem to be a little bit scattered, evaporation hazard seems to be most pronounced during the first 24 hours after the contamination of the animal.

The evaporation hazard was also determined with chemical agent detectors that are currently in use. In the experiments for animals HR013HD and HR014HD an ICAM (provided by US Army) was used to measure the released vapor above the animal. At the time the experiments HR011HD and HR012HD were performed this ICAM was not available and therefore a CAM (from the Dutch Armed Forces) was used; see Table 5 for results. In case of animals HR011HD and HR012HD the response of the CAM was already negligible at t = 2 hours. For animals HR013HD and HR014HD, the

ICAM gave a significant response until t = 4 hours. This is in line with the results obtained for the GC-MS analyses of the collected vapor, which show significantly higher quantities of sulfur mustard for animals HR013HD and HR014HD, and is not necessarily caused by a difference in sensitivity of the two detectors.

Table 5 Response of CAM or ICAM (in number of bars on the display) over time, after measuring released sulfur mustard vapor of the contaminated animal.

Time (h)	HR011HD	HR012HD
1	3-4	2
2	1	0
3	0	0
4	0	0
6	0	0
24	0	0
48	0	0
72	0	0
96	0	0

Time (h)	HR013HD	HR014HD
1	3	3
2	4	4
3	4	4
4	3-4	3-4
6	0	0
24	0	0
48	0	0
72	0	0
96	0	0

With regard to the formation of toxic metabolites under post-mortem conditions, analysis of blood and liver samples from deceased animals that had been exposed (p.c.) to sulfur mustard (Task 3) demonstrated the presence of the sulfur mustard metabolite HD-sulfoxide (see Figures 24 and 25, for ion chromatograms and corresponding mass spectra) in blood, but not in liver samples. This metabolite is considered to be not highly toxic (LD_{50} mouse: 125 mg/kg, subcutaneous) and does not exert vesicant activity (see Lawson and Reid, 1925). The corresponding sulfur mustard sulfone, which still possesses vesicant activity, could not be detected.

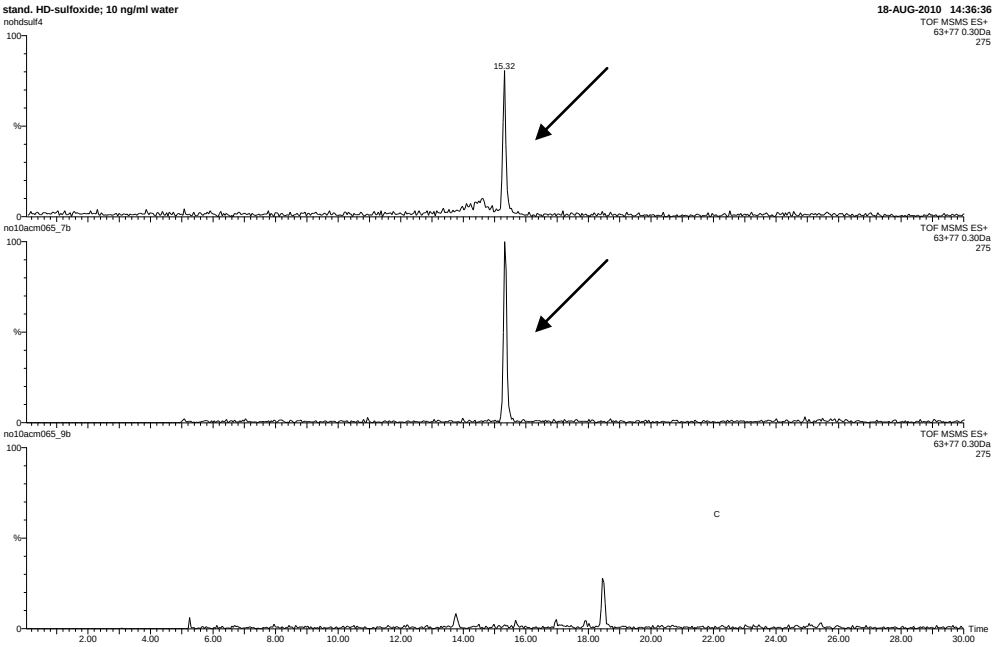

Figure 24 LC ES-MS/MS analysis of HD sulfoxide. Extracted ion chromatograms of the sum of the fragment ions m/z 63 and m/z 73 of MH^+ 175 of HD sulfoxide. Cone voltage 20 V, collision energy 15 eV, Argon gas pressure of approximately 10-4 mBar.
A, upper trace. Standard HD sulfoxide (10 ng/ml),
B, middle trace. Blood sample DA82 (taken at t = 5 hours),
C, lower trace. Liver sample DA84 (taken at t = 72 hours).

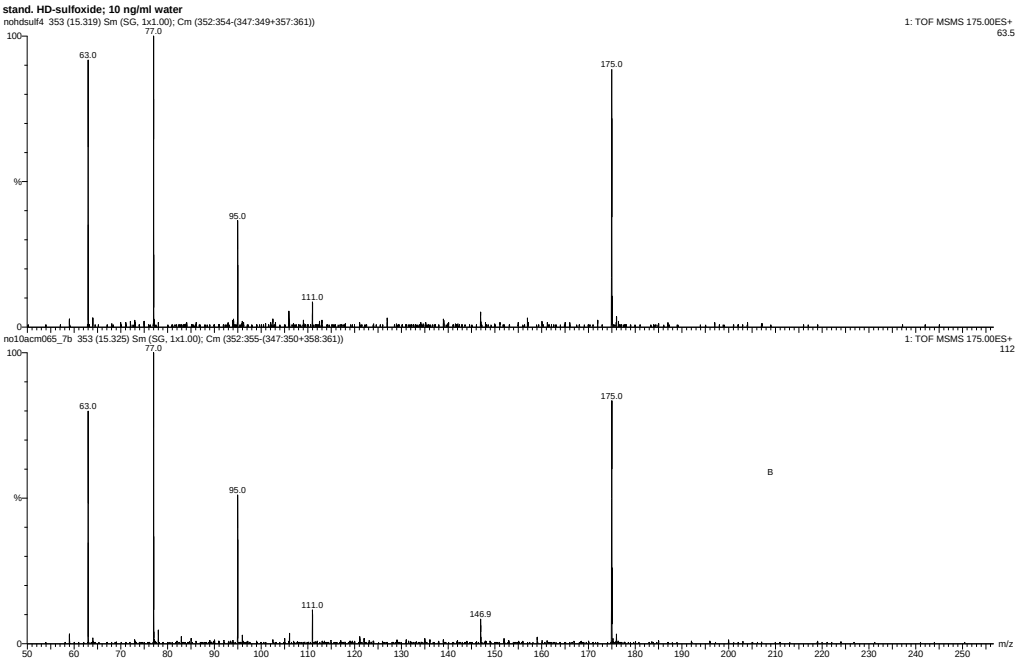

Figure 25 Product ion mass spectra of the protonated molecular ion [M+H]+ 175 of HD sulfoxide.
A, upper trace. Standard HD sulfoxide (10 ng/ml),
B, lower trace. Blood sample DA82 (taken at t = 5 hours).

In addition to potentially toxic metabolites, we have also identified covalent adducts of sulfur mustard with blood proteins. We did not isolate individual proteins from the blood sample, but we digested the entire protein fraction with pronase in order to release any modified amino acid. Mass spectrometric analysis of the resulting digest showed the presence of significant levels of S-(2-hydroxyethylthioethyl) cysteine (see Figures 26 and 27). This adduct is also formed under *in vivo* conditions (Noort *et al*., 2008).

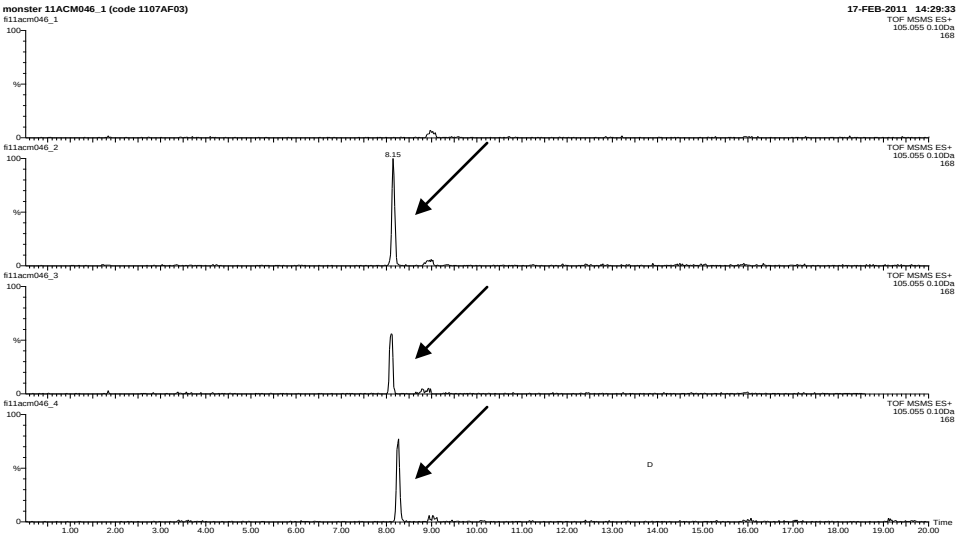

Figure 26 LC-ES MS/MS analysis of S-[2-(hydroxyethyl)thioethyl]-cysteine (MW 225) in pronase digests of guinea pig plasma, isolated after exposure to sulfur mustard (HD). Ion chromatograms of m/z 105.1, base peak in the product ion mass spectrum of the HD-adduct.

A, upper trace. Sample 1107AF03 (T = - 0.5 hours),

B, upper middle trace. Sample 1107AF04 (T = 0 hour),

C, lower middle trace. Sample 1107AF05 (T = 2.5 hours) and

D, lower trace. Sample 1107AF06 (T = 5 hours).

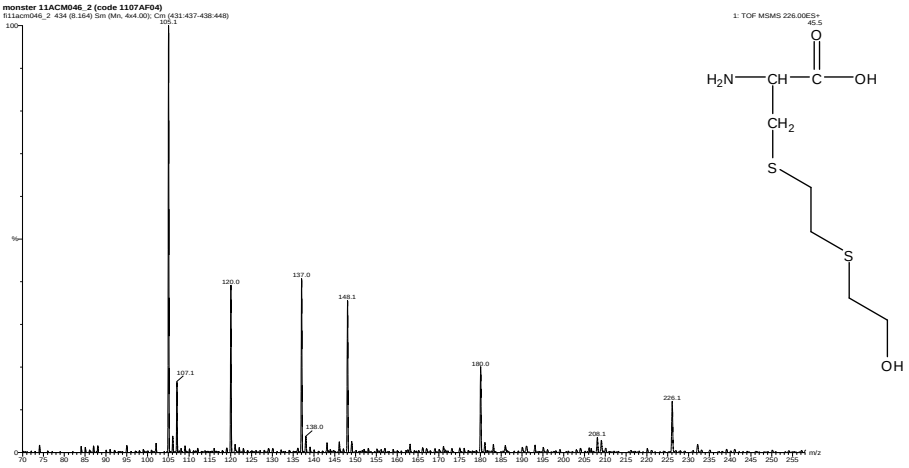

Figure 27 Product ion mass spectrum of the protonated molecular ion (MH+ 226) of S-[2-(hydroxyethyl)-thioethyl]cysteine in sample 1107AF04.

Task 4. Animal study – sulfur mustard vapor exposure

In this task, both the contact and evaporation hazard were studied after contamination of animals by whole body exposure to sulfur mustard vapor. The dose that was applied is 10,000 mg.min.m^{-3} (250 mg.m^{-3} during 40 minutes), equivalent with 1 LCt_{50} (percutaneous) or 12 LCt_{50} (inhalatory). This particular task was highly demanding with regards to the experimental set-up (safety!) and consequently a lot of time was spent in testing of the equipment (see Figure 28 and Figure 29 for a schematic representation), that has been used for several projects in the past (see e.g., Langenberg *et al.*, 1998 for use for exposure experiments with sulfur mustard). Most ignificant and remarkable part of the set-up is the feature of on-line analysis of agent within the exposure chamber, which allows the exact determination of the concentration of agent to which the animal is exposed to.

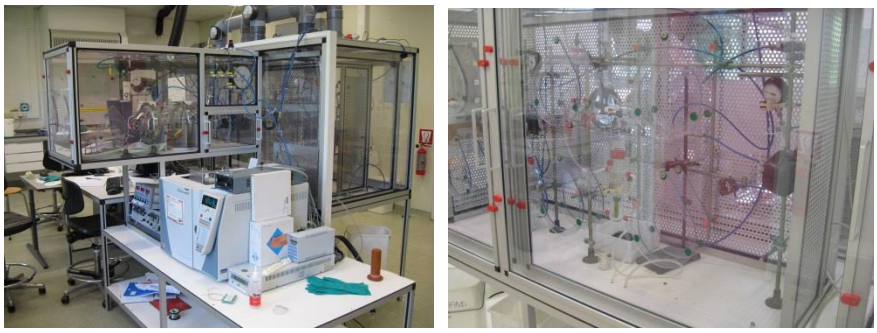

Figure 28 Vapor exposure equipment for whole body exposure of hairless guinea pigs to highly toxic chemicals. The left picture shows the entire set-up, with the on-line GC in the front, the exposure chamber on the left and the vapor generation unit on the right (in the back). The right picture shows the vapor generation unit.

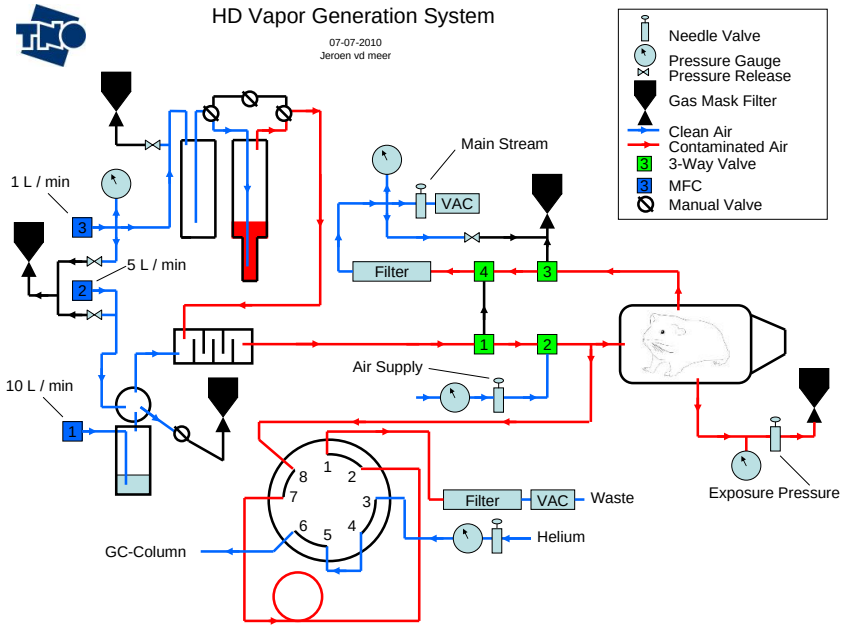

Figure 29 Schematic representation of vapor exposure equipment for whole body exposure of hairless guinea pigs to highly toxic chemicals.

First, the system was tested with the appropriate live agent (sulfur mustard): the desired concentration within the exposure chamber could be reached conveniently in a safe way within 15 minutets; see Figure 30 for a representative vapor concentration profile.

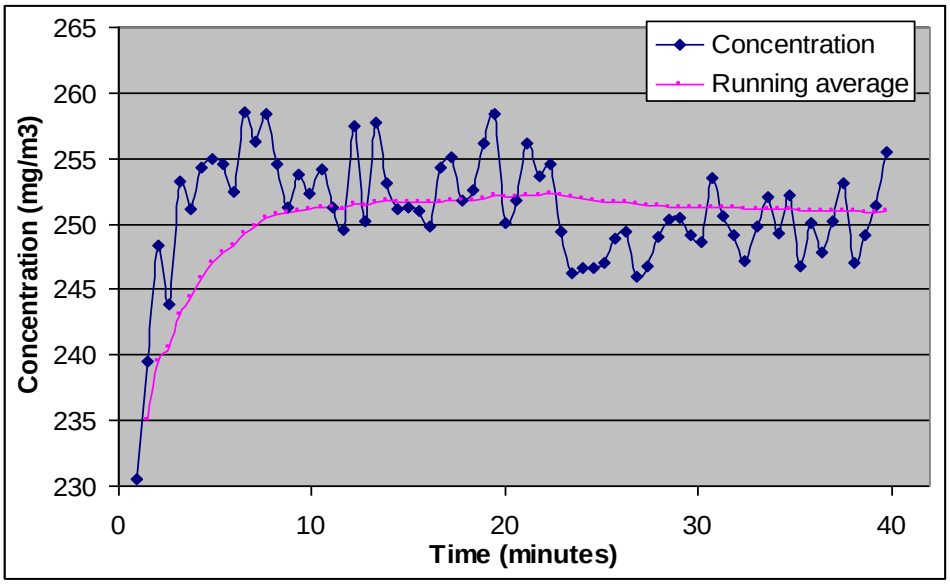

Figure 30 Representative example of a concentration profile of sulfur mustard, as generated during exposure of hairless guinea pigs to sulfur mustard vapor with the equipment depicted in Figures 28 and 29.

Two series of experiments have been conducted within this task. In the first series of experiments, the contact hazard and the hazard posed by biological samples (blood, liver) has been determined. In the second series, the vapor hazard for the vapor-exposed animals has been determined. Animal experiments were conducted on adult, anesthetized hairless guinea pigs, which were contaminated with a supralethal dose of sulfur mustard vapor by whole body exposure.

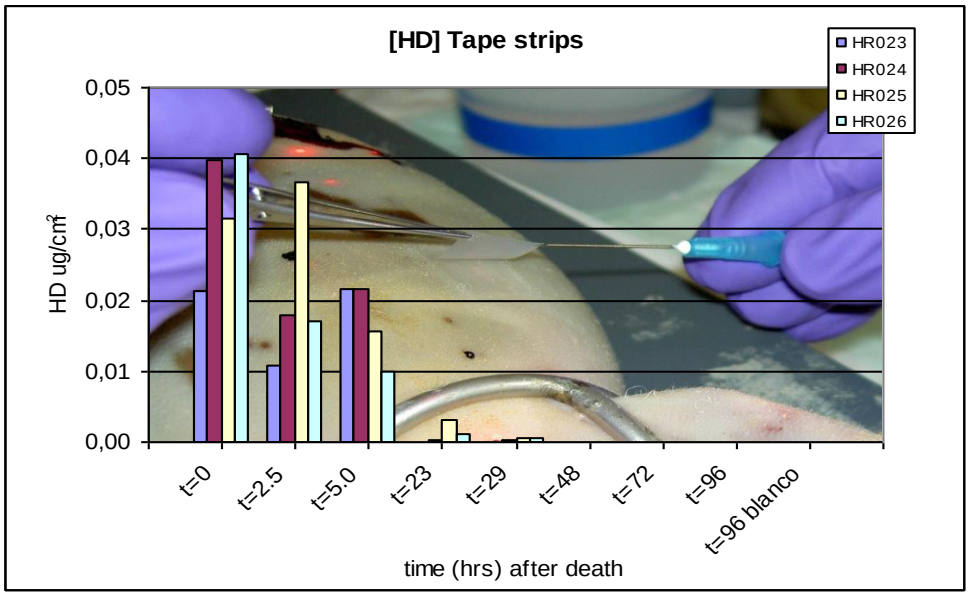

Figure 31 Sulfur mustard levels (expressed as µg sulfur mustard/cm^2) on skin at various sampling points as determined by tape stripping, after exposure of hairless guinea pigs (n=4) to sulfur mustard vapor (250 $mg.m^{-3}$) for 40 minutes.

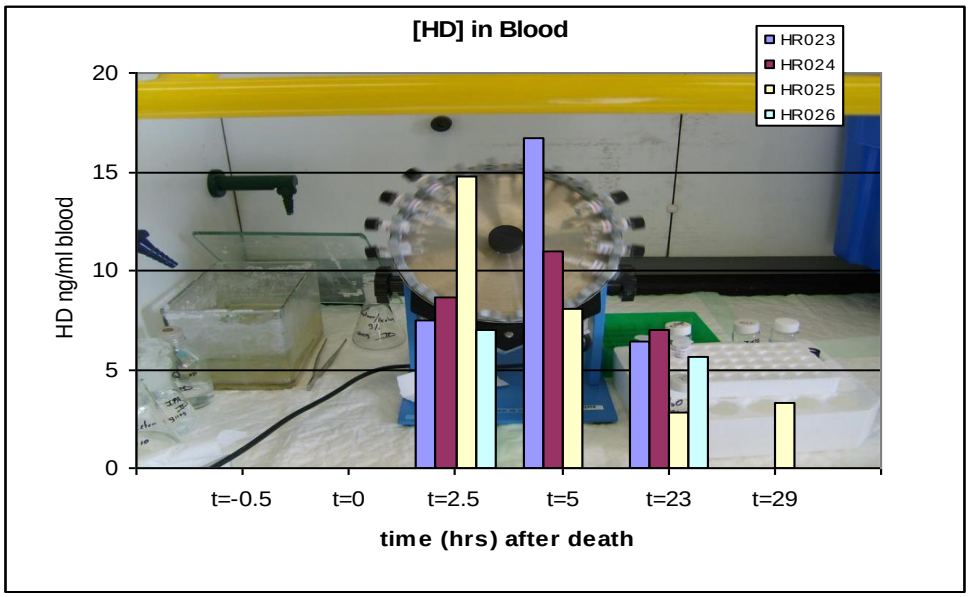

Figure 32 Measured concentrations of sulfur mustard in blood (expressed in ng sulfur mustard/mL blood) at various sampling points, as determined by means of GC-MS, after exposure of hairless guinea pigs (n=4) to sulfur mustard vapor (250 $mg.m^{-3}$) for 40 minutes.

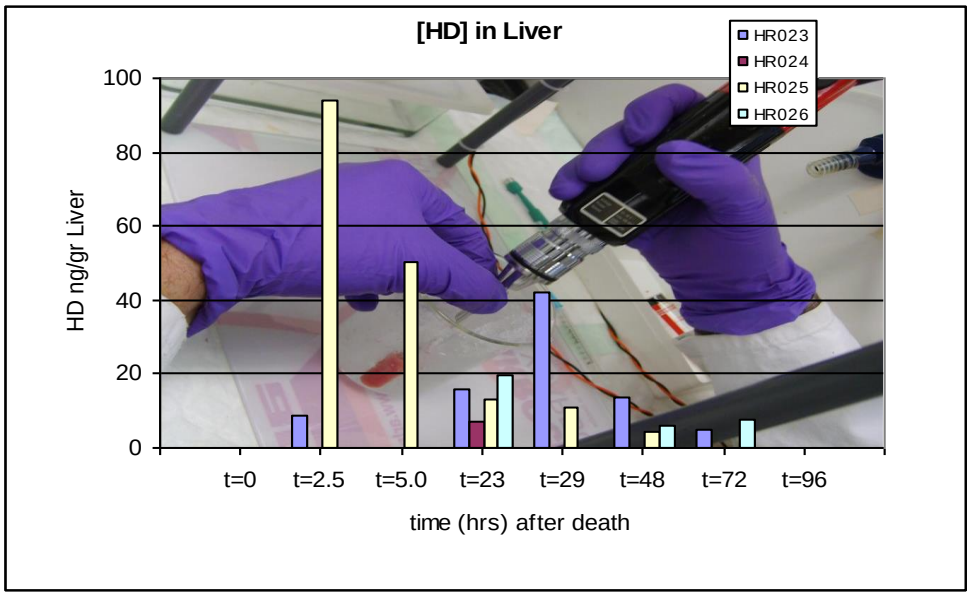

Figure 33 Measured concentrations of sulfur mustard in liver (expressed in ng sulfur mustard/g liver) at various sampling points, as determined by means of GC-MS, after exposure of hairless guinea pigs (n=4) to sulfur mustard vapor (250 $mg.m^{-3}$) for 40 minutes.

Compared to the experiments performed within Task 3 (sulfur mustard liquid exposure), the levels on skin are much lower (see Figure 31). This was to be expected, because in case of liquid exposure a relatively large amount of agent is localized on a limited surface area that is subsequently used for sampling. On the other hand, the internal levels of free agent (blood, liver) are in the same order of magnitude as found after percutaneous liquid exposure (see Figures 32 and 33). Apparently, in the time period that the living animal is exposed to the sulfur mustard vapor, it has been able to capture (through inhalation and through percutaneous uptake) significant levels of agent.

Subsequently, the evaporation hazard was studied after contamination of animals by whole body exposure to sulfur mustard vapor. The contaminated animals were placed in a glass chamber, in which a controlled air flow of 50 ml/min is sweeping over the animal. Animals were euthanized 30 minutes after contamination. The outlet air was lead through two bubble cell in series containing 1 mL solvent (diethyl succinate) each to sample the agent in intervals. The bubble cells were replaced after 1, 2, 3, 4, 6, 24, 48, 72 and 96 hours after euthanization of the animal. A known amount of deuterated sulfur mustard was added to the diethyl succinate as an internal standard. The collected solvent fractions were quantitatively analyzed by GC-MS; see Figure 34 for obtained results.

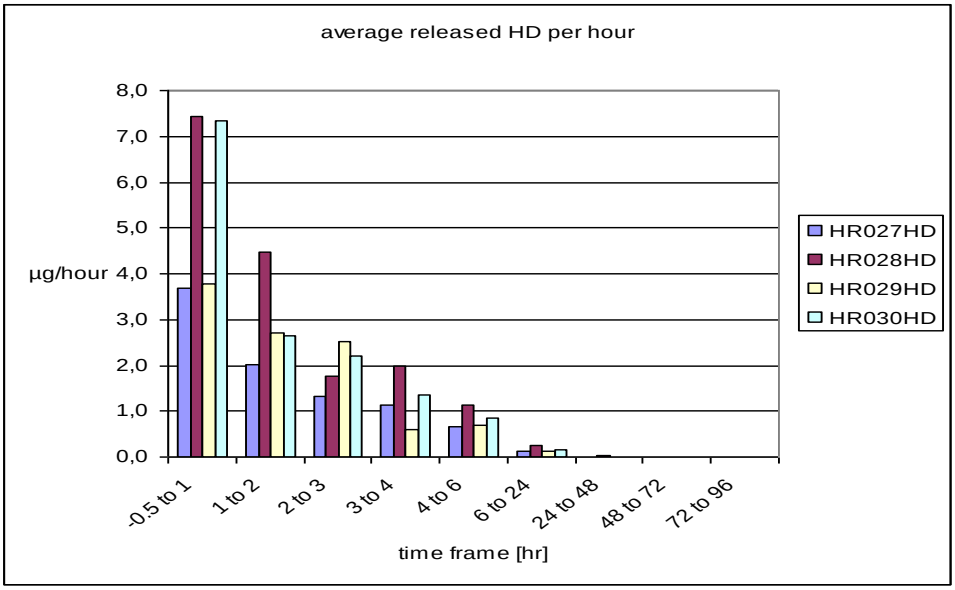

Figure 34 Released amount of sulfur mustard vapor, as determined by means of GC-MS, after vapor exposure of hairless guinea pigs (n=4) to sulfur mustard.

The off-gassing levels are significantly lower in comparison with the levels after liquid exposure, but still detectable amounts of vapor are present in the 48 – 72 hours fraction.
The response of a chemical agent monitor, an ICAM, was used to measure the released vapor above the animal. The ICAM was operated in the H-mode (sulfur mustard-mode). The nozzle of the ICAM was pressed against the air outlet of the glass chamber with the deceased and contaminated animal.

Table 6 Response of ICAM (in number of bars in the display) over time, after measuring released vapor from a hairless guinea pig that had been exposed to sulfur mustard vapor.

time (hour]	HR027HD	HR028HD	HR029HD	HR030HD
1	1	2	1	3
2	1	2	0	2
3	3	1	0	2
4	2	1	0	0
6	2	0	0	1
24	2	0	1	0
48	1	0	5	0
72	*	3	1	0
96	3	4	0	0

*measurement was disturbed by external chemicals

The results of the ICAM measurements are quite scattered (see Table 6) and do not really reflect the off-gassing vapor levels as determined by means of vapor collection followed by GC-MS analysis (see Figure 34). However, we feel that the vapor levels might be close to the detection limits of the detector. In a number of cases there is a significant response at later time points. This might be due to interfering chemicals resulting from extensive putrefaction at these time points, giving a high positive response on the ICAM in the H-mode.

Task 5: Animal study at low temperature – liquid sulfur mustard on skin

Two series of experiments have been carried out during which the contaminated animals were stored in a closed box at low temperature, mimicking the procedure of refrigerated storing of human remains at a mortuary. From the experiments under Task 3 it was clear that especially the skin surface levels rapidly decreased and that already at t = 2.5 hours no intact agent could be analyzed anymore. In addition, it is rather illogical to store the remains of a deceased victim for only 24 hours, and subsequently at ambient temperature. We therefore decided to analyze the agent levels under continuous cooling. Although not part of the original Statement of Work, we have also studied the fate of the agent while the animal was stored at the low temperature, by analyzing blood and liver samples.
Unfortunately, the refrigerator with which the initial two experiments were carried out (animals HR015HD and HR016HD) failed to cool to the desired temperature, for unknown reasons. Maybe this was due to the fact that it was located in a fume hood with a high airflow; the lowest possible temperature with this refrigerator was 10 °C. These experiments have been repeated by using the other refrigerator.

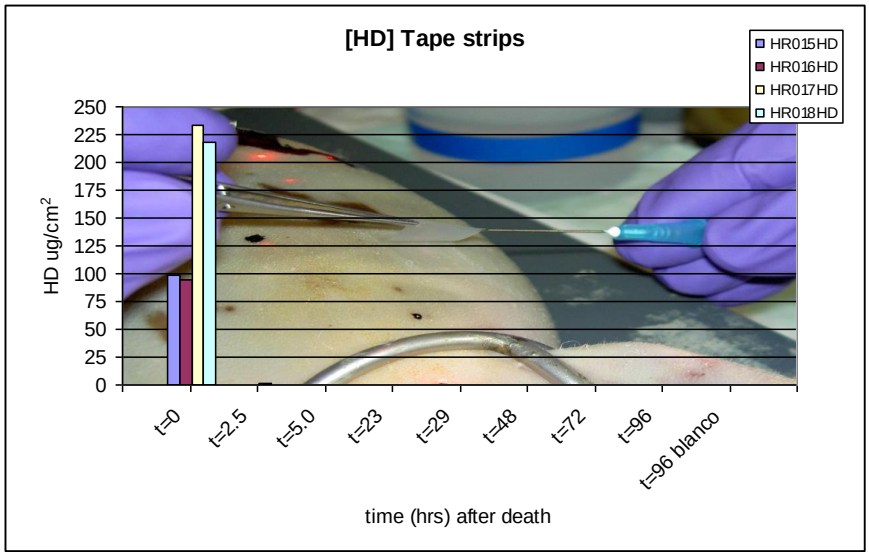

Figure 35 Sulfur mustard levels (expressed as µg sulfur mustard/cm^2) on skin at various sampling points as determined by tape stripping, after percutaneous exposure of hairless guinea pigs (n=4) to liquid sulfur mustard. The remains were stored under cooled conditions: animals HR017HD and HR018HD were kept at 5-7 °C and animals HR015 and HR016 at 10-12 °C.

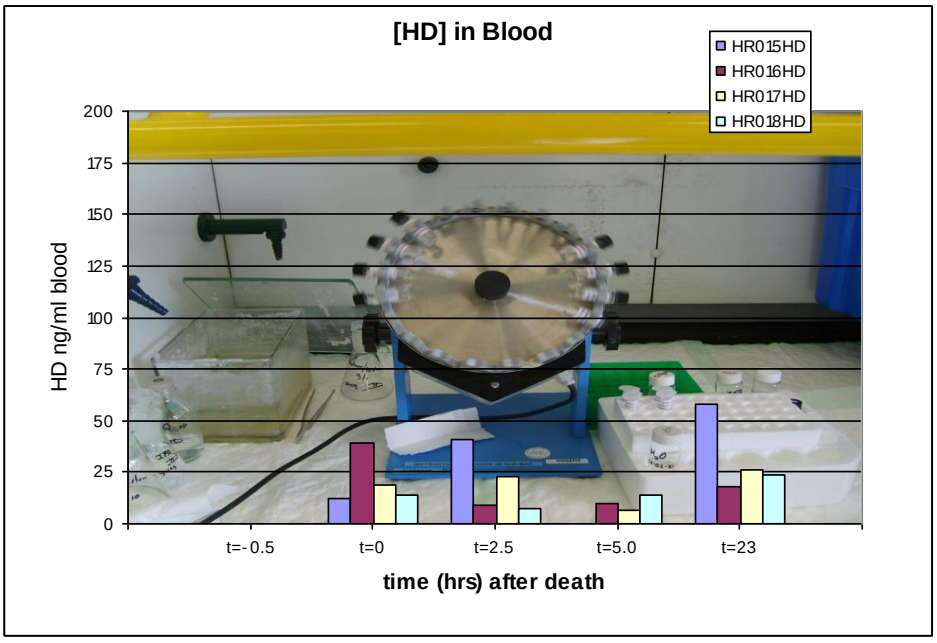

Figure 36 Measured concentrations of sulfur mustard in blood (expressed in ng sulfur mustard/mL blood) at various sampling points, as determined by means of GC-MS, after percutaneous exposure of hairless guinea pigs (n=4) to liquid sulfur mustard. The remains were stored under cooled conditions: animals HR017HD and HR018HD were kept at 5-7 °C and animals HR015 and HR016 at 10-12 °C.

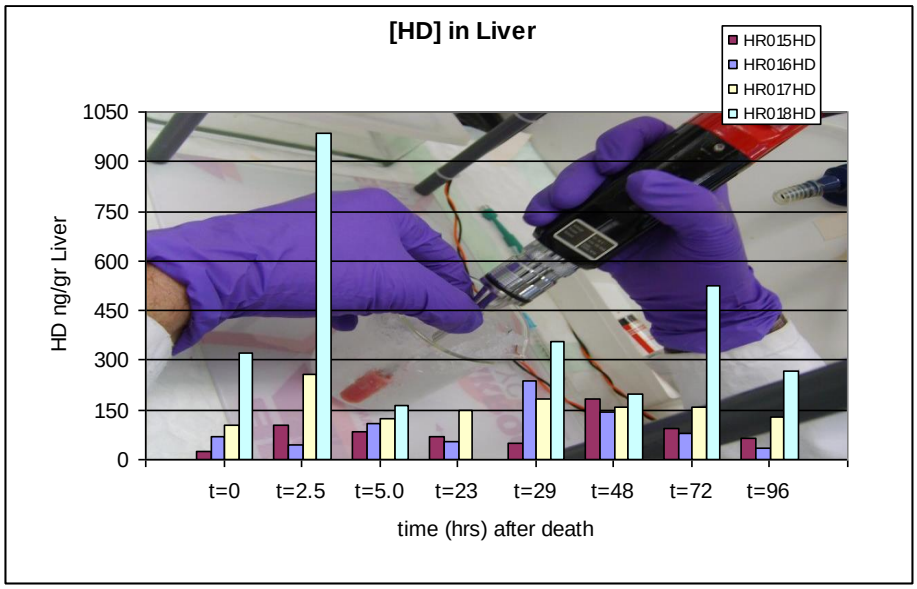

Figure 37 Measured concentrations of sulfur mustard in liver (expressed in ng sulfur mustard/g liver) at various sampling points, as determined by means of GC-MS, after percutaneous exposure of hairless guinea pigs (n=4) to liquid sulfur mustard. The remains were stored under cooled conditions: animals HR017HD and HR018HD were kept at 5-7 °C and animals HR015 and HR016 at 10-12 °C.

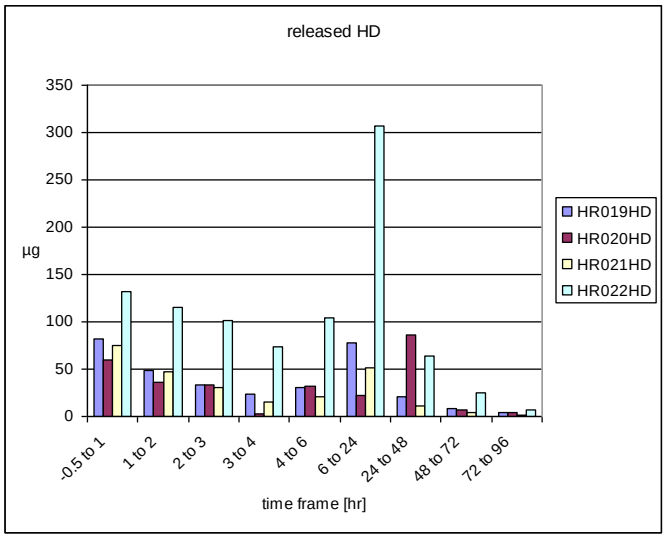

Figure 38 Released amount of sulfur mustard vapor, as determined by means of GC-MS, after percutaneous exposure of hairless guinea pigs (n=4) to liquid sulfur mustard (8 x 1 µL). The remains were kept at 5-7 °C.

Also in this case, intact agent could hardly be detected at t = 2.5 hours (see Figure 35). Also, it appears that the agent levels in blood and liver are significantly higher at lower temperature than at ambient temperature (Figures 36 and 37).
With regard to the vapor hazard, the initial (- 0.5–1 hours) amount of vapor off-gassing from the animal is significantly lower at lower temperature (see Figure 38). On the other hand, based on this limited number of experiments it seems that off-gassing occurs for a longer period of time when the animal is kept under cooled conditions. This is most likely caused by the decrease in volatility/reactivity of the agent at lower temperatures.

As described above, the refrigerator with which the initial two experiments were carried out (animals HR015HD and HR016HD) failed to cool to the desired temperature, for unknown reasons. We here present the data for the animals that had been stored at the right temperature (5-7 °C); see Figures 39-41. Initial experiments had shown that cooling of the remains slightly increased the persistency of the agent, especially in blood and liver, and seems to prolonge the vapor hazard. In case of tape stripping, the results were highly variable. This might be due to the fact that because of the low temperature, the tape stripping procedure is difficult to perform because of the moisture. Also, the moisture will promote the hydrolysis of sulfur mustard; this might also contribute to the significantly lower concentrations at 5-7 °C.

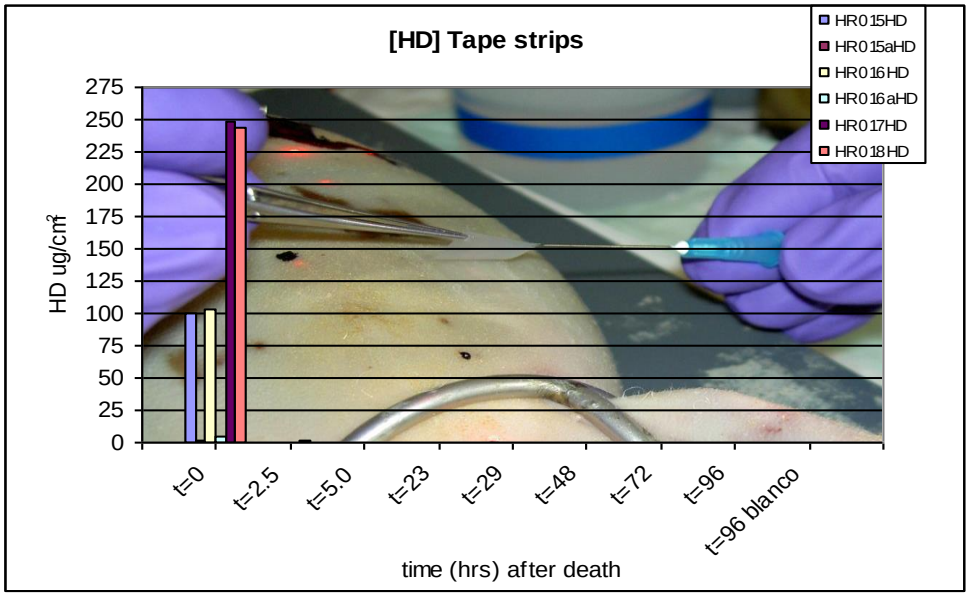

Figure 39 Sulfur mustard levels (expressed as µg sulfur mustard/cm^2) on skin at various sampling points as determined by tape stripping, after percutaneous exposure of hairless guinea pigs (n=4) to sulfur mustard. The remains were stored under cooled conditions: animals HR015a, HR016b, HR017HD and HR018HD were kept at 5-7 °C and animals HR015 and HR016 at 10-12 °C.

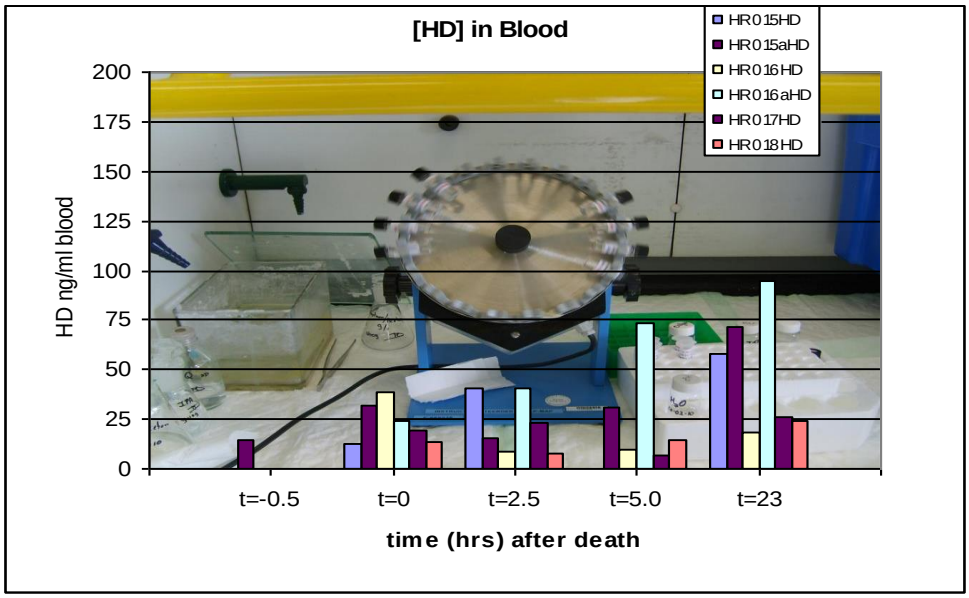

Figure 40 Measured concentrations of sulfur mustard in blood (expressed in ng sulfur mustard/mL blood) at various sampling points, as determined by means of GC-MS, after percutaneous exposure of hairless guinea pigs (n=4) to sulfur mustard. The remains were stored under cooled conditions: animals HR015a, HR016b, HR017HD and HR018HD were kept at 5-7 °C and animals HR015 and HR016 at 10-12 °C.

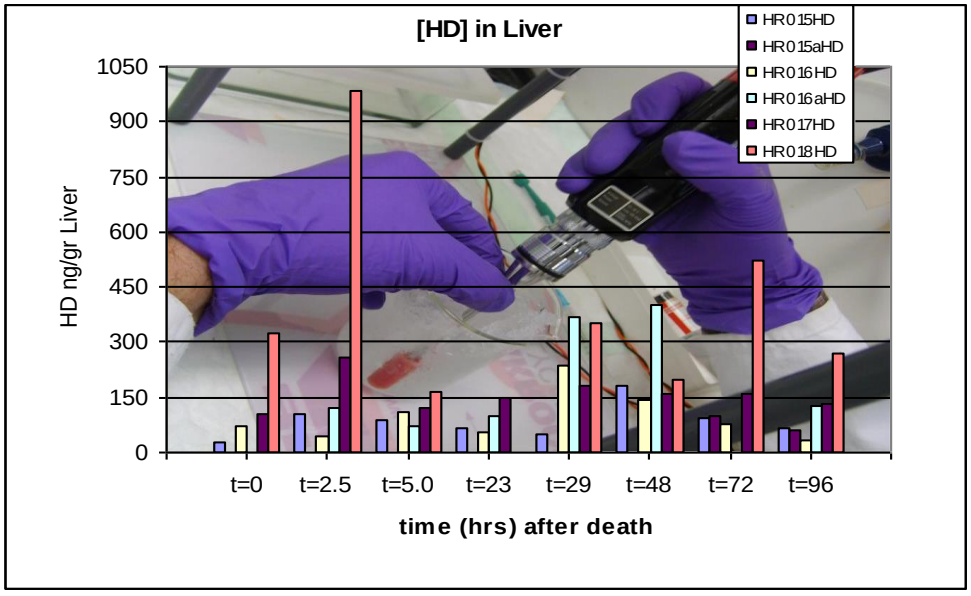

Figure 41 Measured concentrations of sulfur mustard in liver (expressed in ng sulfur mustard/g liver) at various sampling points, as determined by means of GC-MS, after percutaneous exposure of hairless guinea pigs (n=4) to sulfur mustard. The remains were stored under cooled conditions: animals HR015a, HR016b, HR017HD and HR018HD were kept at 5-7 °C and animals HR015 and HR016 at 10-12 °C.

In case of animals HR015HD and HR016HD skin samples were cut out from the exposure sites, after tape stripping for the last sampling point, at t = 96 hours. The samples were processed by extraction and the extracts were analyzed with GC-MS. No intact sulfur mustard could be analyzed. This result is in accordance with the fact that no intact agent could be analyzed anymore after tape stripping at t = 96 hours. Probably, remaining agent has either evaporated, absorbed to underlying tissues, or reacted with cellular constituents within the skin (water, proteins, DNA).

Task 6. Physiologically-based pharmacokinetic (PBPK) modeling

Physiologically Based Pharmacokinetic (PBPK) models are mathematical models that can be used to simulate the pharmacokinetics/toxicokinetics of xenobiotics. The model consists of different compartments inspired from different organs, in which different kinetic conditions exist for the compound of interest. A PBPK model can be used to investigate the influence of dosing on the toxicokinetics, optimize therapeutic strategies and is ultimately useful for the extrapolation to man if the human biochemical parameters are available for each compartment. A PBPK model describing the toxicokinetics of VX in the guinea pig was developed recently. The current PBPK model consists of five compartments: richly perfused tissue, poorly perfused tissue, liver, skin and blood. Since the reactions take place in blood as well, blood is also considered as a compartment in the model. In this project we can consider three stages in the process that is visualized in Figure 42. Stage 1 (0-30 minutes) describes the situation in which the animal is exposed to VX, but is still alive. Characteristic about this situation is that the blood circulation is still intact, which is reflected in the differential equations for each compartment. In stage 2 (30-720 minutes), the animal is dead, which means that the circulation has stopped. In the (postmortem model) the mass transfer component between the compartments has been set to zero, while the other kinetic conditions (enzymatic hydrolysis and binding to enzymes) remain. Stage 3 (12-36 hours) describes the situation in which the degradation of the corpse is in an advanced state. Cellular boundaries between the compartments disappear and residual VX is dispersed through the corpse based on diffusion.

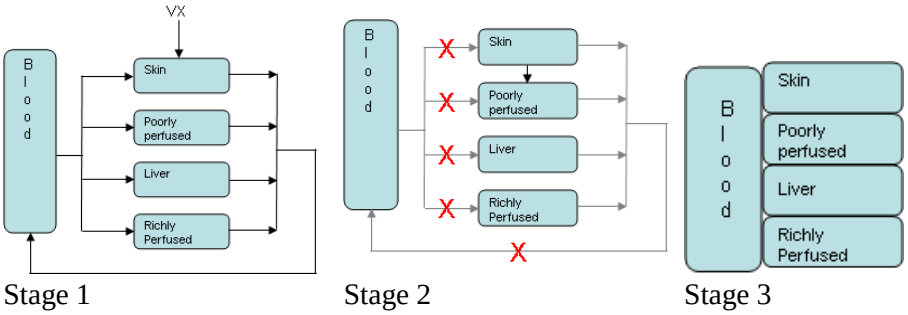

Figure 42 Graphical representation of the mathematical model of the three stages describing the fate of VX in the animal corpse. Stage 1: Animal alive, circulation intact, Stage 2: Animal dead, circulation stopped, enzyme activities in compartment still exist, Stage 3: Further disintegration of the animal corpse, during which boundaries between compartments disappear.

Figure 43 shows the calculated curves of VX isomers and the toxicokinetic data from the publication of Van der Schans *et al*,. (2003). Those toxicokinetic studies were determined in anesthetized, atropinized and artificially ventilated hairless guinea pigs. The i.v. data and the percutaneous data show a rather good fit with the calculated curves.

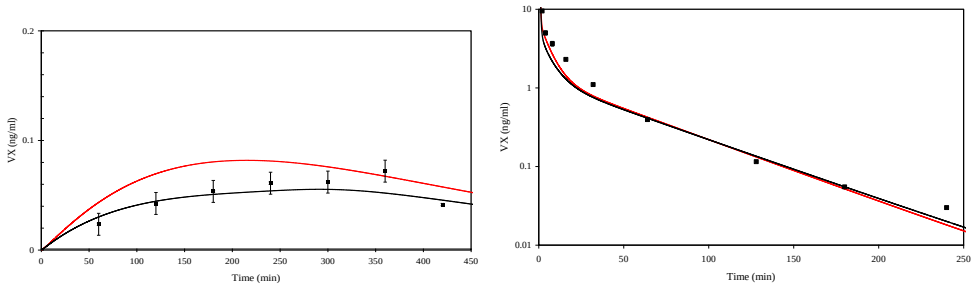

Figure 43 Predicted concentration curves using the PBPK model. Concentration of (+)-VX (red) and (-)-VX (black) in blood of atropinized anesthetized and artificially ventilated hairless guinea pigs. Data points from Van der Schans *et al.*, 2003. Left: IV administration 28 µg/kg, Right: percutaneous exposure to 125 µg/kg VX.

Figure 44 shows the concentration of the VX enantiomers in blood of the hairless guinea pig after percutaneous exposure of VX at a dose of 16 mg/kg. The biochemical parameters were the same as used for the i.v. and p.c. toxicokinetic studies except for the permeability coefficient speed of VX through the skin which was set at 15% of the original value. In the toxicokinetic experiments with living animals, VX was dissolved in isopropanol (IPA) while in the current experiments VX was applied as neat fluid; it is anticipated that the permeability of VX through the skin as neat fluid is lower than when dissolved in IPA. The maximum concentration of VX at 30 minutes is in agreement with the levels of VX in blood of the hairless guinea pig (see Figure 45). The PBPK model also predicts the AChE and BuChE activity. Both enzymes in blood were completely inhibited at 25 minutes after exposure. At 30 minutes after application of the agent the guinea pig was euthanized. At that time the blood circulation stopped but it is assumed that the enzymatic activity remains constant. Once the circulation has stopped (stage 2), the uptake of VX from the skin in blood is ceased, visualized as a rapid decrease of the VX concentration in blood. However, according to the data in Figure 45, the decrease of VX concentration in blood is not that rapid as predicted by the model. Apparently, the enzymatic hydrolytic activity in a deceased animal is much lower than in the living animal.

Figure 46 shows the decrease of VX in blood where the hydrolytic enzymatic activity is set at 1% of the original value. Indeed the decrease of the VX concentration proceeds less rapid, comparable to the concentration presented in Figure 45. Figure 45 showed also that after 2.5 hours the VX concentration in blood remains constant. It was anticipated that the enzymatic hydrolytic activity in the deceased animal is almost negligible and residual VX will persist longer in a dead animal than in a living organism.

The degradation of VX is then determined by chemical hydrolysis only, which is very slow (typical half-life 50 hours).
Stage 3 in the degradation process is determined by the release of VX from the skin compartment, where the diffusion is no longer limited by cellular boundaries. This process is hard to predict in a model since diffusion coefficients in weakened tissue are not available. Accelerated diffusion of VX from the skin to the rest of the body is reasonable in view of the high concentrations of VX that were found in the liver at 24 hours after euthanasia of the animal.

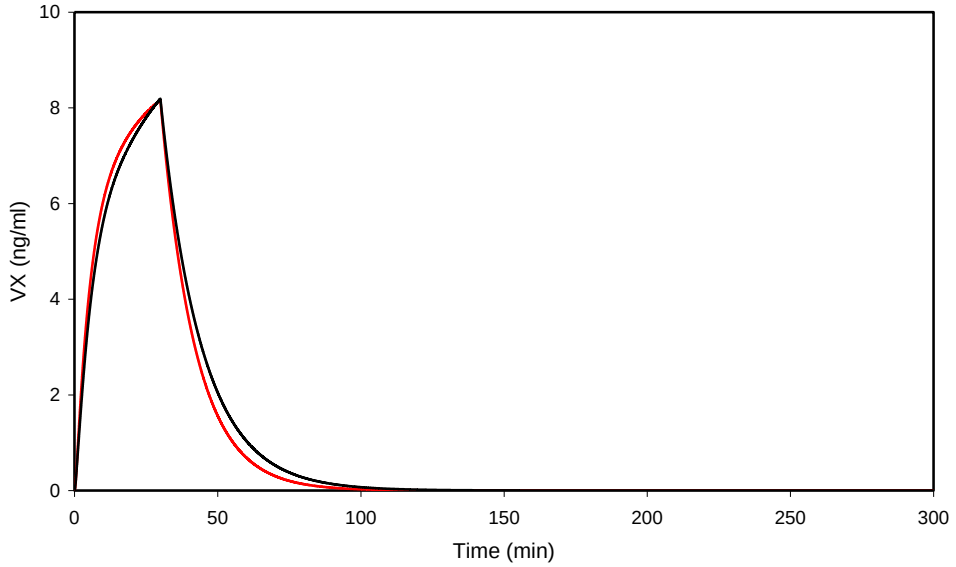

Figure 44 Predicted concentration curves using the PBPK model. Concentration of (+)-VX (red) and (-)-VX (black) in blood of hairless guinea pig after percutaneous exposure of VX (16 mg/kg). Animal was euthanized at 30 minutes after exposure. Note that in this figure t=0 is time of application of agent.

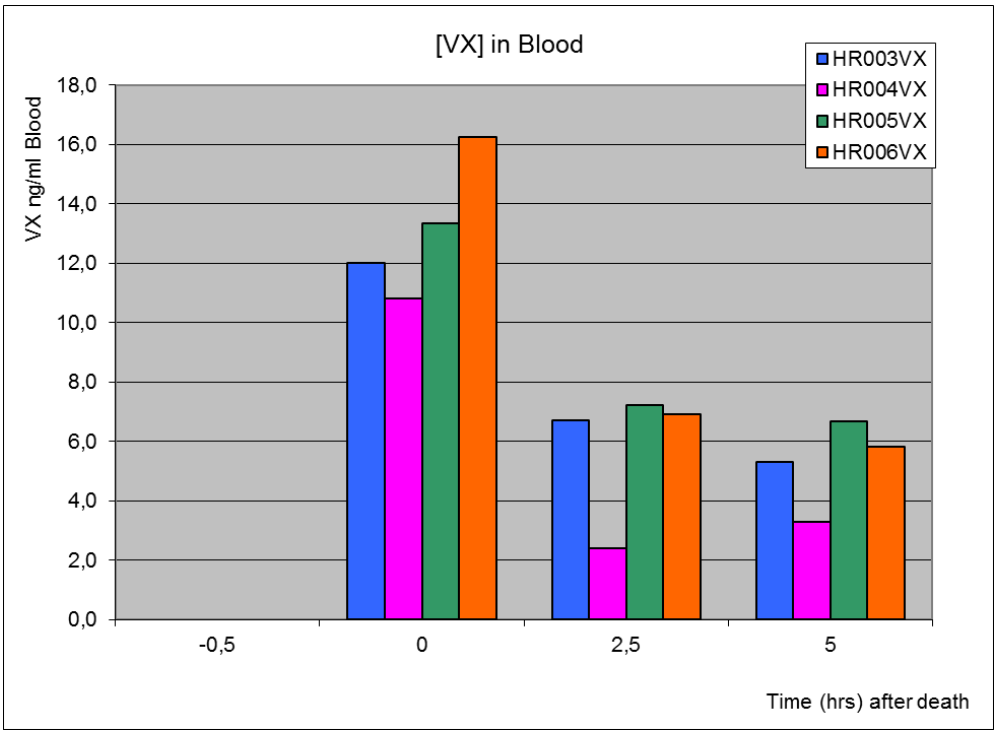

Figure 45 Measured concentrations of VX in blood (expressed in ng VX/mL blood) at various sampling points, as determined by means of LC-tandem MS, after percutaneous exposure of hairless guinea pigs (n=4) to VX. Note that in this figure t = 0 is time of euthanasia.

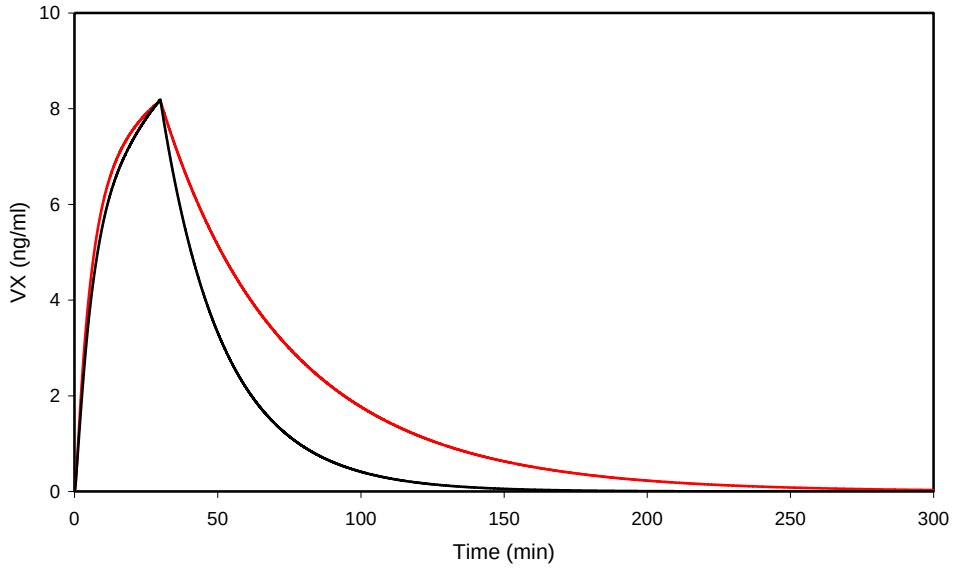

Figure 46 Predicted concentration curves using the PBPK model. Concentration of (+)-VX (red) and (-)-VX (black) in blood of hairless guinea pig after percutaneous exposure of 16 mg/kg. Animal was euthanized killed at 30 minutes after exposure. Hydrolytic activity of VX in blood of a dead animal was set at 1% of the original value. Note that in this figure t=0 is time of application of agent.

Task 7. Hazard posed by contaminated clothing

In order to investigate the hazard of CWA's remaining on personal effects of CWA-contaminated victims, experiments were performed in which two different types of material (US Army clothing and boots; see Figure 47) were contaminated with sulfur mustard, VX and sarin (10 g/m^2, triplicate experiments). The evaporation hazard (sarin and sulfur mustard) was determined in an analogous way as described for Task 3, i.e., with 8 x 1 µL droplets on 8 separate pieces of material of 1.13 cm^2 and by using a similar evaporation chamber.

Figure 47 Examples of test samples prepared from US Army clothing and boots.

Since in case of boots, 3 completely different materials (suede-type leather, synthetic upper material, rubber sole) can be distinguished, it was first determined within a pilot experiment which type of material poses the largest risk in terms of contact and evaporation hazard. Sulfur mustard was selected as most representative chemical agent for these experiments. It was found that suede leather and rubber constitute the largest potential hazard after contamination with sulfur mustard (see Figure 48); consequently, these were selected for the definitive series of experiments. For a limited number of samples, analysis of remaining agent within the material has also been performed.

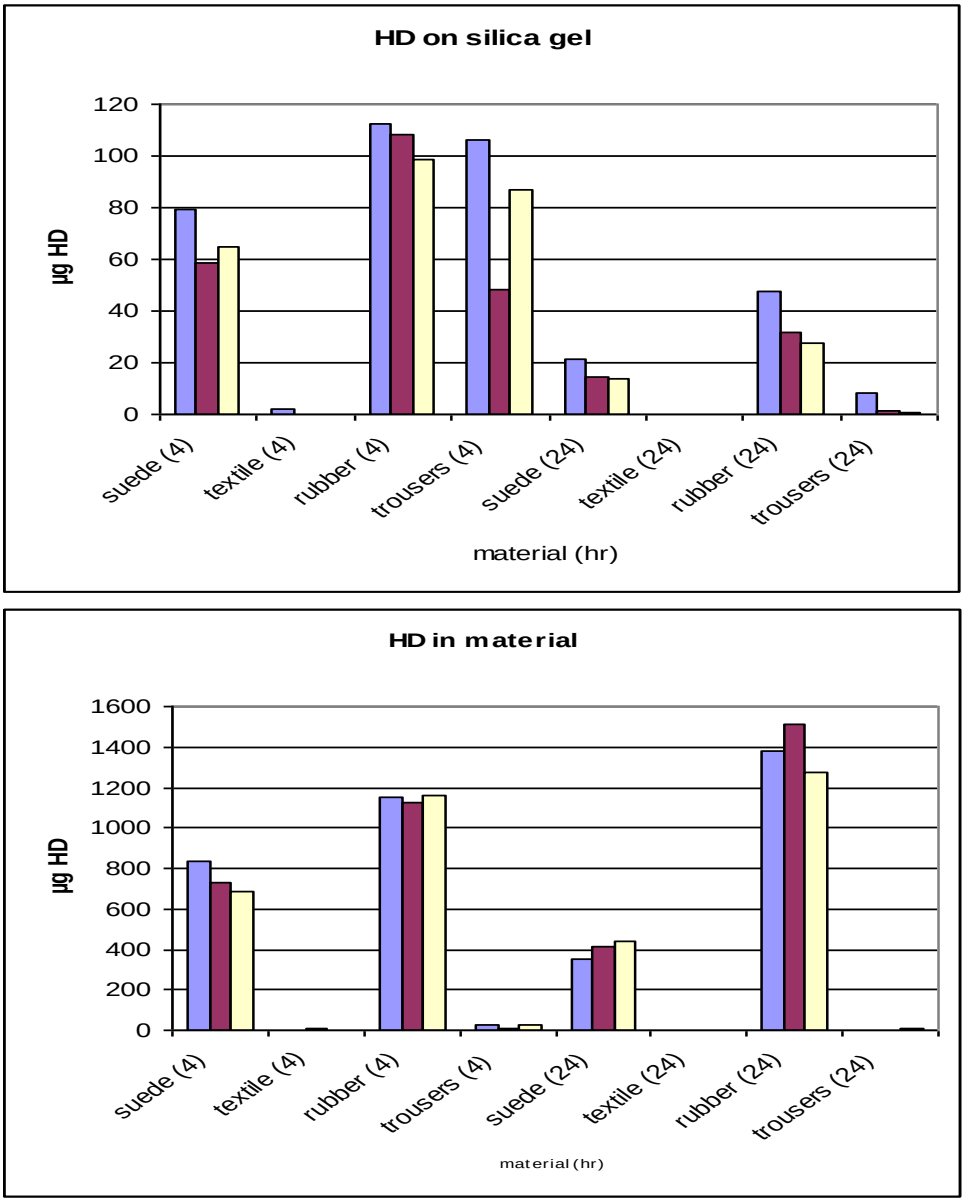

Figure 48 Pilot experiment to select most relevant material for further experiments: contact hazard by sulfur mustard at two different time points (4 and 24 hours) after contamination of various materials (suede leather, textile and rubber soles of US Army boots, and material of US Army trousers) at various time points, as determined by an adsorption procedure with aluminum sheets covered with silica gel, extraction and GC-MS analysis (upper panel). Corresponding levels of remaining sulfur mustard in the various matrices, after extraction with ethyl acetate and GC-MS analyis (lower panel).

Determination of contact risk; sulfur mustard

The contact risk of VX and sulfur mustard was studied by quantification of CWA's on adsorbent material (aluminum sheets covered with silica gel) after contact with contaminated materials under a fixed pressure (20 g/cm^2). One contact time (15 min) was used. Silica gel plates were extracted for 2 hours with ethyl acetate and extract analyzed with GC-MS, using d_8-HD as internal standard. Furthermore, the matrix was extracted for remaining agent by extraction with ethyl acetate for 2 hours (not included in SoW).

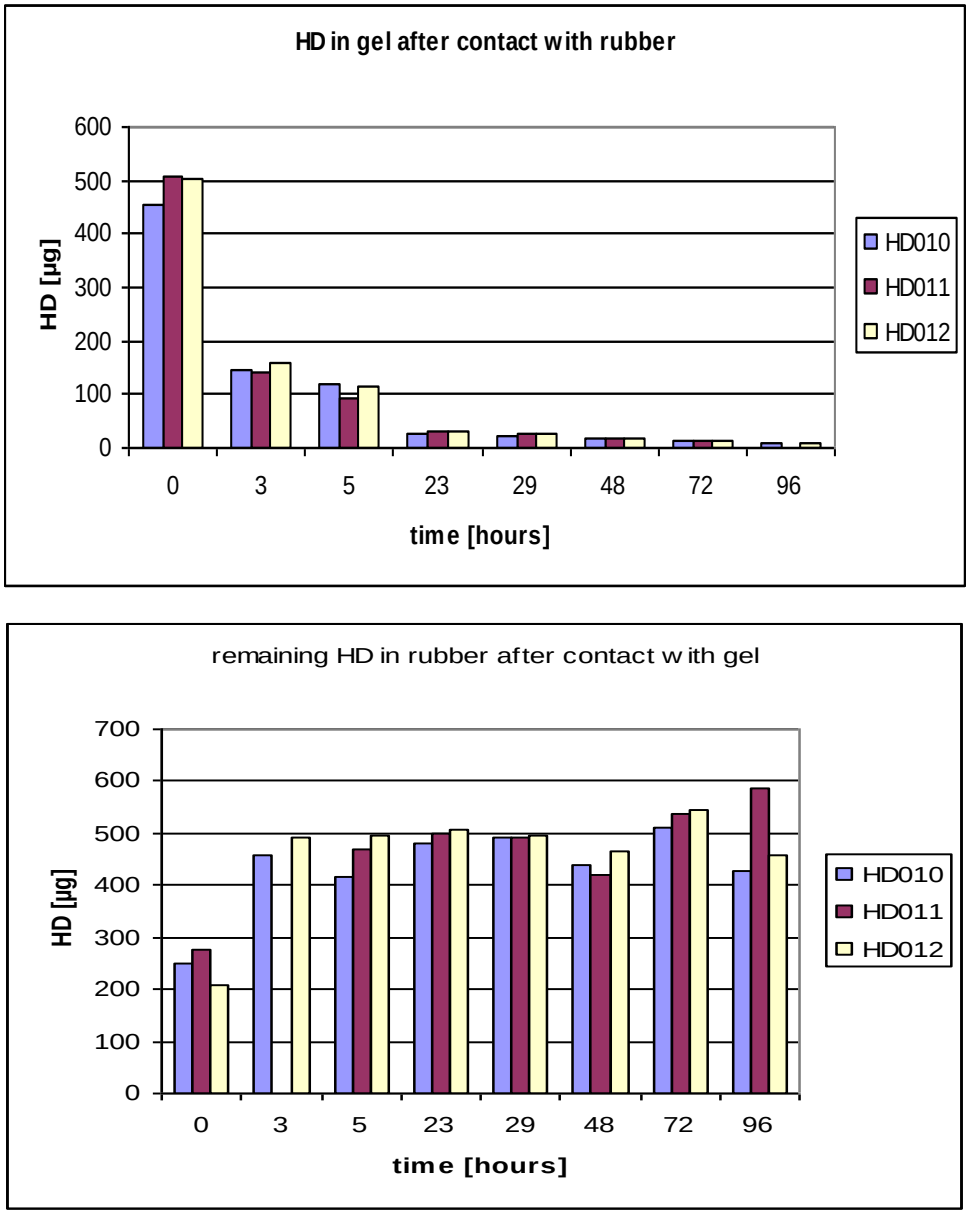

Figure 49 Contact hazard by sulfur mustard after contamination of rubber soles of US Army boots, as determined by adsorption procedure with aluminum sheets covered with silica gel, extraction and GC-MS analysis (upper panel). Remaining sulfur mustard in rubber, after extraction with ethyl acetate and GC-MS analyis (lower panel).

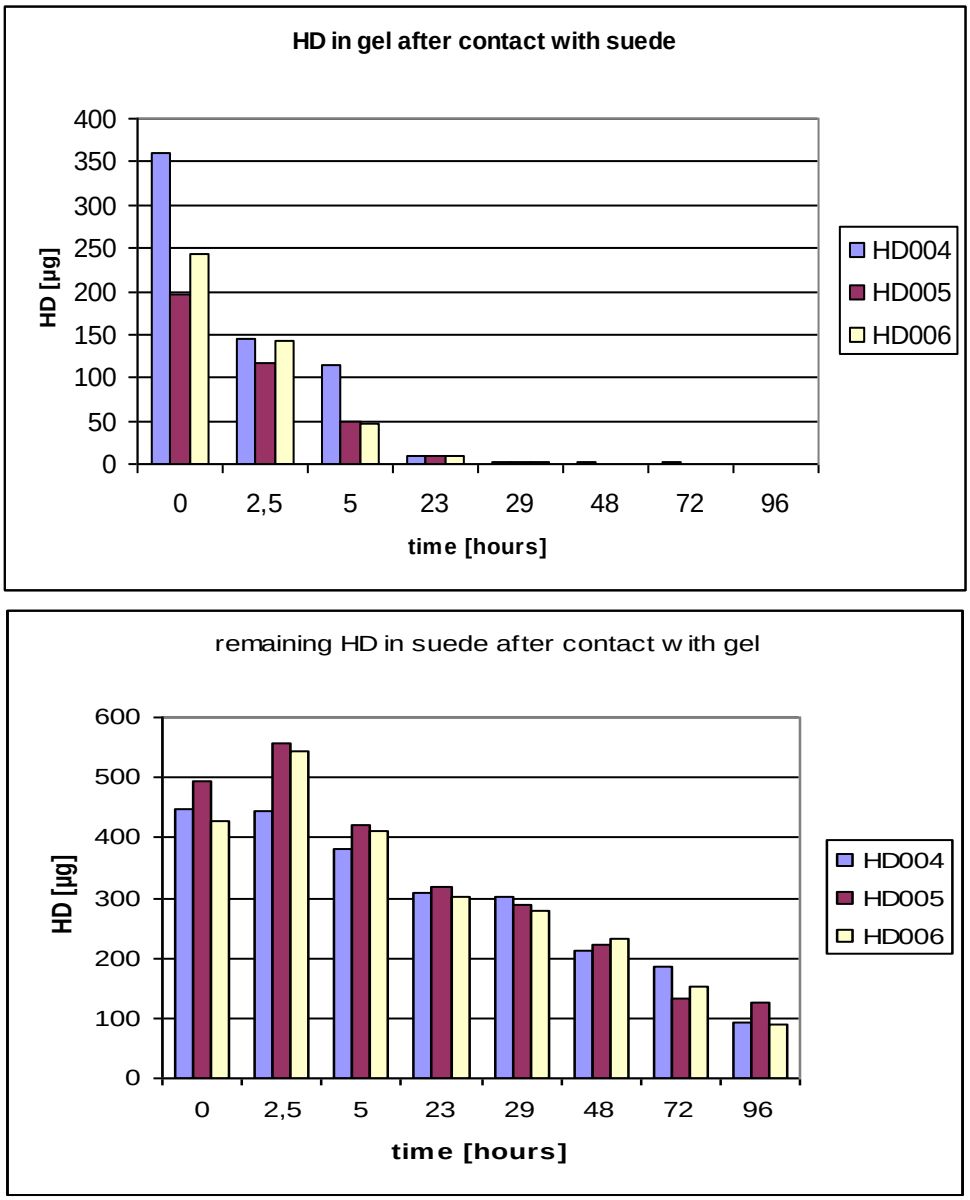

Figure 50 Contact hazard by sulfur mustard after contamination of suede leather of US Army boots, as determined by adsorption procedure with aluminum sheets covered with silica gel, extraction and GC-MS analysis (upper panel). Remaining sulfur mustard in suede leather, after extraction with ethyl acetate and GC-MS analyis (lower panel).

It turned out that sulfur mustard is slightly more persistent *on* rubber of US Army boots than on the suede leather of these boots, and poses a contact hazard for a long period of time (at 96 hours, the agent is still adsorbed on silica at low microgram levels). In addition, sulfur mustard is slightly more persistent *in* rubber of US Army boots than in the suede leather of these boots (see Figures 49 and 50).

Determination of contact risk; VX

In a similar way, experiments with VX were carried out. In case of suede leather, no VX (nor internal standard!) could be analyzed after applying the adsorption procedure. We suspect that something went wrong with these samples. The levels of remaining VX within the suede were fairly high, even after 96 hours (see Figure 51), though much lower than in case of sulfur mustard. The latter observation might also indicate that this particular experiment should be repeated. In case of the rubber soles, the agent levels on the material (causing a contact hazard) decreased more rapidly than in case of sulfur mustard. This might be caused by a different rate in uptake of the agents within the rubber. The amount of agent within the rubber material remained at a high level for up to 96 hours (see Figure 52), similar to sulfur mustard within the rubber material.

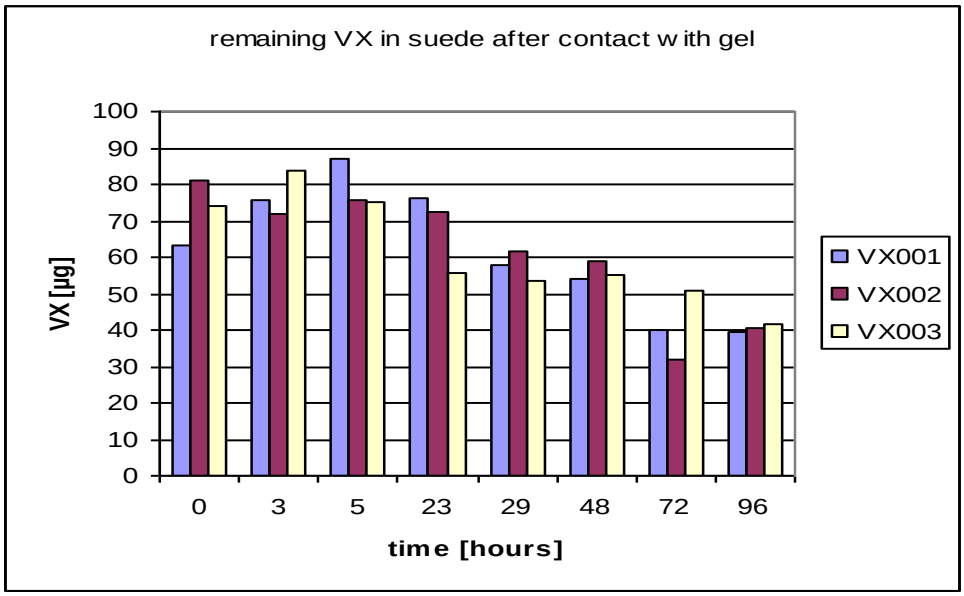

Figure 51 Remaining VX in suede leather of US Army boots, after extraction with ethyl acetate and GC-MS analyis.

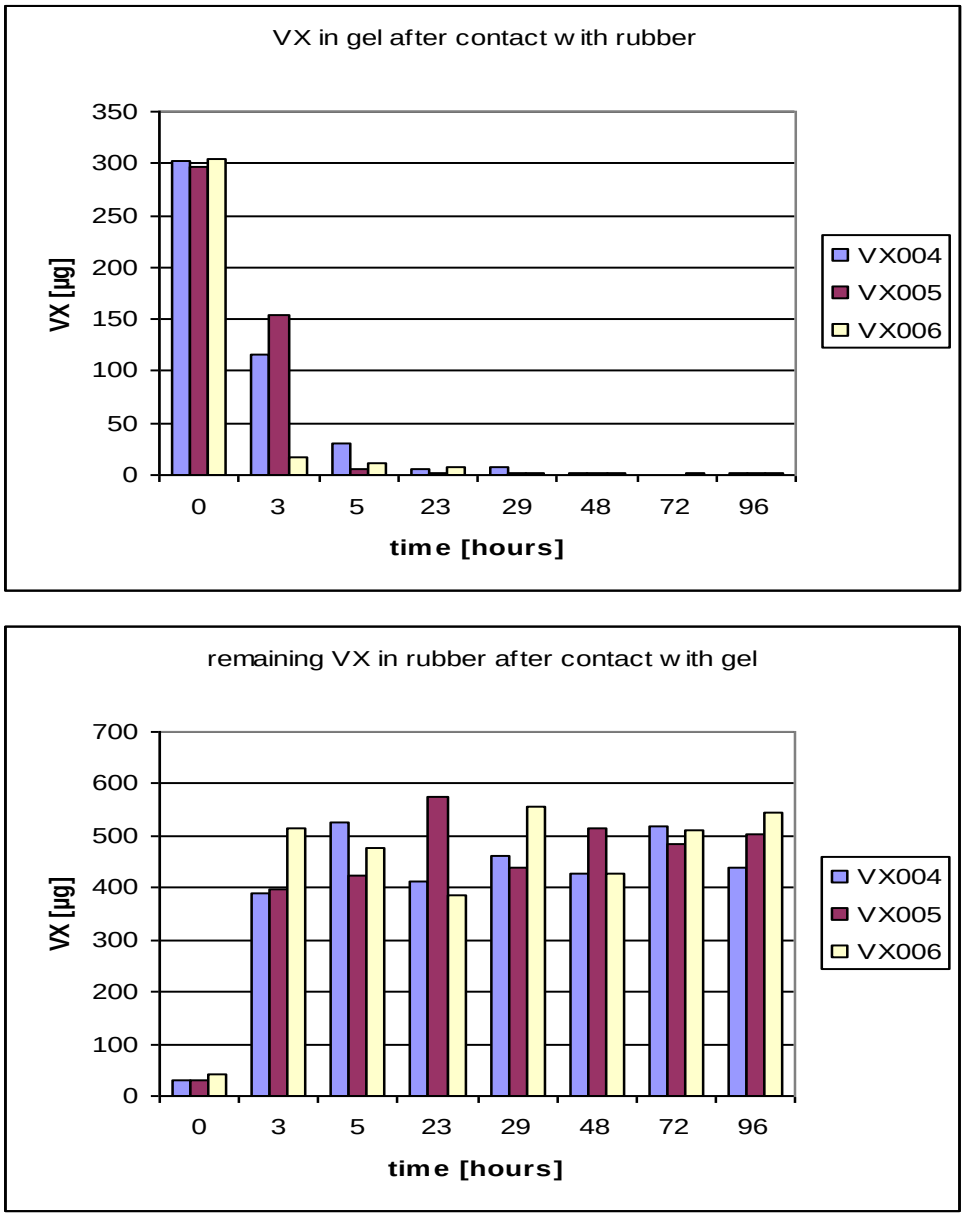

Figure 52 Contact hazard by VX after contamination of rubber of US Army boots, as determined by adsorption procedure with aluminum sheets covered with silica gel), extraction and GC-MS analysis (upper panel). Remaining VX in matrix, after extraction with ethyl acetate and GC-MS analyis (lower panel).

Off-gassing; sulfur mustard

The evaporation risk was determined in an analogous way as described for Task 3, i.e., with 8 x 1 µL droplets on a 8 separate pieces of material of 1.13 cm^2 and by using a similar evaporation chamber (see Figure 53). An air flow of 50 mL/min was applied; air was lead through bubble cell with DES. The bubble cell was replaced at t = 1, 2, 3, 4, 6, 24, 48, 72 and 96 hours. The DES was analyzed with GC-MS, with d_8-HD or d_7-GB as internal standard. Off-gassing of sulfur mustard from US Army clothing and suede of US Army boots slowly builds up, is most pronounced during 2-4 hours after

contamination, and decreases slowly (see Figures 54 and 55). The response of the detector is high, for up to 48 hours (see Tables 7 and 8).

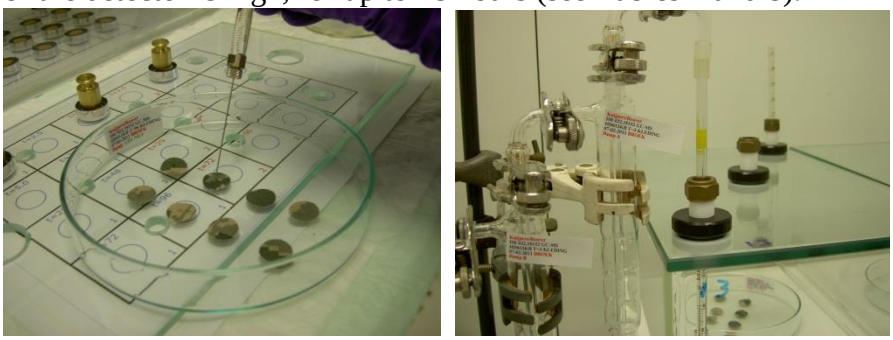

Figure 53 Experimental set-up to determine off-gassing of sulfur mustard from various materials.

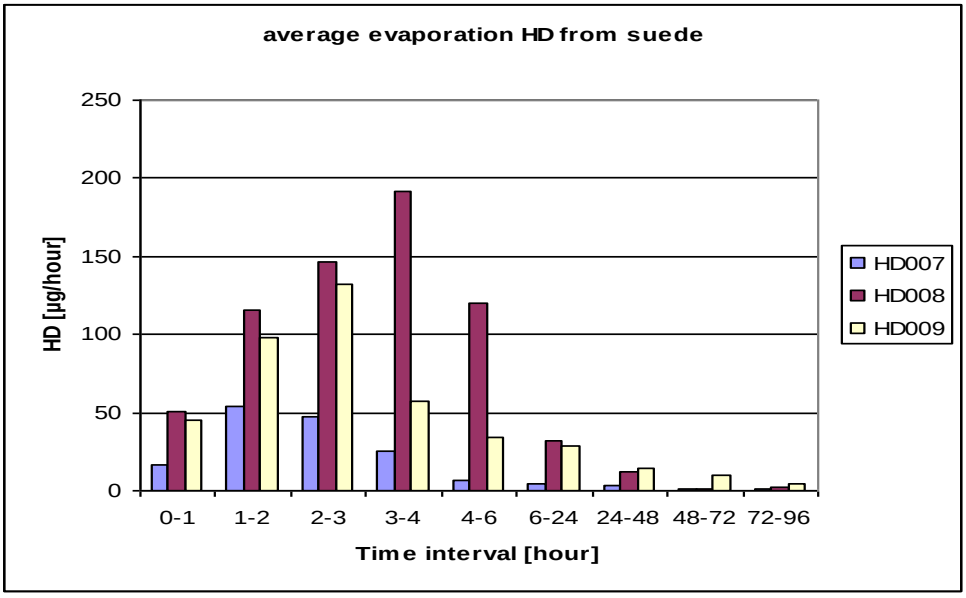

Figure 54 Off-gassing of sulfur mustard after contamination of suede leather of US Army boots.

Table 7 Detector response (in number of bars on the display) after contamination of suede leather of US Army boots with sulfur mustard.

sample	Respons ICAM [bar}		
	HD007	HD008	HD009
vapor suede boot, at t=1 hour	8	8	8
vapor suede boot, at t=2 hour	8	8	8
vapor suede boot, at t=3 hour	8	8	8
vapor suede boot, at t=4 hour	8	8	8
vapor suede boot, at t=6 hour	7	8	8
vapor suede boot, at t=24 hour	5	7	8
vapor suede boot, at t=48 hour	8	8	8
vapor suede boot, at t=72 hour	0	0	5
vapor suede boot, at t=96 hour	2	2	4

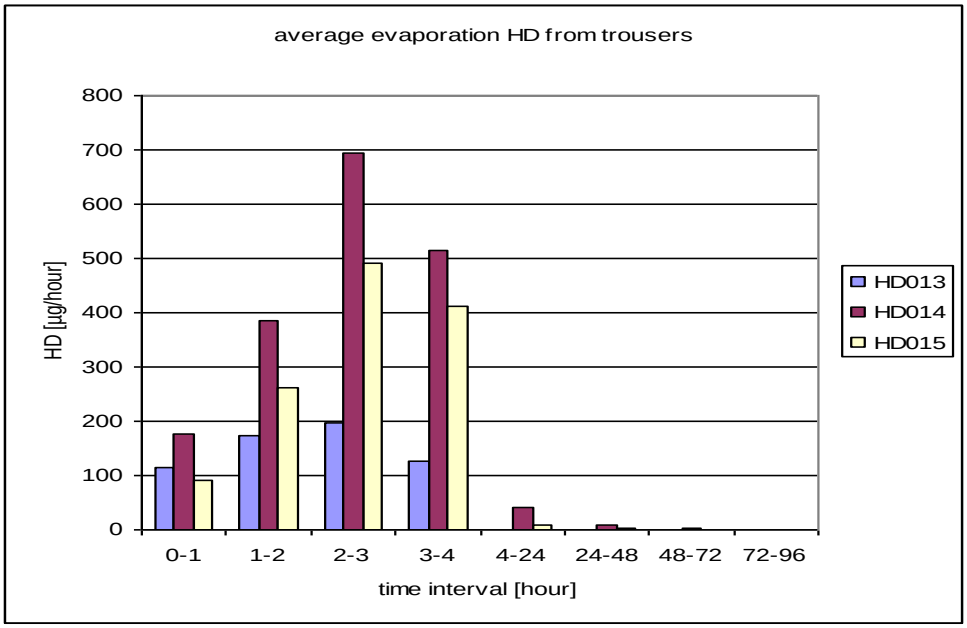

Figure 55 Off-gassing of sulfur mustard after contamination of trousers from a US Army uniform.

Table 8 Detector response after contamination of trousers from a US Army uniform with sulfur mustard.

sample	Respons ICAM [bar}		
	HD013	HD014	HD015
vapor trousers, at t=1 hour	8	8	8
vapor trousers, at t=2 hour	8	8	8
vapor trousers, at t=3 hour	8	8	8
vapor trousers, at t=4 hour	8	8	8
vapor trousers, at t=24 hour	8	8	8
vapor trousers, at t=48 hour	5	8	5
vapor trousers, at t=72 hour	0	7	7
vapor trousers, at t=96 hour	0	6	5
vapor trousers, at t=1 hour	0	5	5

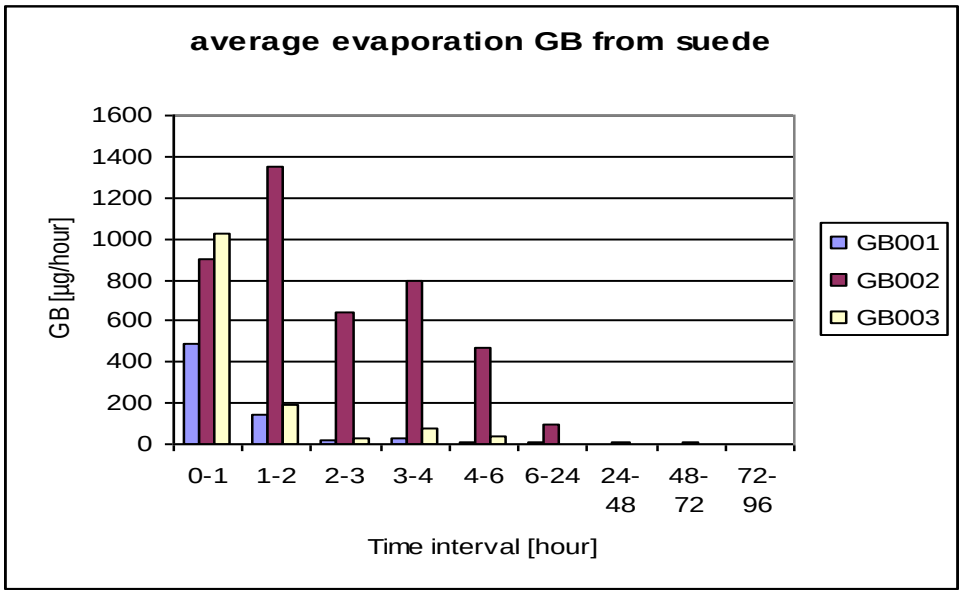

Figure 56 Off-gassing of sarin after contamination of suede leather of US Army boots.

Table 9 Detector response (in number of bars on the display) after contamination of suede leather of US Army boots with sarin.

sample	Respons ICAM [bar}		
	GB001	GB002	GB003
vapor suede boot, at t=1 hour	8	8	8
vapor suede boot, at t=2 hour	8	8	8
vapor suede boot, at t=3 hour	8	8	8
vapor suede boot, at t=4 hour	8	8	8
vapor suede boot, at t=6 hour	8	8	8
vapor suede boot, at t=24 hour	6	8	6
vapor suede boot, at t=48 hour	4	6	5
vapor suede boot, at t=72 hour	0	4	3
vapor suede boot, at t=96 hour	0	0	0

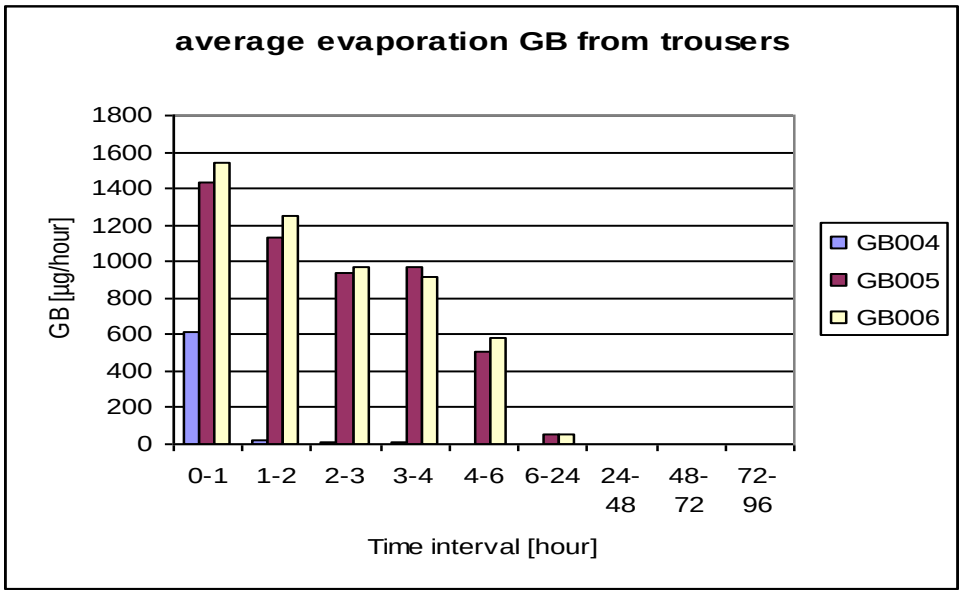

Figure 57 Off-gassing of sarin after contamination of trousers of a US Army uniform.

Table 10 Detector response (in number of bars on the display) after contamination of trousers from a US Army uniform with sarin.

sample	Respons ICAM [bar}		
	GB004	GB005	GB006
vapor trousers, at t=1 hour	7	8	8
vapor trousers, at t=2 hour	5	8	8
vapor trousers, at t=3 hour	4	8	8
vapor trousers, at t=4 hour	1	8	8
vapor trousers, at t=6 hour	1	8	8
vapor trousers, at t=24 hour	0	5	6
vapor trousers, at t=48 hour	0	3	4
vapor trousers, at t=72 hour	0	0	3
vapor trousers, at t=96 hour	0	0	0

Off-gassing; sarin

Slightly different results were obtained with GB: the levels of GB built up more rapidly, while after 24-48 hours, the levels are in general lower than in case of sulfur mustard (see Figures 56 and 57). First of all, this might be caused by the higher volatility of GB. In case of suede leather, the evaporated sulfur mustard levels are significantly lower than the evaporated GB levels; this might be caused by the higher chemical reactivity of sulfur mustard for certain components with the suede leather, comparded to GB. The detector signal (Tables 9 and 10) seems to be in good correspondence with the levels that were assessed by means of trapping and subsequent GC-MS analysis; e.g., see the GB004 series.

It should be remarked, however, that the variability in the evaporation measurements (for both sulfur mustard and GB) is very high. One reason for this might the high reactivity of these agents, e.g. to moisture. We therefore

recommend that in future projects, these experiments should be carried out at least in 6-fold.

Task 8. Extrapolation towards human remains and personal effects

Based on the previous experiments (Task 1-5), the following activities were performed within the framework of Task 8.

1) Estimate secondary contamination hazards for human remains contaminated with liquid VX or HD.
2) Estimate secondary contamination hazards for human remains contaminated with HD vapor.

Skin contamination densities, expressed as mg/cm^2 skin area, were translated into total skin contaminations by multiplying the measured values by skin surface areas of human body parts as reported in the (Dutch) report RIVM 612810 009 (2000), Table 16, and using data for a male of 74 kg.

In order to extrapolate HD releases to the human body(parts), the total skin surface area of a 1 kg guinea pig was estimated at 0,095 m^2 using data from D. Noonan, The Guinea Pig, ANZCCART News 7(1994).

For such an exercise toxicity numbers for humans are needed. One source for such numbers is the NATO AEP-52, which lists effect levels of classical chemical warfare agents applied to the skin for the purpose of deriving design criteria for protective measures (mask, suit, detectors, decontamination procedures,…). We wish to emphasize here that the exposure levels listed in AEP-52 are doses or concentrations to which the skin is exposed, and not for instance the acceptable residual level of agent on materials after decontamination. The latter levels have been estimated in AEP-58, but since they originate from a confidential addendum to this AEP, the numbers are not presented here.

The team that derived the effect levels for AEP-52 has performed a similar study on the effect levels for respiratory exposure of humans, but since the report has not been ratified (yet), the numbers from that study cannot be used here.

According to the AEP-52 the Ct-value of VX vapour for whole body exposure causing no signs of burden of cholinesterase inhibition is 30 mg.min.m^{-3}. A similar effect will occur for a liquid VX dose of 25 µg/kg body weight on the torso, corresponding with 1.75 mg on a 70 kg-man. When applied onto the cheek, liquid VX appears to be 25-50 times more toxic. For sulfur mustard, exposure of the skin to a Ct-value of 50 mg.min.m^{-3} in a period of 10-60 minutes is estimated to cause only minor symptoms. For liquid sulfur mustard a dose of 200 mg per man is estimated to have a negligible military impact.

These toxicity values have been used when interpreting the challenge levels calculated in this study.

Liquid VX contamination

In five contamination experiments the residual amount of VX on the skin of dead guinea pigs was determined as a function of time. From these data, *a worst case vapor hazard* was estimated by extrapolation to human size for various body parts as well as the total body, and by assuming the loss of agent over time is entirely due to evaporation (see Table 10). No metabolism, binding, distribution is included in these results. The concentrations inside a room (5x3x3m) without ventilation were calculated as a result of this evaporation.

Table 10 Concentrations of VX in non-ventilated room, after extrapolation of guinea pig data from exposure to VX liquid.

Concentrations Time (h)	Total human body mg/m^3	Front or Back mg/m^3	Arm mg.m3	2 arms mg/m^3	Leg mg/m^3	2 legs mg/m^3	Torso, entire mg/m^3	Torso, front of back mg/m^3
3	84.89	42.45	6.07*	12.13	13.38	26.76	30.89	15.45
6	122.53	61.26	8.76*	17.51	19.31	38.62	44.58	22.29
22	145.26	72.63	10.38	20.76	22.89	45.78	52.86	26.43
29	151.16	75.58	10.80	21.61	23.82	47.64	55.00	27.50
48	156.37	78.18	11.17	22.35	24.64	49.28	56.90	28.45
72	160.10	80.05	11.44	22.88	25.23	50.46	58.26	29.13
96	155.19	77.60	11.09	22.18	24.46	48.91	56.47	28.24

These concentrations are in most cases higher than the saturated vapor concentration of VX (8.85 mg/m^3 at 25°C). This implies that for the results indicated without an asteriks, the calculated value should be replaced by this saturated concentration. At this concentration, lethal effects are expected within a few minutes of respiratory exposure.

Liquid HD contamination

Four experiments were carried out in which the evaporation of HD vapour from dead skin tissue was monitored over time after applying a liquid HD contamination of 8 µL (10.2 mg) in total. After extrapolation of the animal data to human remains, the agent concentrations inside a room (5x3x3 m) without ventilation were calculated as a result of this release (see Table 11). This was done for the average of the four experiments as well as for a "maximum" exposure case, with levels of the average plus one standard deviation, calculated for the four experiments.

Table 11 Concentrations of HD in non-ventilated room, after extrapolation of guinea pig data from contamination with liquid HD.

Average	Concentration							
	Total human body mg/m^3	Front of Back mg/m^3	Arm mg/m^3	2 arms mg/m^3	Leg mg/m^3	2 legs mg/m^3	Torso, entire mg/m^3	Torso, front of back mg/m^3
1	0.18	0.09	0.01	0.03	0.03	0.06	0.07	0.03
2	0.22	0.11	0.02	0.03	0.03	0.07	0.08	0.04
3	0.25	0.13	0.02	0.04	0.04	0.08	0.09	0.05
4	0.27	0.14	0.02	0.04	0.04	0.09	0.10	0.05
6	0.29	0.15	0.02	0.04	0.05	0.09	0.11	0.05
24	0.32	0.16	0.02	0.05	0.05	0.10	0.11	0.06
48	0.32	0.16	0.02	0.05	0.05	0.10	0.12	0.06
72	0.32	0.16	0.02	0.05	0.05	0.10	0.12	0.06
High	Concentration							
	Total human body mg/m^3	Front of Back mg/m^3	Arm mg/m^3	2 arms mg/m^3	Leg mg/m^3	2 legs mg/m^3	Torso, entire mg/m^3	Torso, front of back mg/m^3
1	0.21	0.11	0.02	0.03	0.03	0.07	0.08	0.04
2	0.29	0.15	0.02	0.04	0.05	0.09	0.11	0.05
3	0.36	0.18	0.03	0.05	0.06	0.11	0.13	0.06
4	0.39	0.20	0.03	0.06	0.06	0.12	0.14	0.07
6	0.44	0.22	0.03	0.06	0.07	0.14	0.16	0.08
24	0.48	0.24	0.03	0.07	0.08	0.15	0.18	0.09
48	0.49	0.25	0.04	0.07	0.08	0.15	0.18	0.09
72	0.49	0.25	0.04	0.07	0.08	0.16	0.18	0.09

These concentrations are below the mild effect threshold, provided the exposure time is limited. In the worst case (after accumulation of the vapor for 72 hours for a liquid contamination covering the entire body), these concentrations are safe to breathe for ca. 1 hour before mild intoxication effects are expected to occur.
After a single contamination with 10 mg of mustard, the concentrations in the room were calculated to reach values of 0.01-0.03 mg/m^3. These are safe to breathe for 16-24 hours.

Vapor HD contamination

In four contamination experiments the residual amount of HD on the skin of dead guinea pigs was determined as a function of time, after contamination with HD vapor. From these data, a worst case vapor hazard was estimated by extrapolation to human size for various body parts as well as the total body, and by assuming the loss of agent over time is entirely due to evaporation. The concentrations inside a room (5 x 3 x 3 m) without ventilation were calculated as a result of this evaporation (see Table 12).

Table 12 Concentrations of HD in non-ventilated room, after extrapolation of guinea pig data (residual liquid on skin) after contamination of animal with HD vapor.

Concentrations Time (h)	Total human body µg/min	Front of Back µg/min	Arm µg/min	2 arms µg/min	Leg µg/min	2 legs µg/min	Torso, entire µg/min	Torso, front or back µg/min
0								
2.5	5.46	2.73	0.39	0.78	0.86	1.72	1.99	0.99
5	6.83	3.41	0.49	0.98	1.08	2.15	2.48	1.24
23	13.55	6.78	0.97	1.94	2.14	4.27	4.93	2.47
29	13.87	6.93	0.99	1.98	2.19	4.37	5.05	2.52

These concentrations are a factor 1000 below the mild toxicological effects threshold of HD. Based on these experiments, no harmful effects are to be expected as a result of exposure to these human remains.

Evaporation of HD vapor from contaminated remains

Four experiments were carried out in which the evaporation of HD vapor from dead skin tissue was monitored over time. After extrapolation of the animal data to human remains, the agent concentration inside a room (5 x 3 x 3 m) without ventilation were calculated as a result of this release (see Table 13).

Two release levels were found: a high (~ 11 µg in the first hour) and a low (~ 6 µg in the first hour). The room concentrations after rescaling to human size were calculated for both exposure levels:

Table 13 Concentrations of HD in non-ventilated room, after extrapolation of guinea pig data (vapor released from dead animal) after contamination of animal with HD vapor

	Total human body µg/m^3	Front of Back µg/m^3	Arm µg/m^3	2 arms µg/m^3	Leg µg/m^3	2 legs µg/m^3	Torso, entire µg/m^3	Torso, front of back µg/m^3
1	2.5	1.2	0.2	0.4	0.4	0.8	0.9	0.4
2	3.5	1.7	0.2	0.5	0.5	1.1	1.3	0.6
3	4.3	2.2	0.3	0.6	0.7	1.4	1.6	0.8
4	4.7	2.3	0.3	0.7	0.7	1.5	1.7	0.9
6	5.3	2.6	0.4	0.8	0.8	1.7	1.9	1.0
24	6.3	3.1	0.4	0.9	1.0	2.0	2.3	1.1
48	6.3	3.2	0.5	0.9	1.0	2.0	2.3	1.1
72	6.4	3.2	0.5	0.9	1.0	2.0	2.3	1.2
High	Concentration							
	Total human body µg/m^3	Front of Back µg/m^3	Arm µg/m^3	2 arms µg/m^3	Leg µg/m^3	2 legs µg/m^3	Torso, entire µg/m^3	Torso, front of back µg/m^3
1	4.8	2.4	0.3	0.7	0.8	1.5	1.7	0.9
2	6.3	3.2	0.5	0.9	1.0	2.0	2.3	1.2
3	7.2	3.6	0.5	1.0	1.1	2.3	2.6	1.3
4	7.9	4.0	0.6	1.1	1.2	2.5	2.9	1.4
6	8.8	4.4	0.6	1.3	1.4	2.8	3.2	1.6
24	10.4	5.2	0.7	1.5	1.6	3.3	3.8	1.9
48	10.6	5.3	0.8	1.5	1.7	3.3	3.9	1.9
72	10.7	5.3	0.8	1.5	1.7	3.4	3.9	1.9

These levels are a factor 1000 below the mild effect threshold concentration, so no harmful effects are expected based on these experiments.

Evaporation risk from an excised liver from a victim of chemical agent exposure

Based on experimental data on accumulation of chemical warfare agents in the liver, the exposure hazard was estimated for a case in which a contaminated human liver, obtained in autopsy was left in an unventilated room, assuming the total amount of agent present evaporates into this space. The results for both VX (after liquid exposure) and HD (after vapor exposure) are depicted below (see Table 14) and are given as the concentration in the room obtained after total evaporation of agent from a human liver which has been absorbing an amount of agent for the time period indicated. (So: 48 hours means that the liver was excised 48 hours after exposure).

Table 14 Concentrations of VX and HD in non-ventilated room, after extrapolation of guinea pig data (excised liver) from contamination with HD (vapor exposure) and VX (liquid exposure).

VX Concentrations Time (h)	HR003VX mg/m^3	HR004VX mg/m^3	HR005VX mg/m^3	HR006VX mg/m^3	Pilot mg/m^3
0	0.00	0.00	0.00	0.00	0.00
2.5	0.00	0.00	0.00	0.00	0.00
5	0.00	0.00	0.00	0.00	0.00
23	0.01	0.01	0.11	0.00	0.00
27	0.00	0.00	0.00	0.00	0.02
29	0.02	0.00	0.05	0.00	0.01
48	0.18	0.10	0.03	0.00	0.02
72	0.08	0.25	0.00	0.00	0.02
96	0.12	0.01	0.02	0.09	0.01
HD Concentrations Time (h)	HR023 mg/m^3	HR024 mg/m^3	HR025 mg/m^3	HR026 mg/m^3	
0	0.00	0.00	0.00	0.00	
2.5	0.30	0.00	3.16	0.00	
5	0.00	0.00	1.65	0.00	
23	0.53	0.23	0.42	0.65	
27	1.40	0.00	0.35	0.00	
29	0.46	0.00	0.12	0.19	
48	0.14	0.00	0.00	0.25	
72	0.00	0.00	0.00	0.00	
96	0.00	0.00	0.00	0.00	

In case of the liquid VX contamination, concentrations are in the range of mild intoxication effects (ECt_{50} ~ 0.1 mg.min.m^{-3}, whereas for HD vapor contamination, the concentration are a factor of 1000 below toxicological relevant values.

In conclusion, it seems from these preliminary 'worst case' estimates that human remains from victims from liquid exposure to chemical warfare agent may present significant vapor hazards, even when only parts of the body are contaminated. It seems worthwhile to study the effects of ventilation, i.e., by using compuret simulations (e.g., using the gCOMIS software package) using such evaporating body(parts) as a source term in a more realistic setting. For victims of vapor exposure, the remaining agent is not enough to exert any hazard.

Task 9: Toxicological evaluation/overall hazard assessment

A rough comparison of the results for contact hazard and off-gassing of deceased animals and personal effects learns the following.
In case of contact hazard of VX, agent levels that could be detected on the skin(by means of tape stripping), posing a contact hazard, were slightly higher than levels on the rubber soles of US Army boots (determined by means of adsorption onto silica gel) at the last sampling point (96 hours). However, in both cases contact risk decreased in a rather gradual way. In contrast, in case of biological remains sulfur mustard agent levels rapidly (> 0.5 hours after application) dropped to relatively low levels (< 2 µg/cm^2), while on rubber soles and suede leather more significant levels (up to 10 µg/cm^2) were present at 96 hours. *Striking is the high stability of VX and sulfur mustard within both suede leather and rubber.*
In case of contaminated animals, no sulfur mustard could be detected anymore within the skin after 96 hours. This pronounced difference can *inter alia* be explained by the high reactivity of sulfur mustard in the presence of water and the presence of numerous other nucleophilic entities such as present in proteins in case of biological remains. It should be kept in mind that in case of contact hazard, different sampling methods were applied for the two types of samples, *i.e.* tape stripping in case of contaminated biological remains and a silica gel adsorption method in case of contaminated protective gear. This might also lead to slightly different results.
With regard to off-gassing, sulfur mustard vapor levels seem to be considerably higher in case of personal gear at equal contamination levels, especially in the 1-4 hours time interval. This might also be due the higher reactivity of sulfur mustard in the biological matrix, as mentioned above. For sarin, high vapor levels were present immediately after the contamination. No comparison between contaminated biological remains and protective gear can be made because in the current project animals have not been contaminated with sarin.
This study has been performed to establish whether or not vapor and/or contact hazards are present when handling chemically or biologically contaminated human remains.
On the basis of the animal experiments that have been performed, the following conclusions can be drawn:

- After contamination with liquid VX the skin poses a contact hazard, likewise contact with blood or liver tissue may also pose a hazard;
- Decontamination reduces the amount of VX in the skin of the deceased animal, and thus the contact hazard. The sooner the decontamination is performed, the more effective it will be;
- At the contamination level of liquid sulfur mustard used we did not find a contact risk from the skin of the deceased animal, A vapor hazard is mainly present in first 24 hours after death. An ICAM only detected the vapor in the first hours after death;

- After exposure to sulfur mustard vapor, the contact hazard posed by skin is low. The vapor hazard is lower than after contamination with liquid sulfur mustard;
- Cooling of the remains reduces the vapor hazard resulting from sulfur mustard contamination. The agent concentration in skin is higher in cooled remains than in those kept at room temperature. As a result, the contact risk will not be reduced by cooling.

On the basis of these findings it is advised to use full personal protection when handling chemically contaminated human remains, also when performing autopsy. Cooling reduces the vapor hazard, but not the contact hazard. Decontamination of the human remains reduces vapor and contact hazard, in particular when performed shortly after death of the chemical victim.

- Military boots and uniforms contaminated with sarin or sulfur mustard pose a vapor hazard, which is easily detected with an ICAM;
- Military boots and uniforms contaminated with VX or sulfur mustard pose a contact risk.

On the basis of these findings it is advised to remove the personal protective gear (or other clothing) from the deceased and to destroy them.

- Skin externally contaminated with *B. anthracis* poses a contact hazard. Decontamination with 5% or 15% bleach does not seem to reduce this contact hazard, whereas the decontaminant PES-solid was only slightly effective;
- Skin from deceased animals internally contaminated with *B. anthracis* does not pose a contact hazard. It is anticipated that the internal organs will pose a contact hazard, albeit that this could not be confirmed with the techniques used.

On the basis of these findings it is advised to use full personal protection when handling human remains contaminated with *B. anthracis*, also when performing autopsy. Preferably such remains should be handled in a biosafety facility. Cooling will most likely not reduce the contact hazard. Decontamination does not seem to reduce the contact hazard.

It has be taken into account that the contamination levels that were used in the current study are fairly high: *ca*. 8 mg of liquid VX or sulfur mustard on the hairless guinea pig and a sulfur mustard vapor exposure 10,000 $mg.min.m^{-3}$. These levels were chosen in order to ensure that we would be able to measure intact agent in and on the tissues after death. It may be argued that such high exposures relate to 'Cold War' scenarios, which nowadays are perceived as being less likely to occur. Obviously, if the challenge doses would be lower, the vapor and contact hazards will also be reduced.

Task 10. Effect of decontamination on the residual chemical hazard by VX

In Task 10 the effect of decontamination on the residual chemical hazard by VX was investigated. Decontamination was performed with commercially available RSDL pouches which are used for skin decontamination by several NATO countries. In a pilot study it was determined to which extent the RSDL-decontamination interferes with the tape stripping procedure and the sample work up and GC-MS analysis by using a non-reactive VX simulant. The experiments were carried out with skin remnants of dead hairless guinea pigs that had been used for other experiments. A non-reactive VX simulant, i.e., diethylmethyl phosphonate (DEMP) was used in order to easily determine the effect of RSDL on tape stripping recoveries.

Tape stripping of DEMP from RSDL- treated skin.

We had concerns that tapes, used to strip contaminated skin, would not stick very well on RSDL-treated skin to remove remaining agent. Therefore, a pilot experiment was performed to compare the removal of agent from RSDL treated and untreated skin. On a cork board two slices of skin were fixated (see Figure 58). Underneath the slices of skin a layer of parafilm was placed. One piece of skin was treated with RSDL. A small piece of a pouch (diameter 6 mm) was immersed in RSDL solution. With a pair of tweezers the RSDL saturated pouch was rubbed on the skin during 30 seconds.

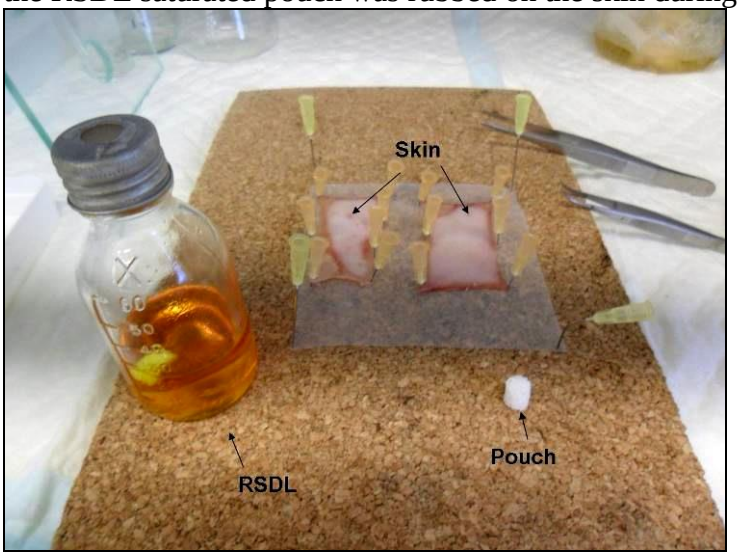

Figure 58 Impression of experimental set-up.

After waiting for 5 minutes two droplets of 1 µL (1 mg) DEMP were put on the RSDL-treated skin, at two different positions. Two droplets of DEMP were also put on the untreated skin. Two minutes after applying the droplets they were still present as a droplet. With the needle of the syringe that was used to apply the DEMP the droplets were spread over a surface of approximately 5 x 5 mm.

The surface whereupon a droplet of DEMP had been spread, was stripped twice with a piece of tape of 10 x 10 mm. Blank samples were obtained by

stripping skin where DEMP had not been applied. It was noted that sticking to the RSDL treated surface was comparable with sticking to the untreated skin.
The strips were extracted in 1 mL ethyl acetate during 30 minutes. The extracts were filtered on a 0.45 µm PTFE filter, diluted (1:100) in ethyl acetate and analysed with GC-MS for the presence of DEMP.

Table 15 DEMP in extracts of strips from skin.

File name	Description	Area (m/z 97)
bl-demp1-strip1	blank skin, DEMP-drop 1, first strip	12682712
bl-demp1-strip2	blank skin, DEMP-drop 1, second strip	3601754
	Total:	16284466
bl-demp2-strip1	blank skin, DEMP-drop 2, first strip	7129739
bl-demp2-strip2	blank skin, DEMP-drop 2, second strip	5260483
	Total:	12390222
rsdl-demp1-strip1	RSDL treated skin, DEMP-drop 1, first strip	8477743
rsdl-demp1-strip2	RSDL treated skin, DEMP-drop 1, second strip	4955003
	Total:	13432746
rsdl-demp2-strip1	RSDL treated skin, DEMP-drop 2, first strip	10031265
rsdl-demp2-strip2	RSDL treated skin, DEMP-drop 2, second strip	6252835
	Total:	16284100

As can be derived from Table 15, the amount of DEMP on strips taken from skin treated with RSDL is comparable to the amount removed from skin that is not treated with RSDL. In future experiments we will consider to apply a third and fourth round of tape stripping.

Pilot experiment with DEMP – Recovery Determination

After establishing that the tape-stripping procedure that has been used before can be applied on RSDL treated skin a pilot experiment with DEMP was conducted. Again two slices of skin were fixated. On both slices of skin three spots were contaminated with a 1 µL drop DEMP.
After 30 minutes the 3 spots on one of the slices of skin, were separately decontaminated by rubbing each spot with a RSDL saturated piece of pouch during 30 seconds. After use the pouches were placed in vials with ethyl acetate (2 mL). After waiting for 10 minutes the RSDL remaining on the spots was removed with a cotton swab. A new swab was used for each spot. The swabs with absorbed RSDL were placed in vials with 2 mL ethyl acetate. Subsequently the spots were stripped twice with a piece of tape (10 x 10 mm). Each strip was placed in a vial with 1 mL ethyl acetate.
The vials with samples and ethyl acetate were placed in a rotator for 30 minutes. The extracts were filtered over a 0.45 µm PTFE filter. Extracts of pouches were diluted 1:100, extracts of the cotton swabs were diluted 1:10 in ethyl acetate. The (diluted) extracts were analysed with GC-MS for DEMP.
After 24 hours the second slice of skin, which was stored in a closed glass container, was treated in the same way.

For the quantification of DEMP, solutions with a known amount of DEMP were prepared and also analysed (see Table 16).

Table 16 Areas measured for standard solutions DEMP in ethyl acetate.

Standard / file	DEMP in ethyl acetate [mg/ml]	Area [m/z 97]
std01	0,001	2273353
std02	0,002	4064462
std03	0,005	9052583
std04	0,010	22746768
std05	0,020	53982016
std06	0,030	78989377

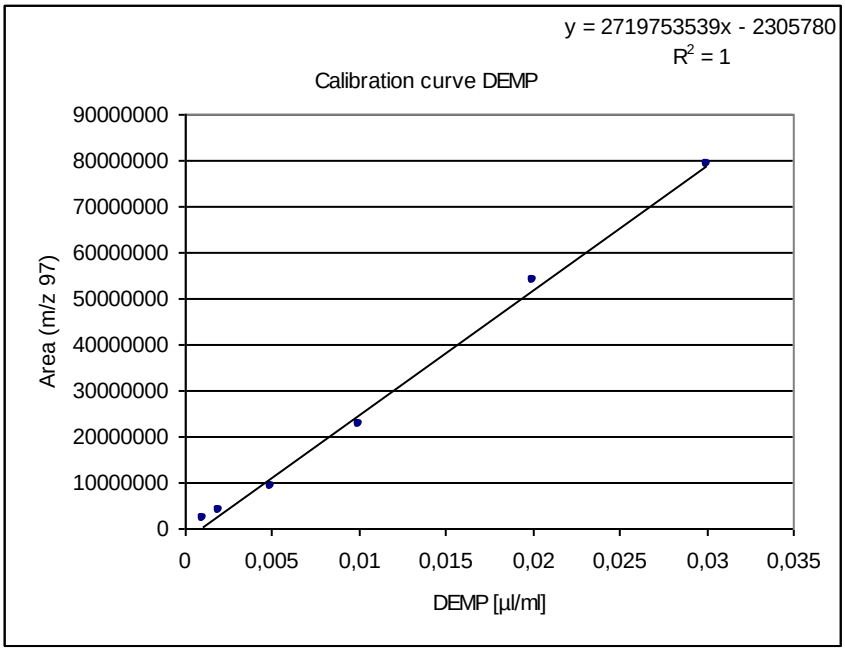

Figure 59 Calibration curve for DEMP in ethyl acetate.

Based on the equation of the calibration curve shown in Figure 59 the amount of DEMP in the samples was calculated. The results are presented in Table 17 and Figures 60 and 61.

Table 17 Calculated amounts DEMP in samples of the pilot study.

Sample/file	Description	Area (m/z 97)	mg DEMP aliquot	Corrected mg DEMP
dec1_str1	decon-1, t=0, strip1	28248582	0,011	0,011
dec1_str2	decon-1, t=0, strip2	15503870	0,007	0,007
dec1-cotton	decon-1, t=0, cotton swab	8356515	0,004	0,078
dec1-pouch	decon-1, t=0, pouch	8666907	0,004	0,807
			Total	0,90
dec2_str1	decon-2, t=0, strip1	38014024	0,015	0,015
dec2_str2	decon-2, t=0, strip2	17023242	0,007	0,007
dec2-cotton	decon-2, t=0, cotton swab	8195562	0,004	0,077
dec2-pouch	decon-2, t=0, pouch	7530986	0,004	0,723
			Total	0,82
dec3_str1	decon-3, t=0, strip1	44195466	0,017	0,017
dec3_str2	decon-3, t=0, strip2	19248175	0,008	0,008
dec3-cotton	decon-3, t=0, cotton swab	9959945	0,005	0,090
dec3-pouch	decon-3, t=0, pouch	8626194	0,004	0,804
			total	0,92
dec4_str1	decon-4, t=24, strip1	7158132	0,003	0,003
dec4_str2	decon-4, t=24, strip2	3988987	0,002	0,002
dec4-cotton	decon-4, t=24, cotton swab	364663	0,001	0,020
dec4-pouch	decon-4, t=24, pouch	0	0	0
			total	0,025
dec5_str1	decon-5, t=24, strip1	5266658	0,003	0,003
dec5_str2	decon-5, t=24, strip2	5020528	0,003	0,003
dec5-cotton	decon-5, t=24, cotton swab	202393	0,001	0,018
dec5-pouch	decon-5, t=24, pouch	0	0	0
			total	0,024
dec6_str1	decon-6, t=24, strip1	7537964	0,004	0,004
dec6_str2	decon-6, t=24, strip2	10862795	0,005	0,005
dec6-cotton	decon-6, t=24, cotton swab	350120	0,001	0,020
dec6-pouch	decon-6, t=24, pouch	0	0	0
			total	0,028

The amount of DEMP in the cotton samples is corrected by a factor 20 due to the fact that the samples are extracted with 2 mL ethyl acetate and the extracts were diluted ten times prior to analyses. For the pouch samples the correction factor is 200 due to an extraction volume of 2 mL and a hundred-fold dilution.

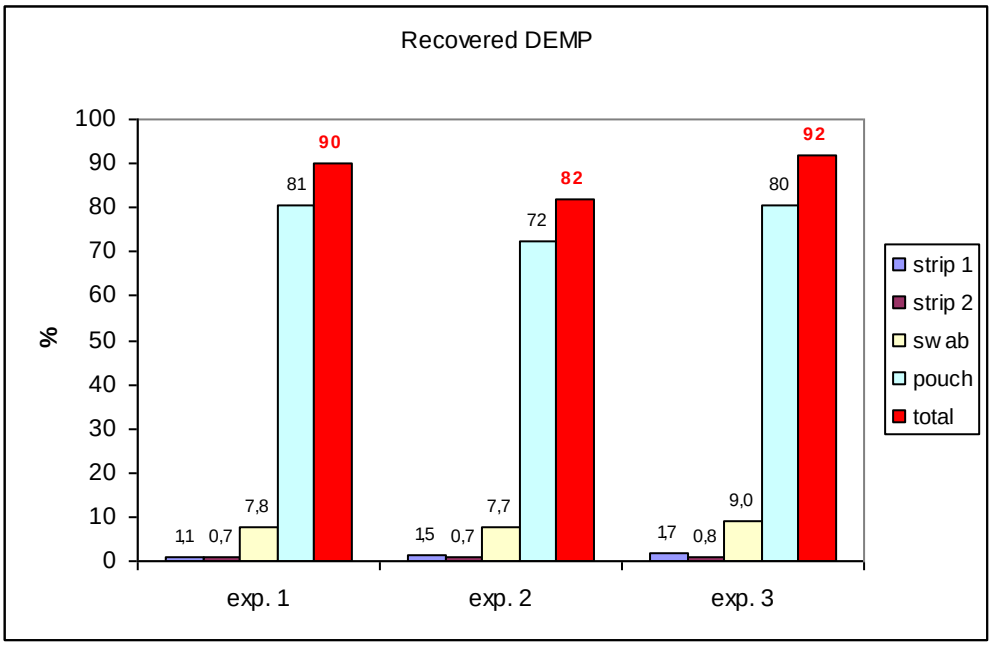

Figure 60 Percentage DEMP in experiments with direct decontamination.

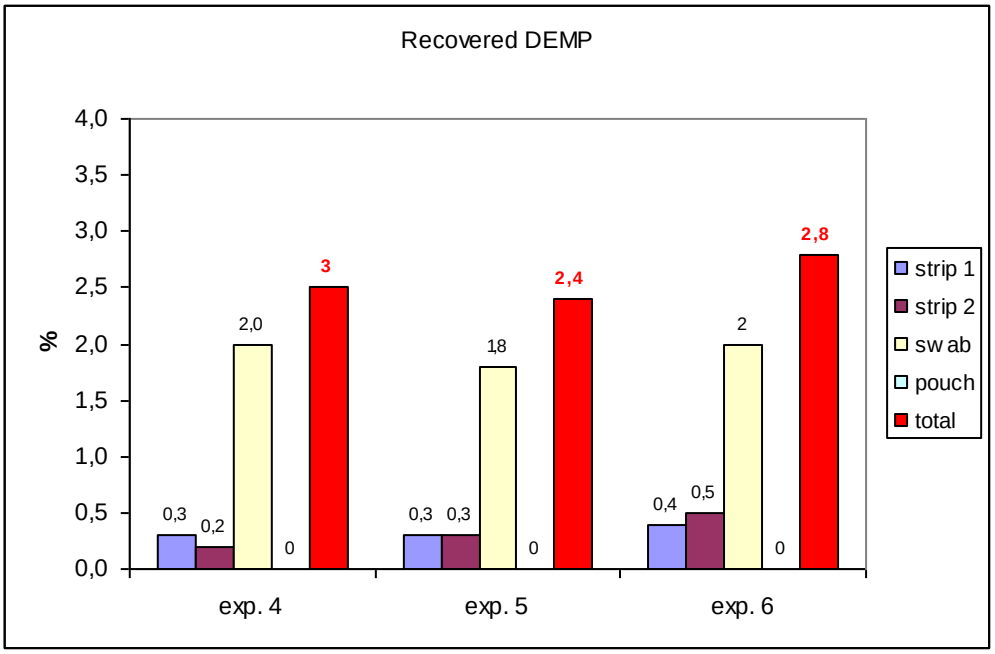

Figure 61 Percentage DEMP in experiments with decontamination after 24 hours.

In conclusion, the results of this pilot experiment show that the efficacy of removal of agents from skin by means of tape stripping is not reduced by treatment of the skin with RSDL. The total amount of DEMP found in the samples directly decontaminated is in the range of 80 – 90 %. In the samples decontaminated after 24 hours 2-3 % of the applied DEMP was recovered; the latter finding is logical, because within the 24-hours time period DMMP can either evaporate or be absorbed in the skin. Although RSDL should decontaminate the live chemical agents it will be interesting to also analyze

extracts of pouches and cotton swabs during the experiments with real agents, in order to determine the residual hazard these items present.

Tape stripping after direct decontamination

Figure 62 shows the accumulated amount of VX sampled by tape stripping for each animal. So, at each time point two spots were tapestripped (4 rounds of tape stripping; for each animal).

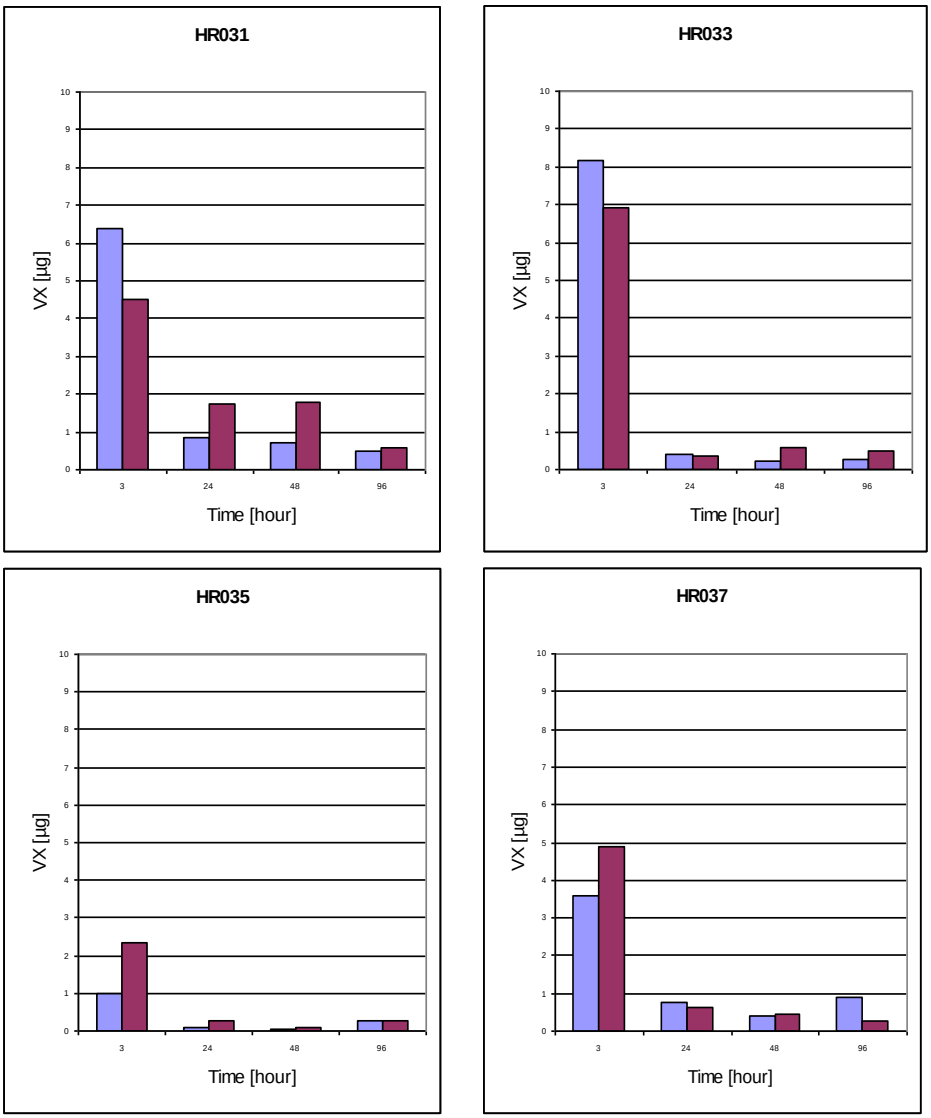

Figure 62 VX levels (in microgram) on skin at various sampling points as determined by tape stripping (4 times) of deceased hairless guinea pigs, after p.c. contamination of the living animals (n=4; HR031, HR033, HR035 and HR037) with liquid VX (8 x 1 µL) and subsequent 'direct' decontamination (after 0.5 hours) with RSDL. On each time point two spots were sampled by means of tape stripping (blue and red bars).

As a control experiment, agent levels were determined without decontamination (see Figure 63). Results are comparable with earlier obtained results (Task 2). It is obvious that decontamination results in much lower levels of VX after tape stripping.

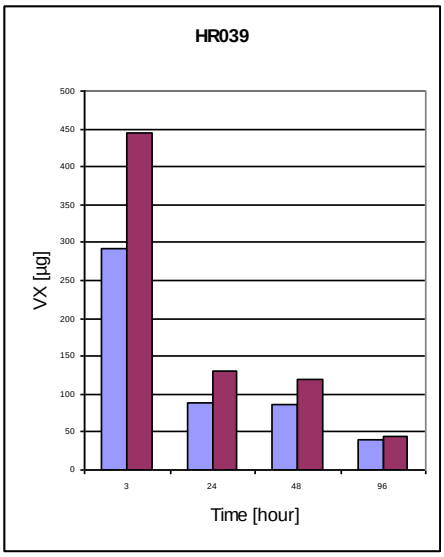

Figure 63 VX levels (in micrograms) on skin at various sampling points as determined by tape stripping of deceased hairless guinea pig, after p.c. exposure of the living animal to neat VX (8 x 1 µL). On each time point two spots were sampled by means of tape stripping (blue and red bars).

After the experiment, the contaminated/decontaminated spots were punched and analyzed for remaining VX (Figure 64). Remarkably, in case of the animal (HR035) for which the levels after tape stripping were lower than for the other animals, the levels within the skin were much higher. This might indicate that the tape stripping procedure had been carried out less efficient, or that the agent had penetrated more deeply in the skin.

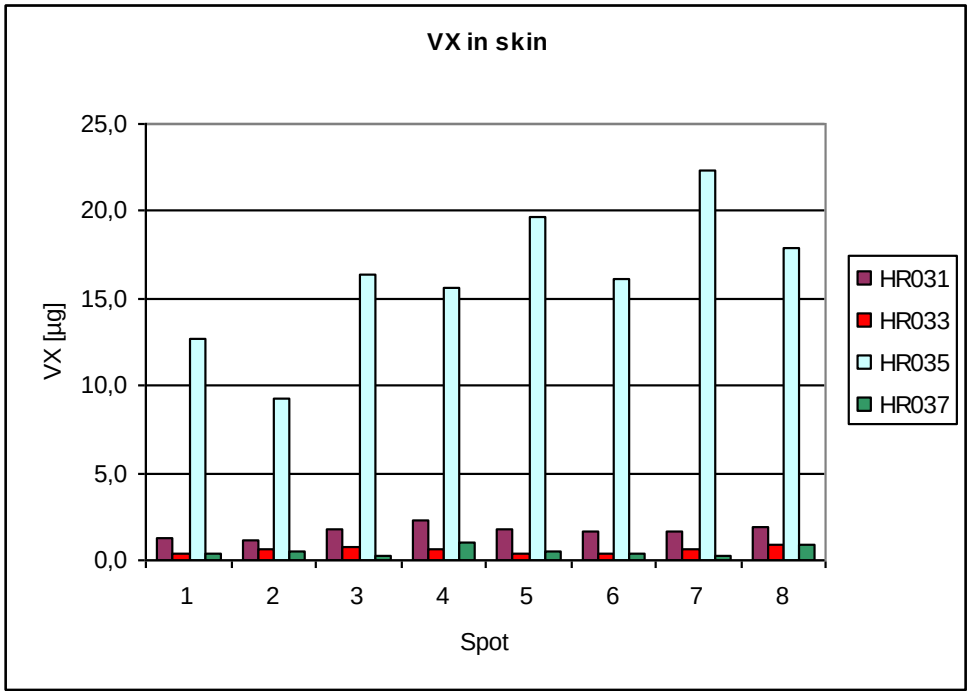

Figure 64 Remaining VX in punched skin spots of deceased hairless guinea pigs, after p.c. contamination with liquid VX and subsequent 'direct' decontamination with RSDL (after 30 minutes).

Tape stripping after delayed decontamination

Figure 65 shows the accumulated amount of VX after delayed (24 hours) decontamination. Figure 66 shows the remaining agent within the skin.

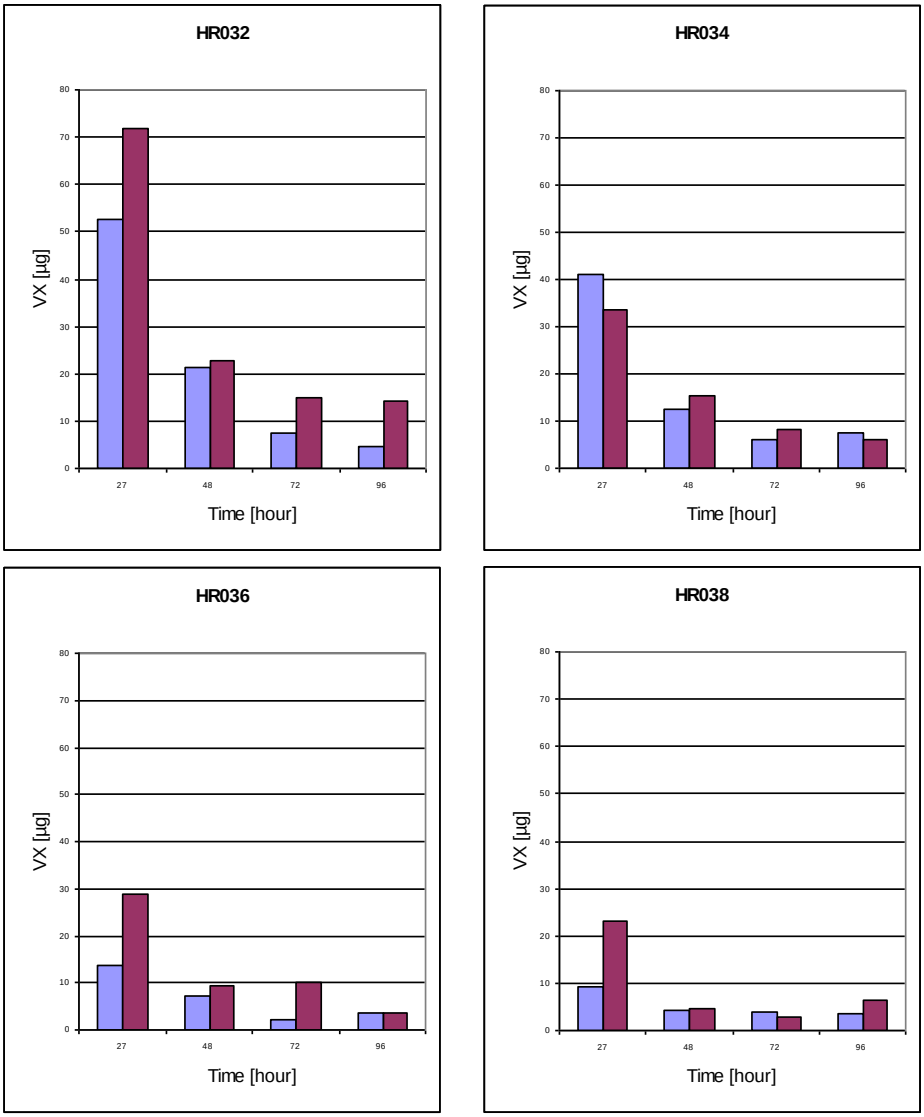

Figure 65 VX levels (in micrograms) on skin at various sampling points as determined by tape stripping (4 times) of deceased hairless guinea pigs, after p.c. contamination (n=4; HR032, HR034, HR036 and HR038) with liquid VX (8 x 1 µL) of the living animals and subsequent delayed decontamination (after 24.5 hours) with RSDL. On each time point two spots were sampled by means of tape stripping (blue and red bars).

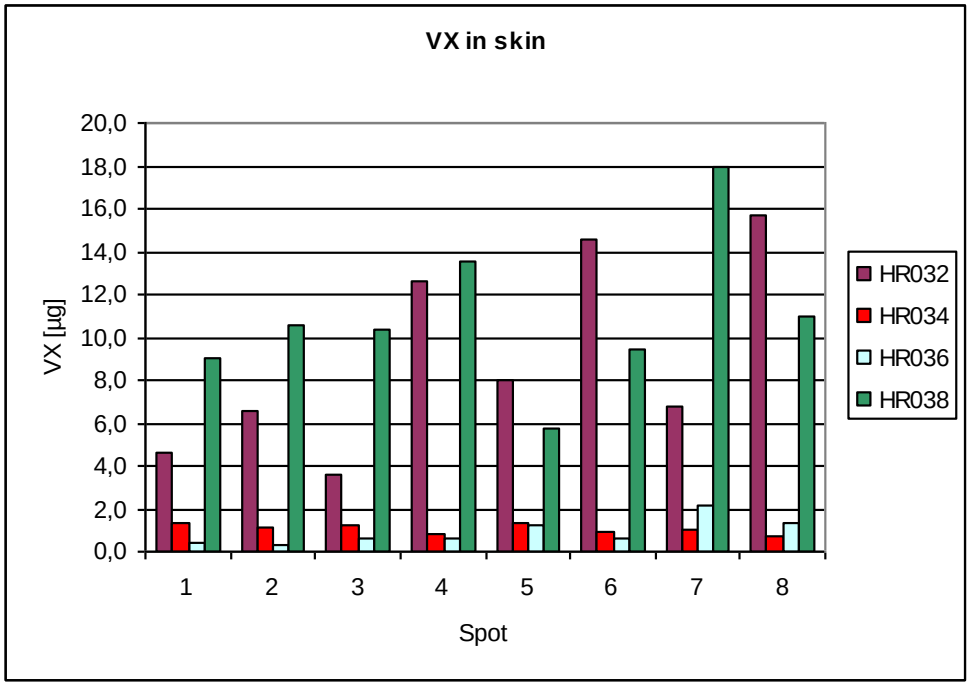

Figure 66 Remaining VX in punched skin spots of deceased hairless guinea pigs, after p.c. contamination with lquid VX and subsequent delayed decontamination with RSDL (after 24.5 hours).

The general trend is that the 'direct' decontamination procedure (i.e. after 30 minutes) with RSDL results in the lowest residual agent levels, in comparison with delayed decontamination. This can be explained by the fact that in case of delayed decontamination the agent can penetrate deeper into the skin, making it less readily accessible to the decontamination agent. Despite of the fact that the agent is not accessible to the decontaminant, it can apparently still be removed by tape stripping and consequently still pose a contact hazard. In this respect, it should be remarked that in a number of instances pieces of skin were completely removed during tape stripping.
In some instances, rather variable results were obtained for the amount of residual agent in skin, determined after completing the experiment (see Figure 66). The general trend, however, was that direct decontamination resulted in lower levels of remaining agent in the skin (compare Figures 64 and 66).
After decontamination the pouches used were analyzed for the presence of remaining agent, in order to determine the hazard of these consumables after use. The pouches were extracted after addition of the internal standard and the extracts were analyzed by means of GC-MS. Neither VX nor the internal standard could be detected. This is not surprising, because of the excess of RSDL compared to VX, causing rapid degradation of the agent. Because of the corrosive character of the extract towards the GC-MS system only a few of the extracts were analyzed.

Task 12 Pilot on feasibility of determination of contact hazard through the use of pig ear skin

It was envisaged that determination of contact hazard by using pig ear skin might be more realistic than by using tape stripping. To this end a pilot experiment with VX and pig ear skin as an alternative for tape stripping was set up.
In these experiments 4 spots on a hairless guinea pig were sampled with pig ear skin, and 4 spots by means of tape stripping to enable direct comparison of the results.
Two different scenarios were evaluated. In the first scenario, no decontamination was performed. In the second scenario, decontamination was performed directly after death (i.e., 30 minutes after application of the agent). The skin decontaminant (RSDL) and procedure used are the same as in Task 10.
Agent levels on skin were determined by means of pig ear skin and tape-stripping, at different time points, up to 96 hours after death (3, 24, 48 and 96 hours). Experiments for both scenarios were conducted in triplicate.

Experiments without decontamination

Amounts of VX in the pig ear samples and the accumulated amount of VX in the 4 tape strips are presented in Table 18 and Figure 66. Amounts of remaining VX in the skin of the hairless guinea pigs at the contaminated spots are presented in Table 19 and Figure 67.

Table 18 Amount VX [µg] in the pig ear samples and the accumulated amount of VX in the 4 tape strips.

			Amount VX [µg]		
spot	sampling time [hr]	sampling method	exp. HR052	exp. HR054	exp. HR056
1	3	pig ear	11,6	47,6	14,6
2		strips	112	129	172
3	24	pig ear	5,02	6,38	4,68
4		strips	50,4	68,6	145,4
5	48	pig ear	2,21	2,76	7,23
6		strips	48,8	49,4	87,9
7	96	pig ear	1,95	2,59	8,36
8		strips	33,4	56,5	85,2

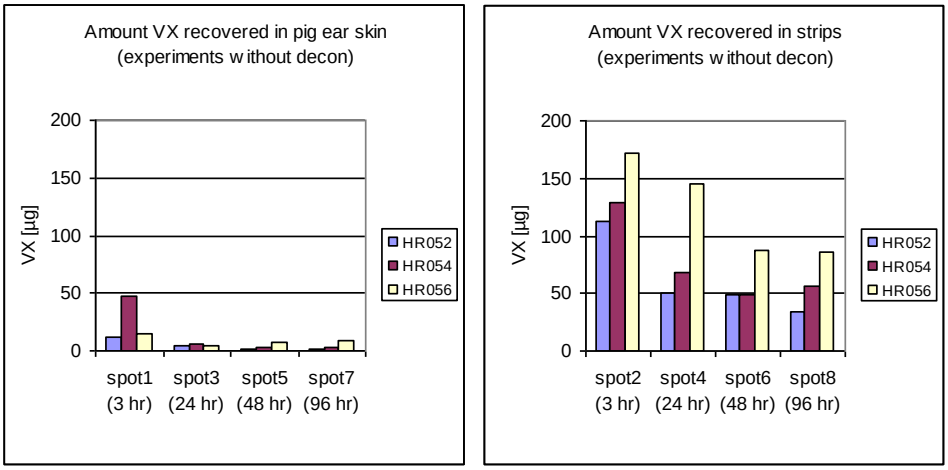

Figure 67 Amount of VX [µg] in the pig ear samples (left panel) and amount of VX in the 4 tape strips (right panel) for experiments without decontamination.

Table 19 Remaining amount of VX in the skin of the hairless guinea pig at the contaminated spots, without decontamination.

	Amount VX [µg] in skin		
spot	exp. HR052	exp. HR054	exp. HR056
1	25.9	7.95	45.4
2	3.09	2.26	5.98
3	21.2	20.0	80.3
4	8.05	6.85	26.8
5	18.6	21.3	46.5
6	9.39	7.58	34.83
7	17.0	20.2	50.9
8	8.89	6.33	18.1

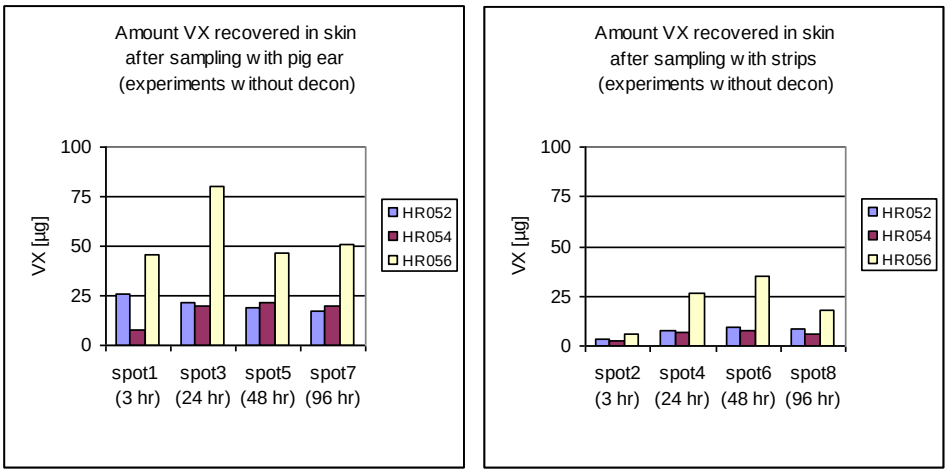

Figure 68 VX [µg] in skin of the hairless guinea pig of spots sampled with pig ear (left panel) or sampled by tape stripping (right panel), without decontamination.

Experiments with decontamination

Amounts of VX in the pig ear samples and the accumulated amount of VX in the 4 tape strips are presented in Table 20 and Figure 68. Amounts of remaining VX in the skin of the hairless guinea pigs at the contaminated spots are presented in Table 17 and Figure 69.

Table 20 Amount VX in the pig ear samples and the accumulated amount of VX in the 4 tape strips, after decontamination with RSDL.

			Amount VX [µg]		
spot	sampling time [hr]	sampling method	exp. HR053	exp. HR055	exp. HR057
1	3	pig ear	0.14	0.55	0.33
2		strips	1.63	1.29	1.21
3	24	pig ear	0.03	0.06	0.05
4		strips	0.24	0.35	0.69
5	48	pig ear	0.06	0.08	0.17
6		strips	0.20	1.74	0.37
7	96	pig ear	0.07	0.15	0.14
8		strips	0.15	0.84	1.21

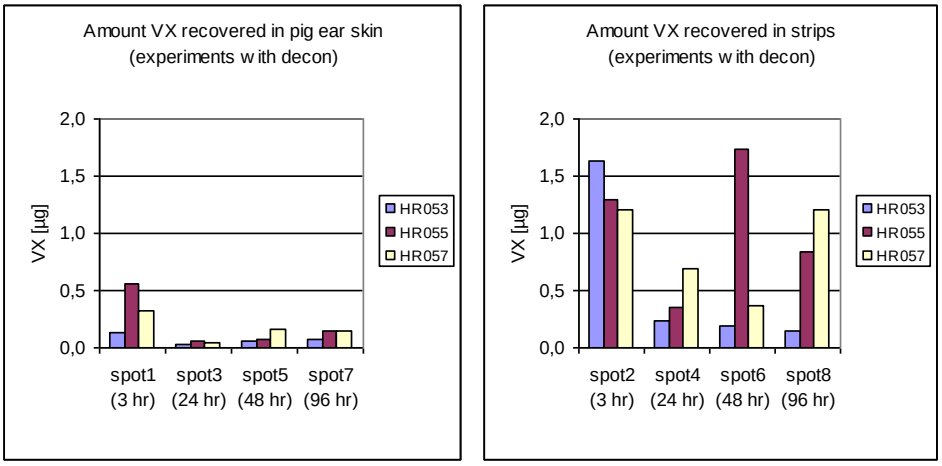

Figure 69 Amount of VX [µg] in the pig ear samples (left panel) and accumulated amount of VX (right panel) in the 4 tape strips, after decontamination with RSDL.

Table 21 Remaining amount of VX in the skin of the hairless guinea pig at the contaminated spots, after decontamination with RSDL.

	Amount of VX [µg] in skin		
spot	exp. HR052	exp. HR054	exp. HR056
1	0.37	1.05	1.21
2	0.37	1.42	0.92
3	0.49	1.15	1.16
4	0.41	1.21	1.30
5	0.09	0.37	0.85
6	0.15	0.93	0.92
7	0.07	0.63	1.32
8	0.03	0.10	1.76

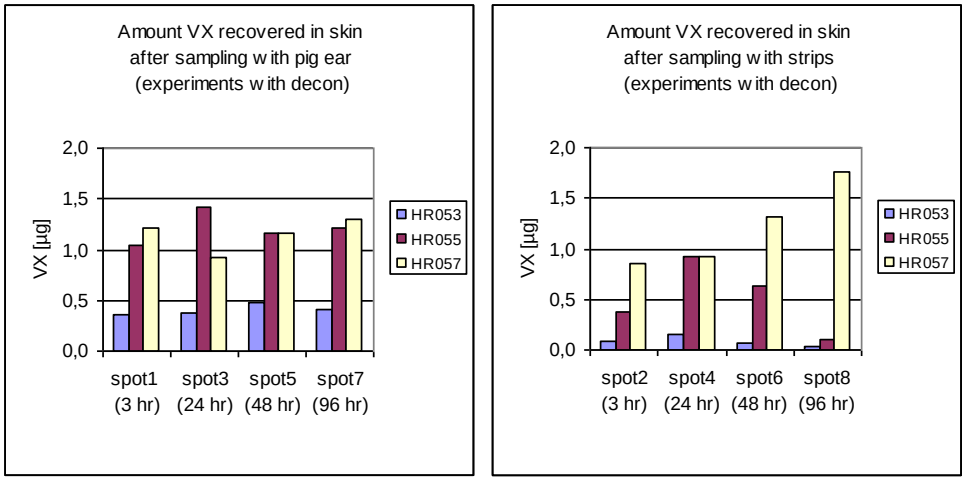

Figure 70 VX [µg] in skin of the hairless guinea pig of spots sampled with pig ear (left panel) or by tape stripping (right panel), after decontamination with RSDL.

As was to be expected, it appears that the quantities of VX in the experiments in which decontamination is applied are lower than in the experiments without decontamination. The amount VX found in the pig ear skins used for sampling is lower than the amount found in the tape strips used for the corresponding spot. This might be caused by various reasons, e.g., because of the smaller contact area in case of sampling with pig ear skin. Also, in case of tape stripping we observed that during sampling at the longer time points part of the guinea pig skin sticked to the tape, but not to the pig ear skin, which could have led to higher levels of VX in case of tape stripping.

Task 12 Hazards from remains contaminated with *Bacillus anthracis*

Before performing the actual experiments with the hairless guinea pigs, a number of introductory tests were conducted for optimization purposes. The efficacy of sampling *B. anthracis* spores (BM233; Vollum strain) from skin using swabs was established by contaminating pig ears (obtained from the slaughter house) with known amounts of spores and applying the swab method, as planned to be used for the actual experiments with the hairless guinea pigs. The efficacy of swabbing the spores from the pig ears was established to be approximately 20%.
Histological investigations showed that there was not much difference between fresh pig ears and frozen pig ears, in case the ears had been frozen in a mild, stepwise manner.
It was assessed that ‘swabbing’ gave better results than tape stripping for sampling spores, so the swabbing method was used for the experiments in Task 13.
It was also assessed that the FRET assay for determining the number of spores still worked properly after decontamination of contaminated pig ears with 5% hypochlorite.

a. Contact hazard from externally contaminated remains

At 8 places on the abdominal skin of the dead hairless guinea pigs small quantities of *B. anthracis* spores were applied (Delta Sterne strain 1221 and Vollum strain BM 233; purity 99%). At various time points (0, 4, 22, 26, 46, 50, 70 and 92 hours after contamination) swab samples were taken to investigate whether the number of spores on the abdominal skin changes over time. After transferring the swab sample into the culture medium, dilution, plating and counting the bacteria colonies, no significant decrease in the number of spores was observed over time (see Figure 71, left two columns), indicating the high persistency of the spores. Blood samples and samples from various organs have also been taken; however, no *B. anthracis* colonies could be detected after plating aliquots of the processed tissues (results not shown).

During the experiment samples were taken for an additional analysis to assess the presence of *B. anthracis*, based on fluorescence resonance energy transfer (FRET). This method is based on a specific protease activity displayed by *B. anthracis* species (see Kaman *et al.*, 2011), which can be used advantageously for diagnostic purposes by cleavage of a specific fluorescent peptide substrate. Also by using this assay no drastic decrease in the number of spores could be observed (see Figure 72, left two columns); only in case of Vollum strain BM233 a slight decrease was noticed for the 70 and 92 hours time points.

b. Contact hazard from externally contaminated remains, after decontamination

In the next series of experiments, decontamination experiments were performed with two different decontaminants; see Figures 71 and 72.

- 5%-bleach solution (adjusted to pH 6.75, according to a US protocol provided by DTRA) proved ineffective (see Figure 71). A control experiment was performed on a glass surface with the same amount of spores. Also in this case it was observed that 5% bleach solution was not effective. In this case, only the Vollum strain has been used. In case the FRET assay was used, a slight decrease was noticed; however, still significant protease-related activity was present in the samples taken (see Figure 72). When 15% bleach was used instead of 5% bleach, decontamination efficacy was quite similar when assessed by standard colony counting. In contrast, according to the FRET assay the decontamination efficacy by 15% bleach was much higher.
- Subsequently, experiments were performed with the decontaminant PES-Solid (50 g PES-Solid in Dahlgren Surfactant Formulation, provided by DTRA). On a glass surface this decontaminant proved to be effective against *B. anthracis* spores. On the hairless guinea pig skin only a small reduction of spores was observed, when used according to the protocol that was delivered by DTRA. In this case, both strains (Delta Sterne and Vollum) have been used; no significant changes were observed in sensitivity of the two strains were

observed. No significant differences between the various time points were observed. In contrast, when we applied the FRET assay to these samples, it seems *B. anthracis* activity (i.e. the protease activity) was significantly diminished for the Sterne group and absent for the Vollum group. Apparently, both assays have a different sensitivity towards the decontaminants.

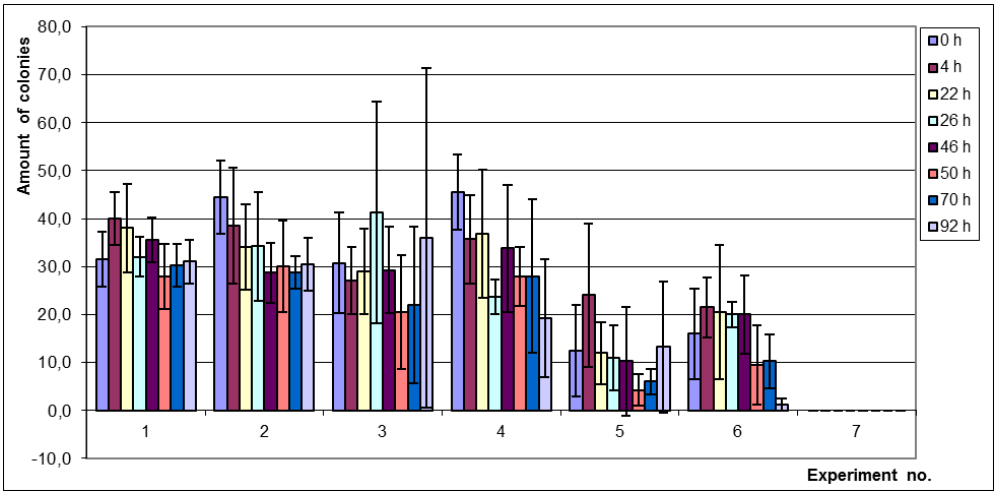

Figure 71 Fate of various *B. anthracis* spores on skin of deceased hairless guinea pigs (n=4 for each experiment), 0, 4, 22, 26, 46, 50, 70 and 92 h after contamination; number of spores quantified by colony counting.
Experiment 1: Control experiment with B.a. Delta Sterne (BM 1221)
Experiment 2: Control experiment with B.a. Vollum strain (BM233)
Experiment 3: Vollum strain (BM233); decontamination with 5% bleach
Experiment 4: Vollum strain (BM233); decontamination with 15% bleach
Experiment 5: Vollum strain (BM233); decontamination with PES Solid
Experiment 6: Delta Sterne (BM1221); decontamination with PES Solid
Experiment 7: Vollum strain (BM233); intranasal contamination (in this case no spores could be detected).

c. Hazard from internally contaminated remains

In another series of experiments living hairless guinea pigs were infected intranasally with *B. anthracis* spores (Vollum strain), using an amount of spores which should be sufficient to cause an infection (see Altboum *et al.*, 2002). The animals showed distinct signs of Anthrax infection after 1 day. The animals were euthanized after 3 days, which should be sufficient time for the infection to spread within the body; swab samples were taken from premarked spots from the belly skin at various time points (0, 3, 6, 22, 29, 48, 72 and 96 hours after euthanasia). No external contamination could be detected at these sampling time points (see Figure 71, right column). After 92 hours lungs, liver, and blood samples were tested for the presence of an infection with *B. anthracis*. However, no colonies of *B. anthracis* could be found after processing the samples. Samples have also been tested by using the more sensitive FRET (Fluorescence Resonance Energy Transfer) assay. Also in this case, the presence of *B. anthracis* could not be ascertained (see Figure 72, right column).

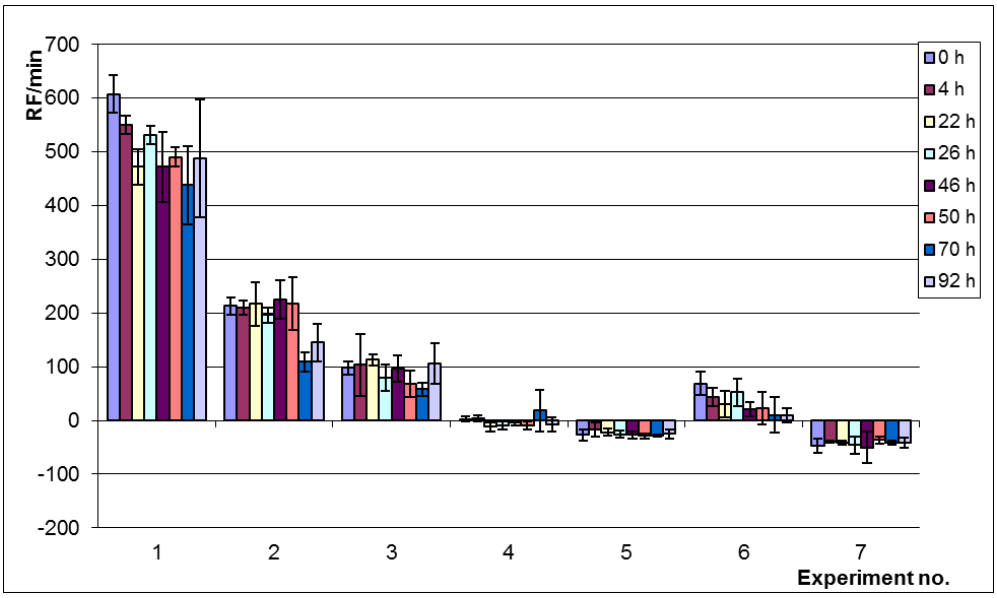

Figure 72 Fate of various *B. anthracis* spores on skin of deceased hairless guinea pigs (n=4 for each experiment) at various time points after death, as assessed by means of the FRET assay.
Experiment 1: Control experiment with B. *anthracis* Delta Sterne (BM1221).
Experiment 2: Control experiment with B. *anthracis* Vollum strain (BM233)
Experiment 3: Vollum strain (BM233); decontamination with 5% bleach
Experiment 4: Vollum strain (BM233); decontamination with 15% bleach
Experiment 5: Vollum strain (BM233); decontamination with PES Solid
Experiment 6: Delta Sterne (BM1221); decontamination with PES Solid
Experiment 7: Vollum strain (BM233); intranasal contamination

V CONCLUSIONS

One of the major conclusions from the literature study (**Task 1**) is that although it is clear that many processes contribute to (slow) degradation of agents after death, it can be expected that the compounds will not completely become detoxified. No examples of toxification reactions were found in the literature.

With regard to the experiments with liquid VX (**Task 2**), the following can be concluded:

- experimental set-up works nicely, and the animal can be sampled for up to 22 hours for blood samples and up to 96 hours for liver samples.
- the amount of VX on skin decreases gradually, but VX is still present after 96 hours at levels between 20 – 90 $\mu g/cm^2$.
- VX is present in blood at the last possible sampling time point (either 5 hours, or 22 hours) and levels seem to be rather constant over time;
- VX is present at much higher levels in liver than in blood, especially after 5 h. This might be an example of agent redistribution;
- As yet, no remarkable results with regard to the formation of toxic metabolites have been obtained. The toxic metabolite V27A (desethyl VX) has been analyzed in one of the blood- and in one of the liver samples, at a level of 0.2 ng/mL (which is very low compared to the VX levels);
- The hydrolysis product EMPA could only be analyzed in liver samples at variable levels (2 – 200 ng/mL), which is in agreement with the high and variable concentrations of VX found in liver.

With regard to the experiments with liquid sulfur mustard (**Task 3**), the following can be concluded:

- Sulfur mustard on skin is not very persistent and at 2.5 after death (3 hours after application) the agent could no longer be detected by means of tape stripping;
- In blood, intact sulfur mustard could be analyzed for up to 23 hours (last possible sampling time point within this particular series), while levels gradually decreased;
- In liver, intact sulfur mustard could be analyzed for up to 48 hours, with gradually decreasing levels;
- Sulfur mustard levels in liver were much lower (depending on sampling time) than VX levels in liver, probably reflecting the much higher intrinsic reactivity of sulfur mustard compared to VX (a much more selectively reacting agent);
- Vapor hazard is most pronounced in the first 24 hours after death.

The relatively non-toxic sulfur mustard metabolite sulfur mustard sulfoxide could be analyzed in the blood of the remains of hairless guinea pigs, after percutaneous contamination with sulfur mustard. In addition, protein adduct formation could be assessed by mass spectrometric analysis of a

cysteine-sulfur mustard adduct, after pronase digestion of whole blood. Similar adduct formation has been observed under normal *in vivo* conditions. From a practical point of view, it is worthwhile to know that detection equipment appears to respond at later time points (> 72 hours), probably because of natural putrefaction products which are formed after death.

With regard to **Task 4**, whole body vapor exposure to sulfur mustard, the following can be concluded. As was to be expected, the levels on skin, expressed in µg/cm^2, are much lower compared to the levels after liquid exposure (as carried out within the framework of Task 3). The off-gassing levels are also much lower. On the other hand, the internal levels of free agent (blood, liver) are in the same order of magnitude as found after percutaneous liquid exposure.

With regard to the animal studies carried out with liquid sulfur mustard on skin at low temperature (**Task 5**), it seems that the agent levels in blood and liver are significantly higher at lower temperature than at ambient temperature. This might be caused by a lower reactivity of the agent at lower temperatures, and/or because more agent had penetrated the skin. The latter explanation is in agreement with the decreased off-gassing as observed during the initial (-0.5–1 hours) time period, right after contamination. Also, it seems that cooling of the remains prolonges the process of off-gassing.

After further evaluation of the VX data by using a PBPK model (**Task 6**), it is plausible that the enzymatic hydrolytic activity in a deceased animal is much lower than in the living animal. This will lead to higher VX concentrations in a deceased organism in comparison with a living organism, and therefore also leads to a larger potential hazard, e.g. during autopsy.

With regard to the contact hazard of contaminated protective gear (**Task 7**), it was assessed that significant levels (up to 10 µg/cm^2) of sulfur mustard could be analyzed after 96 hours on suede leather and rubber soles of US Army boots. The same accounts for VX on the rubber soles (suede leather experiments to be repeated). In case of VX, the agent levels on the rubber material decreased slightly more rapidly than in case of sulfur mustard. Especially *in* the rubber material of Army boots, agent levels remained constant up to 96 hours (last sampling point) at high levels, up to 50% of the applied amount. In the suede of US Army boots, the sulfur mustard levels slowly decreased to approximately 10% (at t = 96 hours) of the originally applied amount. Off-gassing of sulfur mustard from US Army clothing and suede of US Army boots slowly built up, was most pronounced during at 2-4 hours after contamination, and decreased slowly. Slightly different results were obtained with GB: the levels of GB built up more rapidly, while after 24 - 48 hours, the levels are in general lower than in case of sulfur mustard, which might be inter alia caused by the higher volatility of sarin.

Comparison of the results of experiments with contaminated biological remains with those obtained for the contaminated protective gear (**Task 8**

and 9) learns that especially in case of sulfur mustard the hazard posed by contaminated protective gear might be significantly higher. Thus, in case of biological remains sulfur mustard agent levels rapidly (> 0.5 hours after application) dropped to relatively low levels (< 2 µg/cm^2), while on rubber soles and suede leather more significant levels (up to 10 µg/cm^2) were still present even at 96 hours. Striking is the high persistency of VX and sulfur mustard within both suede leather and rubber, which is probably due to the low reactivity of the matrix compared with a biological matrix. In addition, with regard to off-gassing, sulfur mustard vapor levels seem to be considerably higher in case of personal gear at equal contamination levels, especially in the 1-4 hours time interval. This might also be due the higher reactivity of sulfur mustard in the biological matrix, due to the presence of many reactive constituents (e.g., water, proteins).
It should be remarked that the variability in the evaporation measurements (for both sulfur mustard and GB) is very high. One reason for this might be the high reactivity of these agents, e.g. towards moisture. We therefore recommend that in future projects, these experiments should be carried out at least in 6-fold.
It seems from these preliminary worst case estimates that human remains from victims from liquid exposure to chemical warfare agent may present significant vapor hazards, even when only parts of the body are contaminated. It seems worthwhile to study the effects of ventilation, by using computer simulations (for instance by using the gCOMIS software) using such evaporating body(parts) as a source term in a more realistic setting. For victims of vapor exposure, the remaining agent is not enough to exert any hazard.

Direct decontamination with RSDL results in the lowest residual VX levels, in comparison with delayed decontamination (**Task 10**). This might be explained by the fact that in case of delayed decontamination the agent can penetrate deeper into the skin, making it less readily accessible to the decontamination agent. Nonetheless, in the latter case the agent can still be removed by tape stripping and consequently still poses a contact hazard.

The amount of VX found in the pig ear skins used for sampling is lower than the amount found in the tape strips used for the corresponding spot. This might be caused by various reasons, e.g., because of the smaller contact area in case of sampling with pig ear skin. Also, in case of tape stripping we observed that during sampling at the longer time points part of the guinea pig skin stuck to the tape, but not to the pig ear skin, which could have led to higher levels of VX in case of tape stripping (**Task 12**).

When *B. anthracis* spores (Delta Sterne and Vollum strain) were applied topically onto the animal, hardly any decrease of the number of spores over time (until 92 hours after death) was observed (**Task 13**). It was assessed that treatment with neither 5% bleach nor 15% bleach did result in a significant decrease of the number of spores (determined after colony counting), while the decontaminant PES-solid proved to be only slightly effective. In contrast, according to the protease activity-based FRET assay, the PES solid and 15% bleach proved to be quite effective for the virulent Vollum strain. The striking difference in response of both assays remains an issue for further research. After intranasal contamination of the animal with spores (Vollum strain), *B. anthracis* colonies could not be detected after plating swab samples taken from the skin of the belly and after plating processed samples of various organs, despite of the fact that the animals displayed clear physical signs of a *B. anthracis* infection. Also when the FRET assay was used, no response was detected for these swab samples.

Overviewing all results obtained in this study it can be concluded that scenarios can be envisaged in which vapor and or contact hazards are posed by human remains contaminated by VX or sulfur mustard. Decontamination of the remains with RSDL shortly after death will reduce both the vapor and contact hazards significantly. We do not think that new decontamination technology is required to counteract these hazards. With regard to contamination of the inner parts of the body, one might consider the use of embalming fluids containinig a proper decontaminant. To some extent the vapor hazard can be detected by chemical agent monitors. Both the vapor and contact hazard can be mitigated by using personal protective equipment: mask, gloves, suit.

Chemically contaminated personal assets such as uniforms and boots also pose a considerable hazard, due to the high persistence of CWA on and in these materials, in particular the rubber and suede of the boots. We did not study the effect of decontaminants on contaminated personal effects. However, it can be expected that agent that has been absorbed in rubber materials (e.g. the soles of boots) is quite resistant against decontaminants. It might be worthwhile to pursue the development of more gaseous decontaminants that penetrate more deeply into these materials.

For remains contaminated with *B. anthracis* a similar level of personal protection is required. Autopsy on such remains is preferably performed in a biosafety environment. According to one assay used, the spores were quite refractory against decontamination, so this might be a subject for further exploration, e.g., by evaluation of additional decontaminants.

The result of this study may help in adjusting existing protocols for handling contaminated humqan remains. It has to be taken into account that the contamination levels that were used in the current study are fairly high: *ca*. 8 mg of liquid VX or sulfur mustard on the hairless guinea pig and a sulfur mustard vapor exposure 10,000 $mg.min.m^{-3}$. These levels were chosen in order to ensure that we would be able to measure intact agent in and on the tissues after death. It may be argued that such high exposures relate to ‘Cold War’ scenarios, which nowadays are perceived as being less likely to occur.

Obviously, if the challenge doses would be lower, the vapor and contact hazards will also be reduced.

Further research in this field could be aimed at similar ‘postmortem’ studies for NTA’s in various physical states, with the emphasis on agent persistence, distribution and possibilities for decontamination. In addition, the biological fate of agents that cause contagious diseases (e.g., *Yersinia pestis*) under postmortem conditions and possibilities for decontamination, should be addressed.

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VI TECHNICAL OBJECTIVES/STATEMENT OF WORK

Objective

The objective of the study is to obtain knowledge - by literature search, experimental data and modeling - on the biological and chemical fate and residual hazard of chemical warfare agents (CWAs) on contaminated human remains and personal effects. The study is anticipated to provide input for the development of a model to predict the residual hazard in time after death, as well as new decontamination technology and protocols for the safe handling of contaminated human remains and personal effects.

1.0 Scope

The residual hazard of contaminated human remains will be studied in three phases. In Phase I, the biological and chemical fate of CWAs in human remains will be studied by literature search and animal experiments on hairless guinea pigs. The biological and chemical fate of CWAs (concentration and metabolism) will be determined in intoxicated and euthanized animals in time after death. Obtained experimental data will be used in a Physiologically-Based PharmacoKinetic (PBPK) model to predict the kinetics and detoxification of CWA in human remains in time after death.
In Phase II, the residual vapor and contact hazard will be measured from intoxicated, deceased animals, as well as from contaminated clothing material and rubber boots. The government indicated there should be no problem in providing the requested uniforms items in support of the proposed work. It will be attempted to extrapolate the obtained experimental data towards the situation of contaminated human remains and personal effects.
Finally, in Phase III, results will be integrated and evaluated against available toxicological data. It will be shown whether secondary exposure may lead to negligible or significant toxicological effects. Advise shall be provided concerning future research and the way ahead. The study is anticipated to provide input for the development of a model to predict the residual hazard in time after death, as well as new decontamination technology and protocols for the safe handling of contaminated human remains and personal effects.

2.0 Background

Intoxicated, deceased victims of an attack with CWAs will need to undergo recovery, decontamination, possibly autopsy, transportation into the home land, and finally interment. Several guidelines are available that describe these procedures, with a focus on the safety of personnel. During the handling of contaminated human remains and personal effects, the agent that is still present on (or inside) the contaminated body may pose a hazard to unprotected personnel. Therefore, the presence of (residual) agent is continuously monitored by detection equipment.
Limited attention has been paid to the scientific background of this secondary hazard. Agents may remain present on the skin as a liquid, be released as a vapor or in body fluids, thus presenting both a contact and a vapor hazard. Agents may be

present for a longer time in human remains compared to living human beings, due to postmortem changes of physiological processes that influence the biological and chemical fate of the agents.

Biological fate in healthy human beings

The distribution and metabolism of CWAs in living organisms have been studied extensively. After exposure, agents will be absorbed and distributed, depending on the route of administration. Some useful data was obtained from experiments on human volunteers in the 1950s, as well as from extensive toxicokinetic studies in laboratory animals.
Following distribution, natural detoxification occurs by binding of nerve agents to scavenging enzymes such as acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and carboxylesterase(CaE). Metabolism occurs mainly by spontaneous and enzymatic hydrolysis of nerve agents and sulfur mustard. Furthermore, particularly for sulfur mustard scavenging to proteins will occur. Enzymatic activities and hydrolysis rates of CWAs in plasma and tissue homogenates have been well documented. These data has been used to develop a PBPK model to predict the toxicokinetics in intoxicated (living) human beings.

Postmortem biological and chemical fate

After death, physiological processes start to change rapidly, many of which have an effect on the biological and chemical fate of CWAs. It is anticipated that CWAs will remain present for a longer period of time in human remains compared to healthy human beings, because of the slowed down or terminated processes of natural detoxification. On the other hand, certain biochemical mechanisms may be activated which may enhance destruction of CWAs. Several postmortem physiological changes are of particular interest in this respect: termination of blood circulation, decrease of body temperature, redistribution of agents and putrefaction. Three mechanisms of reaction between CWAs and physiologically active materials found in human remains are anticipated:

- *Postmortem Redistribution* Agent concentrations in body fluids and organs may change, similar to drugs, due to postmortem redistribution. Agents may be redistributed from ‘reservoir’ organs such as the gastrointestinal tract, liver, and lungs to surrounding tissues.
- *Postmortem Scavenging* As mentioned above, natural detoxification of nerve agents occurs by binding to scavenging enzymes. However, victims that suffered from a lethal dose of nerve agent will have enzyme activity levels that approach zero. It is estimated that postmortem scavenging will play a limited role in the detoxification of nerve agents; in case of sulfur mustard, alkylation of proteins will contribute to the post-mortem detoxification.
- *Postmortem Metabolism* The natural metabolism of CWAs is expected to continue postmortem, but at a lower rate due to the decreasing body temperature and due to the fact that metabolism in the liver will stop. Alternative, yet unknown degradation pathways may arise, e.g. by bacteria during putrefaction. It can not be excluded that under postmortem conditions the metabolism is incomplete or slightly altered, and consequently might give rise to the formation of toxic reaction products.

Hazard of contaminated human remains

The presence of toxic agents on or inside the body of deceased victims, as well as on their clothes, poses a threat to (unprotected) personnel handling the remains. Several (civil) cases are known from history, in which handling contaminated victims lead to intoxication of rescuers. These cases include the exposure to sulfur mustard in Bari (1943), and the sarin attacks in Matsumoto (1994) and Tokyo (1995). In the later case, it has been assumed that the secondary exposure resulted from off-gassing of sarin from the clothing of victims.

It has been estimated that 80 % of the contamination will be present on the clothing of victims after a chemical incident. Removed clothes may be collected with other personal effects, stored and/or destroyed. These contaminated items may present both a contact and vapor hazard. Especially porous materials, such as rubber, are known to absorb CWAs, leading to a prolonged hazard.

The secondary hazard from (decontaminated) human remains may seem less important than the hazard from contaminated clothing. However, after removal of clothing and decontamination of skin and hair, the hazard may not be completely removed as deceased victims may continue to emit fluids or gases after decontamination. A vapor concentration may build up when a body has been temporarily stored in a body bag or a (refrigerated) coffin at a morgue. An essential tool for the determination of the actual hazard of contaminated human remains and personal effects, is the use of chemical agent monitors.

3.0 Tasks/Technical requirements

Task 1 Literature Study on Postmortem Processes (FY 09)

A literature study will be conducted on the postmortem changes in physiologically relevant processes such as agent redistribution and metabolism. The possible formation of toxic degradation products will be addressed. Activity data of relevant enzymes (such as cholinesterases) will be gathered, preferably determined in postmortem blood or tissue samples, or otherwise *in vitro* at ambient temperatures. Postmortem research on organophosphate pesticide intoxications may provide information that also applies to nerve agent intoxications. Data from case studies of exposure to CWAs shall be looked up, both incidental and during experiments performed on volunteers in the 1950s.

The study shall include consultation of the Dutch Forensic Institute in The Hague (The Netherlands), which are experts in the area of postmortem processes and with which TNO has close contacts.

Task 2: Animal Study – Liquid VX on skin (FY 09)

Animal experiments will be conducted on adult, anesthetized hairless guinea pigs, that will be contaminated with a supralethal dose of liquid VX on the skin applied on multiple spots. The proposed dose is 8 droplets of 1 µl, equivalent with 128 LD_{50}. Animals that have survived the contamination after 30 minutes will be euthanized at that time point.

To establish the contact hazard, the deceased animals (n=4) will be placed in a ventilated glass chamber, after which the contact hazard is measured in time after death. For each time interval, a different spot on the skin will be sampled. Samples

will be taken up to a maximum of 96 hours after death, at 2.5, 5, 23, 29, 48, 72 and 96 hours. The contact hazard will be established by skin tape-stripping combined with GC analysis. Tape-stripping is a frequently used method to quantify residual contamination on the skin (including liquid droplets and agent penetrated in the first layer of the skin).

As a control for analytical measurements, two experiments will be performed on animals that were not exposed to VX. It was chosen not to use contaminated living animals as a control group. Tissue and/or blood samples will be taken at various time intervals (2.5, 5, 23, 29, 48, 72 and 96 hours) to monitor the amount of intact agent (n=4) and metabolites (n=2; at 5 and 72 hours, qualitative analysis) by GC-MS and/or LC-MS-based methods that have been developed during earlier toxicokinetic studies on VX in the contractor's laboratory. Since postmortem may be difficult to sample, tissue will be sampled that is rich in blood, such as liver and lung. The exact sampling procedure has to be established during a pilot study. The postmortem degradation rate of CWA in the biological samples will then be determined by GC-MS and/or LC-MS analysis, Special attention will be paid to the search for unknown and/or toxic metabolites. The anticipation, that cholinesterase activity will be near zero, will be checked by butyrylcholinesterase (BuChE) activity determinations.

Task 3: Animal Study – Liquid Sulfur Mustard on Skin (FY 09)

In Task 3, both the contact and evaporation hazard will be studied after contamination of animals by liquid sulfur mustard on skin. Animal experiments will be conducted on adult, anesthetized hairless guinea pigs, that will be contaminated with a sublethal dose of liquid sulfur mustard on the skin applied on multiple spots. The proposed dose is 8 droplets of 1 µl, equivalent with < 1 LD_{50}. Animals will be euthanized 30 minutes after contamination. In the first series (four animals) the deceased animals will be placed in a glass chamber, in which a controlled air flow is sweeping over the animal. The outlet air will be lead through a bubble cell containing solvent to sample the agent in intervals, followed by quantitative analysis by gas chromatography (GC). In addition, the response of the chemical agent monitor ICAM (to be provided by US Army; otherwise a CAM used by the Dutch Armed Forces will be used) to the released vapor above the animal will be measured. Sampling and measurements will take place until vapor is no longer detected, with a maximum of 96 hours. This sampling technique seems more suitable in this set-up than the originally proposed SPME (solid phase micro extraction).

In the second series (four animals), the contact hazard will be determined as described under Task 2. Tissue and/or blood samples will be drawn at various time points (2.5, 5, 23, 29, 48, 72 and 96 hours) to monitor the amount of intact agent (n=4) and metabolites (n=2; at 5 and 72 hours, qualitative analysis), according to earlier developed GC-MS-based methods in case of intact agent and literature-based LC-MS methods for metabolites (see Task 2). The postmortem degradation rate of CWA in the biological samples will thus be determined. Special attention will be paid to the search for unknown and/or toxic metabolites. Furthermore, the reaction of albumin with sulfur mustard will be determined by MS analysis of adduct peptides obtained after proteolytic digestion at various time intervals.

Task 4: Animal Study - Sulfur Mustard Vapor Exposure (FY 09)

In Task 4, both the contact and evaporation hazard will be studied after contamination of animals by whole body exposure to sulfur mustard vapor. Animal experiments will be conducted on adult, anesthetized hairless guinea pigs, that will be contaminated with a sublethal dose of sulfur mustard vapor by whole body exposure. The proposed dose is 10.000 $mg.min.m^{-3}$ (250 $mg.m^{-3}$ during 40 minutes), equivalent with 1 LCt_{50} (percutaneous) or 12 LCt_{50} (inhalatoir). Animals that have survived the contamination shall be euthanized. Furthermore, experiments will be carried out as described under Task 3.

Task 5: Animal Study at Low Temperature - Liquid Sulfur Mustard on Skin (FY 10)

Based on the obtained results in Tasks 2-4, two series of animal experiments will be performed in which contaminated, euthanized animal will be temporarily stored in a closed box at low temperature, thus mimicking the procedure of refrigerated storing human remains at a mortuary. Animal experiments will be conducted on adult, anesthetized hairless guinea pigs, that will be contaminated with a sublethal dose of liquid sulfur mustard on the skin applied on multiple spots. The proposed dose is 8 droplets of 1 µl, equivalent with < 1 LD_{50}. Animals will be euthanized 30 minutes after contamination. Next, animals will be stored in a closed glass box for 24 hours at 4 °C. Then, experiments will be determined as described under Task 3 to determine both the vapor and contact hazard in two series of experiments (four animals for each series).

It is expected that the low temperature and storage in a closed box will be mainly of influence on the residual hazard, but not on blood/tissue levels and metabolism. Therefore, and to limit the amount of work, no biological sampling and analysis shall be performed in this task.

Task 6: Physiologically-Based PharmacoKinetic (PBPK) Modeling (FY 09)

Physiologically-based pharmacokinetic modeling (PBPK) is a mathematical modeling technique for prediction of the absorption, distribution, metabolism and excretion (ADME) of a compound in humans and other species used in pharmaceutical research. A PBPK model, that was previously developed to model the *in vivo* toxicokinetics of CWAs, will be adapted to predict the kinetics of VX in blood and / or tissues of deceased, intoxicated humans. To limit the amount of work, modeling shall focus on the kinetics of VX. Strictly speaking, the modeling that is performed is not PBPK-modeling, as the contractor's PBPK-model for VX is flow-limited. In fact, the contractor will perform a kind of biochemical-kinetic modeling of the toxicant within the various compartments. However, the contractor will use their PBPK-model for this purpose. In the postmortem model, parameters such as the cardiac output and alveolar ventilation will be set to zero. Input on expected cholinesterase activities will be obtained from the literature study (Task 1), whereas measured VX concentrations in postmortem blood/tissue will be obtained from the animals experiments (Task 3).

Task 7: Hazard of Contaminated Clothing (FY 10)

The hazard of CWAs remaining on personal effects will be investigated. Experiments will be performed in which two different types of materials (proposed are clothing and rubber boots from the U.S.Army) will be contaminated with sulfur mustard, VX, and sarin (10 g/m^2, triplicate experiments). The evaporation of sulfur mustard and sarin in time will be measured using a similar evaporation chamber as described for the animal studies (Task 3) followed by GC analyses of collected vapor. In addition, the response of chemical agent monitor ICAM (to be provided by US Army; otherwise a CAM used by the Dutch Armed Forces will be used) to the released vapor above the animal will be measured. The evaporation risk of VX will not be considered due to the low volatility (previous results have shown that VX hardly poses an evaporation risk).

In a second series of experiments, the contact risk of VX and sulfur mustard will be studied by quantification of CWAs on absorbent material (aluminum sheets covered with silica) after contact with contaminated materials under a fixed pressure. One contact time will be used (e.g., 15 minutes). The contact risk will be determined in time up to 72 hours after contamination. The contact risk of sarin will not be considered due to the high volatility (previous results have shown that sarin hardly poses a contact risk).

Task 8: Extrapolation towards Human Remains and Personal Effects (FY 10)

It will be attempted to extrapolate the obtained experimental data on contact and vapor hazard towards the situation of contaminated human remains and personal effects. The amount of released CWA from intoxicated, deceased animals will be translated to that of an average human being using the released amount of CWA per body weight. Low temperature experiments shall not be considered. If required, a correction for the difference in exposed dosage will be made. A similar calculation will be made for contaminated clothing (one material) using the amount of released CWA per area of clothing. The vapor concentration will be calculated in time, based on the experimental data obtained in previous tasks, using the software program Gcomics. Gcomics is a multi-zonal infiltration model based on modules, and has been previously used (Boone et al., 2008) to estimate residual vapor hazards. Different parameters will be used, such as distance to the contaminated object and the degree of ventilation. Obtained data will provide operational insight in the vapor hazard from human remains and clothing.

The contact hazard will be determined as follows. The amount of liquid agent that will be transferred after contact of skin with contaminated human remains and personal effects will be extrapolated from the experimental data obtained in previous tasks. It is anticipated that contact will occur only by the hands (contact area 200 cm^2).

Task 9: Integration: Toxicological Evaluation and Way Ahead (FY 10)

Finally, in Phase III, results from Phases I and II shall be integrated. Conclusions obtained in all previous tasks shall be summarized. The relationship will be studied between the postmortem biological and chemical fate of CWAs (Phase I) and the hazard resulting from the handling of contaminated human remains and effects (Phase II). For instance, a prolonged vapor hazard could be related to persistency of

agent and possible toxic metabolites measured in biological tissues. The results will be evaluated against available toxicological data (both inhalation and dermal) that are available from literature, including NATO reports. Thus, a first conclusion shall be drawn on the actual hazard arising from handling contaminated remains and personal effects. It will be shown whether secondary exposure may lead to negligible or significant toxicological effects.
Finally, advise shall be provided concerning future research on the subject and the way ahead. It is anticipated that the study will provide input for the development of a model to predict the residual hazard in time after death, as well as new decontamination technology and protocols for the safe handling of contaminated human remains and personal effects.

Task 10: Effect of decontamination on the residual chemical hazard (optional year 2; FY 11)

It can be envisaged that the most convenient way to significantly reduce the contact hazard of contaminated human remains would be a single skin decontamination step. On the other hand, it can also be envisaged that after decontamination the agent might still pose a residual contact hazard, e.g. by redistribution from a skin depot or from organs/tissue/fat.
It is proposed to explore this assumption in more detail via a study with liquid VX and hairless guinea pigs, with n=4, in analogy with Task 2. It is proposed to use RSDL as skin decontaminant. A commercially available RSDL pouch will be used. The lotion will be removed with a gauze at 10 minutes (arbitrary) after application. It will be determined to which extent the RSDL-decontamination interferes with the tape stripping procedure, by determination of the recovery of VX after skin decontamination, by using a non-reactive VX simulant that has a similar octanol/water partition coefficient as VX. Two different scenarios will be evaluated. In the first scenario, decontamination will be performed directly after death (i.e., 30 minutes after application of the agent). Subsequently, agent levels on skin will be determined at different time points, up to 96 hours (e.g., 3, 24, 48 and 96 hours; 2 spots per time point). In the second scenario, decontamination will be performed after a longer period of time with an additional series of animals (n=4), in order to allow the agent to penetrate the skin more thoroughly and allow the formation of skin depots of the agent. Thus, it is proposed to study the effect of decontamination at 24 hours, and sample at various time points, up to 96 hours. It is proposed to use one animal as a positive control (VX exposed, no decontamination). No blood and liver samples will be analyzed, because we focus on direct contact hazard by contaminated skin.

Task 11: (optional year 2; FY 11)

This part is classified.

Task 12: Pilot on feasibility of determination of contact hazard through the use of pig ear skin (optional year 2; FY 11)

Also, it would be more beneficial to look at contact hazards on skin rather than the contact hazard on tape. We propose to perform a pilot experiment (n=3) with VX, with pig ear skin as alternative for tape. We propose to sample 4 spots (4 time points, i.e. 3, 24, 48 and 96 hours) on the contaminated hairless guinea pig with pig ear skin, and sample 4 spots by means of tape stripping. This enables direct comparison of results.

Pieces of pig ear skin will be placed on (de)contaminated skin sites of the hairless guinea pig under a pressure of 20 $g.cm^{-2}$ for 15 minutes (pressure and time subject to change). Next, the agent will be extracted from the pig ear skin sample and analyzed with GC-MS or LC-tandem MS. The results will be compared with the tape stripping experiments that have been performed in the first stage.

Task 13: Hazards from remains contaminated with *Bacillus anthracis* (optional year 2; FY 11)

a. *Contact hazard from externally contaminated remains*

Hairless guinea pigs (n=4) will be euthanized, transferred into an isolator in our BSL-3 facility and subsequently contaminated with *B. anthracis* Delta Sterne (as a simulant for *B. anthracis*) on 8 premarked spots on the belly. Each spot will be contaminated with 10 µl of medium containing approximately $1*10^8$ spores. Prior to this, it will be thoroughly ascertained that these are exclusively robust spores.

An indication of the contact hazard in time is obtained by taking swabs from the sites at 0, 3, 6, 22, 29, 48, 72 and 96 hours after contamination. In addition, samples will be drawn from blood (as long as possible), liver and lung, to check for possible internal contamination. The efficiency of sampling spores/bacteria/spores from skin using swabs will be established by contaminating pig ears with known amounts of spores/bacteria and applying the swab method as planned to be used on the hairless guinea pigs.

The number of bacteria/spores will be determined via a FRET-assay (‘fluorescence resonance energy transfer’) using an in-house designed (patent pending) substrate. The assay is selective, sensitive and rapid and is based on protease-activity of the bacterium. Read-out of the assay will be the fluorescence signal of the cleaved substrate. The identity of this product will be confirmed via mass-spectrometric analysis of a few samples. Since the assay requires vegetative bacteria, spores have to be cultured in BHI for 3 hours before they exhibit protease activity.

After these experiments have been performed successfully, the procedure will be repeated (also n=4) with ‘hot’ Anthrax, i.e. *B. anthracis*, Vollum or Ames strain.

b. *Contact hazard from externally contaminated remains, after decontamination*

Similar experiments will be performed as described under 4a, but now decontamination will be performed with 5 % hypochlorite solution. The time point for decontamination will be 1 hour after contamination. It will be verified that traces of 5% hypochlorite do not interfere with the FRET assay. In the subsequent experiments with euthanized hairless guinea pigs (n=4 both for *B. anthracis* Delta

Sterne and *B. anthracis*, Vollum or Ames strain) sampling points will be at 0, 3, 6, 22, 29, 48, 72 and 96 hours.
A second decontaminant (material, MSDS and test protocol to be provided by DTRA) will also be tested.

c. *Hazard from internally contaminated remains*

Hairless guinea pigs (n=4) will be placed in the isolator in our BSL-3 facility. *B. anthracis*, Vollum or Ames spores will be administered intranasally, a technique which we have already applied successfully in mice. After 72 hours the animals will be euthanized, which should be sufficient time for the infection to spread within the body, although the animals may not show clear clinical signs. At 0, 3, 6, 22, 29, 48, 72 and 96 hours after euthanasia swabs are taken from premarked spots from the belly. Also samples will be taken from blood, liver and lung. *B. anthracis* will be analyzed using the FRET-technology.

4.0 Contract Data Requirement Lists / Other Deliverables

Technical reports

Quarterly technical reports are due on April 5, July 5, October 5 and January 5 of each year. A quarterly technical report will not be required the month an annual or final report is due. Reports shall contain a concise summary of the work performed and any issues relating to performance, schedule and cost shall be identified.

Financial reports

Quarterly financial reports are due on April 5, July 5, October 5 and January 5 of each year. The first part shall update the POA&M planned expenditure rate graph with the actual expenses added to it. The second part shall update the task identification of the POA&M. The third part shall provide updates to the critical path diagram.

Annual reports

An annual report shall be provided on the 15^{th} day of the 13^{th} month of the effort; for format requirements etc, see Contract Data Requirements List in contract.

Final report

A draft final report is initially due on the 15^{th} of the following month after the end of the contract. Comments from DTRA will be provided to the PI within 30 days of report receipt. A final report is due 2 months later, which shall be delivered to DTIC as well as DTRA. For further requirements, see Contract Data Requirements List in contract.

Appendix A

Literature study Task 1

Postmortem Biological Fate of Chemical Warfare Agents.

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A.1. Aim of the Study

Like any regular drug or food compound, Chemical Warfare Agents (CWAs) are subject to various physiological processes upon entrance of the human body, including absorption, distribution, metabolism and excretion. These processes, referred to as the biological fate, contribute to the natural detoxification of the agent. After death, physiological processes change completely. Regular distribution through the blood stream will stop. Normal metabolism will slow down or even stop, whereas putrefaction and agent redistribution will start. The postmortem changes will have an effect on the natural detoxification of CWAs, which may therefore remain present in the toxic form for a longer time in human remains compared to living human beings. The agents may thus present a hazard to personnel handling contaminated bodies.

The aim of this literature study was to describe the postmortem changes in physiologically relevant processes and their (possible) effect on the natural detoxification of CWAs. Literature of postmortem changes and tissue distribution of drugs and organophosphate (OP) pesticides was included to estimate the behavior of nerve agents in human remains.

In this report, the in vivo biological fate of CWAs is discussed briefly in Chapter 2, and the postmortem biological fate of CWAs is discussed in Chapter 3, including redistribution and metabolism. Concluding remarks are given in Chapter 4.

A.2. *In vivo* biological fate of CWAs

The distribution and metabolism of CWAs in living organisms have been studied extensively. After exposure, agents will be absorbed and distributed, depending on the route of administration. The biological fate of nerve agents is relatively simple (Noort et al., 2002, Noort et al., 2005, Black et al., 2005, Black 2007). The predominant process consists of hydrolysis (either spontaneous or enzymatic) to relatively non-toxic phosphonic acids that are readily excreted in urine. In addition to binding to its physiological target acetylcholinesterase, scavenging by butyrylcholinesterase (BuChE), carboxylesterase (CaE), and even albumin has been well documented (Williams 2007). The metabolism of sulfur mustard is complex, *inter alia* because of the agent's bifunctional alkylating character, resulting in a wide range of mixed hydrolysis/oxidation products and glutathione-derived conjugates in various oxidation stages. Also, extensive binding to proteins occurs (Noort, 2002, Noort 2005, Black 2005, Black 2007, Noort 2008). It has recently been hypothesized that metabolism of sulfur mustard depends on the route of exposure. For instance, metabolism in skin cells might result in toxic metabolites (Vijayaraghavan et al (2005).
Hydrolysis rates of CWAs, and enzymatic acitivities in plasma and tissue homogenates have been well documented. These data has been used to develop a PBPK model to predict the toxicokinetics in intoxicated (living) human beings (e.g., Langenberg 1997).
Within the framework of the current study, two of the most relevant questions are: how long can intact agents persist in an organism and to which organs does distribution predominantly occur? Toxicokinetic studies in laboratory animals have provided some very useful data on the distribution and persistency of various agents in living organisms (see e.g., Van der Schans 2003 and 2008), e.g. for VX and sulfur mustard (the focus agents of the current project). This will be

discussed in slightly more detail below; also, some relevant studies with human volunteers are addressed.

A.2.1. VX

In a number of toxicokinetic studies performed by Van der Schans et al (see Van der Schans et al, 2008 for an overview), it has been assessed that VX is more persistent *in vivo* than G-agents (sarin, soman). Independent of the exposure route, VX can be detected in the blood for up to 6 hours after the exposure.

In a highly relevant study reported by Chilcott et al. (2005) domestic pigs were exposed to radiolabelled VX (2 LD50; 120 ug/kg) and distribution of the agent was subsequently studied.

Only 1.2 ± 0.2 % of the applied dose was not absorbed. Approximately one-fifth of the applied dose (as ^{14}C-VX or its radiolabeled metabolites) remained in the skin exposure site which suggests that postmortem decontamination procedures might lower the risk of contact hazards. From the sampled tissues, 53.3 ± 3.5% of the dose could be recovered. It was estimated that the total systemic absorption was approximately 77.1 ± 2.9 %. The average area over which the total amount of ^{14}C-VX could be detected was 3.0 cm ± 0.5 cm^2 (1.2 cm ± 0.5 cm^2 per mg VX). Studies with human volunteers showed that VX spreads over human (forearm) skin to ca. 6 m^2/mg of applied agent (Craig et al, 1977).

Table A.1: Distribution of VX after application on pig skin (from Chilcott et al, 2005).

Compartment	Location	Recovery (%)	Concentration 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 ± 51
Total recovery	(Unabsorbed + local + systemic)	76.2 ± 3.5	-
Unaccounted	(Total recovered – applied dose	23.6 ± 3.5	-

In a study with human volunteers, VX was applied to the skin of 139 male human subjects at environmental temperatures of -18°,2°, 18°or 46°C. After 3 hours, the skin was decontaminated after which the men spent the next 21 hours at about 27°C. Based on the inhibition from red blood cell cholinesterase, the amount of VX penetrating the skin was estimated (Craig et al, 1977).

Table A.2: Penetration of VX during 3 hours at indicated temperature before decontamination and subsequently at 27°C [a] (from Craig et al, 1977).

Room temp (°C)	No. of men	Dose range (µg/kg)	Penetration fraction and standard error of mean P1 (%)	P3 (%)	PM (%)	PM-P3 [c] (%)	Time of PM (hr)
				Cheek			
46	12	3-4	9.8 ± 2.5	31.9 ± 3.2	34.8 ± 2.8	2.9 ± 0.7	5.6 ± 0.9
1	12	4-8	3.4 ± 1.1	15.1 ± 1.6	19.7 ± 1.3	4.6 ± 1.0	8.5 ± 2.0
2	12	8	1.3 ± 0.4	7.3 ± 1.5	15.8 ± 1.4	8.5 ± 1.3	10.4 ± 1.7
-18	12	8	0.4 ± 0.5	3.5 ± 0.9	15.5 ± 1.3	12.0 ± 1.1	12.2 ± 1.8
				Forearm			
46	46	2-25	0.70 ± 0.34	2.98 ± 0.63	5.75 ± 0.51	2.77 ± 0.51	16.2 ± 0.8
18	24	10-50	0.00 ± 0.10	0.38 ± 0.12	2.00 ± 0.25	1.62 ± 0.20	16.1 ± 1.1
18[b]	21	15-30	0.15 ± 0.13	0.60 ± 0.19	4.14 ± 0.47	-	16.8 ± 1.3

[a] Penetration fraction is (P) calculated as 0.02 times the inhibition in percent divided by the dose in µg/kg . P1, P3 and PM are the fractions at 1 and 3 hr (at decontamination) and at the time of greatest inhibition. The factor 0.02 µg/kg percent inhibition is derived from data from another report (Kimura et al, 1960) that are presented in Table A.3;

[b] In this series, the men remained at 18°C for 6 hr and were not decontaminated;

[c] PM-P3 is the amount of VX remaining in the skin at the time of decontamination.

The penetrated fractions of VX can be compared with other compounds that are applied to intact human skin. In case of parathion applied to the skin, 36% of the applied dose for forehead and 9% of the applied dose for forearm was excreted in urine, presumably at room temperature (Maibach et al, 1971). In comparison, the fraction of applied VX at the time of greatest cholinesterase inhibition at 18°C was 20% for cheek and 2% for forearm skin.

Table A.3: Red blood cell cholinesterase activity during and after intravenous infusion of VX in 4 human volunteers (from Kimura et al, 1960).

Time (hr)	Accumulated dose (µg/kg)	Activity as percent of preinfusion 1	2	3	4	Average inhibition (%)	Dose to produce 1% inhibition (µg/kg)
0	0	100	100	100	100	0	-
1	0.25	92	90	80	90	12	0.021
2	0.50	78	60	67	67	32	0.016
3	0.75	71	57	63	58	38	0.020
4	1.00	56	42	35	54	53	0.019
6	1.00	52	50	48	53	49	-
12	1.00	60	54	62	63	40	-

Further increase in cholinesterase inhibition after decontamination was evidence of a deposit of VX in the skin. The amount of VX remaining in the skin after decontamination was larger in the forearm and less in the cheek at higher temperatures.

The cheek and the volar surface of the forearm represent areas of high and intermediate permeability to VX (Sim, 1962; Maibach et al, 1971).

When VX was applied to the skin of the cheek, forehead, abdomen and volar surface of the forearm the dose to produce a 70% depression of ChE was estimated to be 5.1, 11.2, 31.8 and 40.0 µg/kg, respectively (Sim, 1962).The difference in permeability between forearm and cheek

might be due to differences in blood flow. The inter-individual variability of penetration through the skin at the same dose was profound. For the forearm, at a dose of 10 µg VX/kg the cholinesterase inhibition in blood ranged from 0% in one person to 100% in another person (Kimura et al, 1960). This has also been observed in experiments with laboratory animals that were exposed percutaneously to VX (Joosen et al., manuscript in preparation).

A.2.2. Sulfur mustard

In order to provide a quantitative basis for pretreatment and therapy for intoxications with sulfur mustard, the toxicokinetics and distribution of this agent has been studied extensively in laboratory animals (see Langenberg et al, 1998). The high persistency of the agent in tissue samples is significant: independent of the exposure route, intact sulfur mustard could be detected up to 6 hours after the exposure, at ng/ml levels. In contrast with G-type nerve agents, sulfur mustard partitions strongly to various organs, especially the lung, spleen, liver and bone marrow. This is probably caused by the lipophilic nature of sulfur mustard.
Several studies in which human volunteers were exposed to sulfur mustard have been reported; see Young and Still (2003) for a highly valuable compilation. However, these studies were mainly focused on toxicological effects following the exposure, and hardly any data on sulfur mustard concentrations or distribution were provided. One of the in vivo studies focused on the penetration rate of radioactively labeled sulfur mustard through human skin (Nagy et al., 1946). It was *inter alia* found that penetration was enhanced with increasing temperature.

A.3. Postmortem Biological Fate of CWAs

After death, physiological processes start to change rapidly, many of which will have an effect on the biological fate of CWAs. When a victim dies slowly, some changes may start even before death (such as a reduced blood circulation and a changed percutaneous uptake).
After death, the following physiological changes occur (Pélissier-Alicot 2003, Drummer 2004, Yarema 2005, Flanagan 2005, Drummer 2007, Ferner 2008) which can affect the biological fate of agents:

- The blood circulation stops immediately after death;
- The body temperature gradually cools from 37 °C to ambient temperature (*algor mortis*);
- Postmortem redistribution of the agent can occur;
- Movements of blood within the vessels possibly occur early after death and may be responsible for physical redistribution of agents between different vascular compartments. This can possibly occur because of gradual muscular stiffening (*rigor mortis)* and putrefaction, but also by movement of the body;
- Putrefaction (decomposition) and eventual liquefaction of tissues by enzymes and bacteria occurs during postmortem periods. Degradation and/or synthesis of xenobiotics by bacteria are part of the process;
- Blood changes such as a decrease in blood pH, blood coagulation and hypostatis;
- Saponification is the hydrolysis of fatty tissues with the release of free fatty acids. This process starts within weeks after death and is therefore not considered relevant for this study.

The fate of CWAs is dependent mostly on postmortem redistribution and metabolism (including scavenging, putrefaction and degradation). These processes are discussed in detail in the following paragraphs.

A.3.1. Postmortem Redistribution

A.3.1.1. Factors influencing postmortem redistribution

After death, agent concentrations in body fluids and organs can change, similar to drugs, due to postmortem redistribution (Hilberg 1999, Leikin 2003, Pélissier-Alicot 2003, Drummer 2004, Yarema 2005, Drummer 2007, Ferner 2008). The extent of these changes varies significantly between drugs. Compounds may be redistributed by passive drug release from 'reservoir' organs such as the gastrointestinal tract, liver, lungs and myocardium to surrounding tissues and organs. Redistribution occurs by passive diffusion immediately after death, and later on by cell autolysis (leakage of compounds into the extracellular space), blood movements and the putrefaction process.

Redistribution depends on various factors, of which the most important are:

- Chemical and pharmacokinetic properties of the compounds;
- Concentration and volume in reservoir organs such as stomach, lungs and skin;
- Distance between reservoir organs to other organs and body parts;
- Time between death and sampling of body fluids and tissues (i.e. postmortem interval);
- Temperature of the corpse and surroundings;
- Body handling (including reanimation).

One of the main properties that determines postmortem redistribution are the chemical properties of the involved compounds, such as lipophilicity, acid/base properties, ionization, etc. For example, postmortem redistribution particularly occurs with basic lipophilic compounds with a large distribution volume.

Furthermore, postmortem redistribution depends on the pharmacokinetic properties, i.e. biological fate including absorption, distribution, metabolism and elimination (Pélissier-Alicot 2003). *Absorption* of compounds can occur by passive diffusion and/or active transport. Passive diffusion is affected by postmortem changes such as intracellular pH and by loss of the membrane integrity. Active transport requires energy (usually ATP) as well as carrier system to transfer molecules. The cessation of ATP production, rapidly after death, probably blocks this process. After absorption the compounds are *distributed* throughout the body. Some compounds can bind to proteins in the blood. Total protein blood concentration decreases after death (breakdown in amino acids and peptides due to acute anoxia and from proteolysis by autoenzymes) resulting in increase of in intravasculair concentration of free form of the drugs. Furthermore, compounds are distributed to solid tissues and organs. The postmortem redistribution of a compound cannot be predicted only by its lipophilic properties and its apparent volume of distribution (Vd). Other factors such as the absorption route, dose, or particular affinity of the drug for some tissues must be envisaged as well as the possibility of a residual metabolic activity in the first hours after death. Besides distribution, compounds may undergo *metabolism*. The metabolizing system may persist several hours after death, inducing the breakdown of a drug and the synthesis of its metabolites. Postmortem metabolism will be discussed in more detail in 3.2. Similar to absorption, hardly any research is done on postmortem *elimination*. Glomerular filtration of

compounds probably stops at the time of death since a blood flow needed is for this process. Similar, tubular secretion is an active process which needs ATP, thus will probably stop shortly after death. In contrast, tubular reabsorption is a passive process, which probably still occur after death.

At the time of death, the concentration of a drug or agent will be higher in some organs than others. Agent will redistribute from the 'reservoir' organ to the surrounding tissues and fluids, which will obviously occur more rapidly when the agent concentration and volume are high.

The distance of a reservoir organ to surrounding organs also plays a role in the redistribution. It is well-known that concentrations in heart blood may increase after death due to redistribution from surrounding organs such as the stomach and lungs. Femoral blood (i.e. peripheral blood from the upper leg) is less susceptible for this phenomenon, since the distance to reservoir organs is larger.

Obviously, the postmortem interval, or time between death and sampling, is a factor that determines the amount of redistribution. During a longer period of time, the extent of redistribution will increase. However, postmortem metabolism will also have more time to occur, as discussed in A.3.2.

Postmortem processes are enhanced by higher temperature of the surroundings and thus of the corpse. Finally, body handling, transport and reanimation attempts can cause the blood to flow and thus accelerate redistribution.

Research to determine the extent of postmortem changes of compounds in humans is difficult. Postmortem redistribution can be studied by comparing drug or agent concentrations in multiple specimens i.e. body fluids and tissues. If concentrations are for instance higher in heart than peripheral sites then the possibility of redistribution exists. It would be ideal to compare a clinical specimen taken shortly prior to death and compare this with postmortem specimens. However, this is rarely possible. Animal models (such as rats, rabbits and pigs) could be used to investigate the postmortem changes because sampling can occur at defined times before and after death. In most studies conducted so far conclusions generally support the human postmortem studies (Hilberg 1999, Drummer 2007). The main limitation with animal models is the assumption that animals can mimic humans particularly when it comes to nuances such as diffusion of drugs from lungs and gastric content after death and the possibility of altered drug distribution with depletion of blood volume (especially with smaller animals) (Drummer 2007). Recently quantitative structure-activity relationship (QSAR) methodology was evaluated as a tool to estimate the ability of drugs to redistribute across tissue barriers postmortem on the basis of their molecular, physicochemical and structural properties. Due to the high complexity of postmortem redistribution process further QSAR studies are needed (Giaginis 2009).

A.3.1.2. Postmortem body fluid and tissue collection

After a suspected intoxication, various body fluids and tissues are collected during autopsy for toxicological analysis. Most commonly, blood, liver and urine are analyzed, when available. In case of a suspected oral intoxication, the stomach contents can also be investigated. Furthermore, tissues such as lung, kidney, brain, bile and fat tissue will be collected. In case of extreme putrefaction, muscular tissue, hair and bone could be useful, although the psychical state of the body determine what specimens are available for collection (Drummer 2004, Drummer 2007).

Blood is always the preferred sample matrix for toxicological analysis and allows concentrations to be compared with clinical and pharmacokinetic data (Hilberg 1999, Drummer 2002, Pélissier-Alicot 2003, Skopp 2004). Importantly, blood must be taken from a peripheral site (i.e. femoral

blood) to avoid excessive postmortem changes due to redistribution. A difficulty of postmortem blood is the bioanalytical procedure. For example, it is almost impossible to obtain serum or plasma from postmortem blood because of postmortem hemolysis, hence whole blood is the most common specimen. Solid Phase Extraction (SPE) of whole blood is usually more difficult than of serum because whole blood is more viscous (Drummer 2007b).
In urine, the accumulation of drugs and metabolites usually results in high concentrations facilitating detection of drugs use or exposure (Skopp 2004). Therefore, urine has a great potential to provide information on antemortem exposure. Generally, there is no correlation between urine drug concentration and pharmacological effect and hence, urine may not be indicative for acute poisoning.
Liver is the main metabolic organ and is one of the preferred specimens in postmortem toxicological analysis, besides blood (Drummer 2004, Skopp 2004). Ideally the right lobe of the liver must be sampled because it less prone to postmortem reductribution (Pélissier-Alicot 2003).

A.3.1.3 Postmortem redistribution of CWAs and organophosphorus pesticides

In this study, data was collected to explore the possible postmortem redistribution of CWAs and OP pesticides in humans after (fatal) intoxications. OP pesticides were chosen since they are chemically similar to nerve agents and are frequently used for suicidal and homicidal intoxications, mainly by ingestion. Although these compounds are less toxic and slower inhibitors of cholinesterase, they can be used as a model for nerve agents, for which less data is available.
Besides the study of open literature, the database of forensic reports of the NFI from the years 2004 to 2009 was investigated to possibly obtain additional information on OP investigations in human remains. Unfortunately, no reports of the NFI were present involving the measurement of an OP pesticide intoxication in human remains.
To our knowledge no literature is available on postmortem redistribution of nerve agents in animals or humans. Drasch et al (1987) described a fatal case with sulfur mustard. Several tissues and body fluids were stored after autopsy at -20°C for twelve months until they were analyzed. In Table A.4, the concentrations of sulfur mustard in the tissues of an Iranian soldier who died 7 days after inhalation and/or dermal exposure to sulfur mustard are depicted (Drasch 1987, ATSDR 2003). Similar to other highly lipophilic compounds, according to this data sulfur mustard seems to accumulate in the lipid compartments of the body. Because of this, postmortem redistribution of sulfur mustard seems to be likely. However, *intravenous* injection of sulfur mustard in rats revealed a different distribution pattern, i.e. kidney>lung>liver>spleen>brain. Besides difference in measurement methods, species variation and postexposure time, the route of exposure seems also to be significant factor (ATSDR 2003).

Table A.4: Sulfur mustard concentrations found in fluid and tissues of a dead soldier (Drasch 1987).

Tissue	Concentration (mg/l or mg/kg)
Blood*	1.1
Urine	Negative
Liver	2.4
Kidney	5.6
Splee	1.5
Lung	0.8
Muscle (thigh)	3.9
Fat (thigh)	15.1
Abdominal skin	8.4
Skin with subcutaneous fat	11.8
Liquid from a skin blister	Below dectection limit
Brain	10.7
Cerebrospinal fluid	1.9

* It is unclear where the blood was taken (for instance from the heart of peripheral)

Based on these concentrations, it can be concluded that sulfur mustard seems to accumulate in the lipid compartments of the human body, including skin with subcutaneous fat. Since these concentrations were measured in samples which were taken seven days after exposure, redistribution of sulfur mustard from the lipid depots occurs at a rather slow rate. The concentrations found suggest, at least in the lipid tissues, ante mortem and postmortem stability of sulfur mustard in tissue.
Any lipid rich tissue can be used for the detection of sulfur mustard in a corpse, and, therefore poses more hazards for secondary contamination.

Postmortem OP pesticide concentrations from literature are shown in Table A.5 for (heart) blood, urine, stomach contents, liver and various other tissues. The data shows a high variation of concentrations between different OP pesticides cases, but also between cases with the same OP pesticide. This variation is mainly dependent on the type of OP, the ingested amount, the exposure route, and the intervals between the time of exposure and death, and between time of death and the autopsy. Substantial postmortem variability is also reported for drug concentrations (Drummer 2004).

Most studies that are summarized in Table A.5 reported (heart) blood concentrations. It is often unclear if blood was sampled from the heart or from peripheral sites. As mentioned before, concentrations in heart blood may increase after death due to redistribution from surrounding organs such as the stomach and lungs. Femoral blood (i.e. peripheral blood) is less susceptible for this phenomenon. Nonetheless, the presence of the different OP pesticides in the blood indicates that not all of the OP pesticides are degraded or detoxified.
In suicidal intoxications, OP pesticides are often orally ingested. This may result in high concentrations in the stomach contents as can be seen from Table A.5. Redistribution from the stomach to surrounding organs was shown by the toxicological results after a suicidal ingestion of the OP insecticide dichlorvos (DDVP). High concentrations of the OP had diffused from the stomach to the heart and spleen within one day after death (Shimizu 1996). Concentrations in

blood and liver were relatively low, which was attributed to the fast hydrolysis of dichlorvos. These findings are confirmed by other reported dichlorvos cases (Klys 1997, Moriya 1999).
A case of omethoate poisoning was described (Pavlic 2002). The concentration in the stomach was very high and nearly matched the concentration of the pesticide solution that was ingested. Apart from the gastric content, the highest omethoate levels were found in bile and kidney tissue, which indicates that elimination of omethoate had already started.

Klys (1997) studied the OP pesticides poisoning in clinical and medicolegal cases in Krakow. They found higher concentrations of OP pesticides in postmortem blood compared to antemortem blood. This indicates that postmortem redistribution occurs with OP pesticides.

In a few cases, OP pesticides concentrations in fat tissue were reported (for example, Morgade 1982, Klys 1997, Akgür 2006). OP pesticides are lipophilic compounds and diffuse into fat tissue, given time. It is known that highly lipophilic drugs are concentrated in adipose tissue by simple physical dissolution in neutral fats (Pélissier-Alicot 2003). This redistribution occurs slowly, as the blood flow is low and the equilibrium between blood and adipose tissue concentrations may not have been achieved at the time of death.
Akgür et al. (2003) reported OP insecticide concentrations in blood, liver and fat tissue of 32 suicide cases. Methamidophos was detected in all cases except two. Methamidophos was found in a higher concentration in fat tissue than in blood. There was a significant positive correlation between blood and liver methamidophos concentrations ($r=0.518$, $n=25$). No correlation was found between fat and blood, nor between fat and liver. In some cases, the OP pesticides parathion methyl, diazinon and dimefox and/or dichlorvos were detected, mainly in fat tissue. It was stated that blood concentrations were low since OP pesticides do not remain in the circulation for more than a few minutes to hours after exposure. It was concluded that insecticides are retained in fat to a far greater extent than in liver or blood following OP poisoning. It is important to note that mortality in the reported cases occurred from one to twenty days after exposure, which allowed for distribution.
In another study, a single case of diazinon suicidal intoxication was reported and included fat analysis. Diazinon was found in fat in a low concentration compared to blood but in a higher level than in liver and kidney (Poklis 1980). The highest concentrations of diazinon were found in the stomach contents, blood and bile. The time between exposure and death was less than one day in this case.
The differences in blood/ fat concentration ratio between the two studies above may be explained by the time between exposure and death. In the first hours after exposure, blood concentrations are high, whereas subsequent slow distribution towards fat tissue occurs. This is confirmed in the study of Klys (1997), who showed that pesticides concentration in the human brain increased with a longer survival time before death.

Table A.5: Organophosphate pesticide concentrations found in human fluid and tissues after autopsy.

Organophosphate		Organophosphate concentration (µg/ml or µg/g)													Ref
	n	Heart blood	Blood (1)	Urine	Stomach contents	Intestinal contents	Bile	Liver	Kidney	Adipose tissue (fat)	Brain	Spleen	Heart	Lung	
Acephate	1	146		270	2,200										Tanaka 2005
Acephate metabolite: methamidophos	1	3.0		1.9	3.2										Tanaka 2005
acephate (2)	1	46		107	1,000										Tanaka 2005
acephate metabolite: methamidophos	1	ND		1.6	ND										Tanaka 2005
Chlorfenvinphos		0,3-10,1 (n=5)						0,32-15,9 (n=5)	0,16-15,8 (n=3)	1,1-10,7 (n=2)	0,1-3,9 (n=4)			0,8-15,9 (n=3)	Klys 1997
Chlorpyrifos	6		0.72												Park 2009
chlorpyrifos-methyl		1.01; 1.71	0.615 to 4.15	ND	2041		ND	1.41	0.472		0.379	0.666	0.491	8.6	Moriya 1999
demeton-S-methyl	1		10	121	trace			17	5						Baselt 2008
Diazinon	1		277	Not available	219		200	4	0.1	15	2				Poklis 1980
Diazinon	4		1.03												Park 2009
Diazinon	3		104 (0,7-277)		443 (44-1200)			126 (4-345)	1,5 (0,1-3)	26 (15-37)	25 (2-62)				Baselt 2008 (3)
Diazinon	1		3.68					3.2	1.64	15.67	1.07		2.91		Klys 1997

Table A.5: Organophosphate pesticide concentrations found in human fluid and tissues after autopsy., continued.

Organophosphate		Organophosphate concentration (µg/ml or µg/g)													Ref
	n	Heart blood	Blood (1)	Urine	Stomach contents	Intestinal contents	Bile	Liver	Kidney	Adipose tissue (fat)	Brain	Spleen	Heart	Lung	
Dichlorvos	1		29	4.5	1200000 (300 g in 250 ml)			20	80		9.7	3340	815	81	Shimizu 1996
Dichlorvos			0,91-4,33 (n=2)					0,75-4,88 (n=2)	3,94 (n=1)	1,42-5,8 (n=2)	0,66 (n=1)				Klys 1997
Dichlorvos	1	ND; 0.043; 0.082	ND	2929		8.99	ND	ND		ND	ND	0.542	ND	ND	Moriya 1999
Dimethoate	1		49,8 (N=1)					71.57	98.8	116.4	28.2				Klys 1997
Fenitrothion			1,9-9,49 (n=5)					0,8-11,5 (n=5)	3,91-4,54 (n=2)	5,1 (n=1)	0,04-1,15 (n=5)			0,15 (n=1)	Klys 1997
Malathion	1			ND	ND	2,100	98,000	570,000							Chaturvedi 1989
Malathion	1			1.8		978			ND						Thompson 1998
Malathion	4			0.82											Park 2009
Malathion	2		0,16-3,1					0,08-3,0	0,16-11,4	0,43-3,27	0,07-1,7				Klys 1997
Malathion (4)	1	ND		ND						ND					Zivot 1993
Malathion	1	0,3			8621		1,04	ND	17,5	76,4	4,4	1,3			Morgade 1982
Malathion/malaoxon metabolite	1	ND			2.5		<0.10	ND	1.2	8.2	1.4	<0.10			Morgade,1982

Table A.5: Organophosphate pesticide concentrations found in human fluid and tissues after autopsy, continued.

Organophosphate		Organophosphate concentration (µg/ml or µg/g)													Ref
	n	Heart blood	Blood (1)	Urine	Stomach contents	Intestinal contents	Bile	Liver	Kidney	Adipose tissue (fat)	Brain	Spleen	Heart	Lung	
Methamidophos	25		0.27 ± 0.44					0.39 ± 0.61		1.24 ± 3.88					Akgur 2003
Mevinphos	1		360	8				240	20						Baselt 2008
Omethoate	1	208		225	48,223		524	341	505						Pavlic 2002
Parathion	17		2.90												Park 2009
Parathion	19		9,0 (0,5-34)	10 (0,4-78)				11 (0,1-120)	3,3 (0,2-12)		4,9 (0,9-13)				Baselt 2008
Parathion methyl	1		375							333					Akgur 2003
Phosphamidon	1		40					135	160				280	200	Baselt 2008
Phozalone			0,08-0,19 (n=3)					0,05-0,76 (n=3)	0,11-0,2 (n=3)	0,23-0,67 (n=3)	0,003-0,08 (n=3)			0,29-2,4 (n=2)	Klys 1997
Trichlorphon			0.27 ± 0.44					0.39 ± 0.61		1.24 ± 3.88					Klys 1997

ND: not detected.
(1) Unknown whether heart blood or femoral blood;
(2) Cause of death: bleeding (hemorrage) from injuries;
(3) Baselt 2008 is a combination of Poklis 1980 and Wall 1982;
(4) Death followed 12 days after intoxication.

A.3.2. Postmortem Metabolism

The metabolism of CWAs is expected to continue postmortem by partly different mechanisms than antemortem (prior to death). In this section, first scavenging of CWAs will be discussed. Then, the postmortem degradation of drugs and CWAs will be outlined, including postmortem metabolism, chemical degradation and putrefaction.

A.3.2.1. Scavenging

In the human body, CWAs and related compounds will rapidly bind to proteins and other binding sites. It is well-known that binding of nerve agents and OP pesticides to the enzyme acetylcholinesterase (AChE) leads to immediate intoxication. After binding to this enzyme and other proteins (such as butylcholinesterase (BuChE) or albumin), the agent looses its toxic properties and therefore these binding sites are referred to as scavengers. In deceased victims, scavenging of residual agent could still occur to some extent, thus detoxifiying the agent and decreasing the danger for personnel handling the body.

In case of a fatal intoxication by nerve agent or OP pesticides, it is estimated that postmortem scavenging by esterases will play a limited role in detoxification, since it is assumed that the received dosage will be far higher than the amount of available cholinesterase. The total amount of available cholinesterase (BuChE and AChE) is normally limited to about 60 nM in human blood.
This assumption is confirmed by reported data of fatal OP pesticide intoxications in which AChE and/or BuChE activity in whole blood, serum or plasma was determined after death (Poklis 1980, Kusu 1990, Moriya 1999, Pavlic 2002, Tanaka 2005). In Table A.6 the postmortem ChE activity levels of these cases are depicted. Enzyme activity levels were determined using different techniques. However, the data show that after fatal OP intoxications the enzyme activity approaches zero, compared to antemortem activity levels. When there was an alternative cause of death (no OP intoxication), ChE activity levels were comparable to those of healthy controls (Kusu 1990, Klette 1993, Uemura 2008). One case was reported in which a victim was intoxicated with a non-fatal dose of the OP acephate whereas the cause of death was hemorrhage (bleeding) from injuries (Tanaka 2005). The ChE level was in the low end of the normal range.

A study showed that there was no significant decrease in ChE activity up to 72 hours after death and heart blood concentrations were comparable with femoral blood concentrations (Uemura 2008). Hence, the ChE activity in blood does not seem to be subjected to postmortem changes within a postmortem interval of 72 hours.

Besides esterases, binding to sites with less affinity (e.g. the tyrosine-411 residue in albumin) might occur (Noort 2009). In case of sulfur mustard, alkylation of proteins (e.g. hemoglobin and albumin) and DNA might contribute to the postmortem detoxification of this agent. Although (to our knowledge) no data is available to underline this hypothesis. Moreover, Drasch et al (1987) have established the presence of sulfur mustard in postmortem human tissues. Therefore, if postmortem scavenging of sulfur mustard occurs, the contribution might be limited. Anyhow, this does not completely detoxify sulfur mustard.

It should be kept in mind that non-covalently bound agent may be released from e.g., proteins. This phenomenon has been described for drugs. For example, it is known that drugs bound to albumin are rapidly released, leading to higher concentration of the free drug (Pélissier-Alicot 2003). Furthermore, proteins are broken down into amino acids and peptides after death, during which bound drugs or agents can be released. However, the speed of this process is unknown and experiments confirming an increase in drug concentration in time after death have not been described.

Table A.6: Postmortem cholinesterase (ChE) enzyme activities after intoxication with organophosphate (OP) pesiticides. For comparison, antemortem ChE levels are given and postmortem levels after alternative causes of death.

OP	N	ChE enzyme (1)	Postmortem ChE level	Antemortem ChE level (normal range)	Unit (2)	Reference
Acephate	1	BuChE (serum pseudo ChE)	78	3500-8000	U/L	Tanaka 2005
Diazinon	1	BuChE (plasma ChE)	0	40-80	Rappaport units/ml	Poklis 1980
Omethoate	1	AChE (AChE in serum)	10	3500-8500	U/L	Pavlic 2002
OP	1	BuChE (pseudo ChE)	10	1000-2800	IU/L	Kusu 1990
OP	1	AChE + BuChE (serum ChE)	3	203-460	IU/L	Moriya 1999
Acephate, not fatal (3)	1	BuChE (serum pseudo ChE)	3539	3500-8000	U/L	Tanaka 2005
No OP (4)	11	BuChE (pseudo ChE)	1670 (1130-2130)	1000-2800	IU/L	Kusu 1990
No OP (4)	53	AChE + BuChE (whole blood ChE)	0.48 (0.20 to 0.74)	0.60	pH units	Klette 1993
No OP (4)	15	AChE + BuChE (whole blood ChE)	0.39	0.60	pH units	Klette 1993
No OP (4)	153	BuChE (pseudo ChE)	204.1 ± 120.7	200-495	IU/L	Uemura 2008

(1) AChE and/or BuChE, between brackets reported details.
(2) Reported units were different for the different techniques used.
(3) Cause of death: hemorrhage (bleeding) from injuries.
(4) No OP pesticide intoxication or intoxication with other cholinesterase inhibitors.

A.3.2.2. Postmortem degradation of drugs

Postmortem degradation of drugs has been described and occurs by different processes simultaneously: continued metabolism, chemical degradation and putrefaction.

The metabolism of drugs continues several hours after death, inducing the breakdown of a drug and the synthesis of its metabolites (Pélissier-Alicot 2003). Postmortem changes in drug-metabolizing enzymes of rat liver and human liver are described in literature (Gallenkamp 1981, Yamazaki 1994). Diverse parameters were measured, such as microsomal protein, cytochrome P-450, NADPH cytochrome C reductase and glucose-6-phosphatase. Nearly all parameters based on microsomal protein decreased during autolysis. However, there was a marked difference in decrement between the parameters. Thus, a residual enzymatic activity, variable with the nature of the enzymes involved, is likely during the first hours after death.

A well investigated drug for postmortem metabolism is cocaine. Cocaine is hydrolyzed to benzoylecgonine at physiological pH and by plasma cholinesterase to ecgonine methylester. After death, cocaine is gradually broken down and benzoylecgonine and ecgonine methylester concentrations rise in blood. This is probably due to postmortem hydrolysis and residual plasma (or hepatic) esterase activity (Logan 1997, Pélissier-Alicot 2003). This indicates that besides postmortem metabolism by endogenous enzymes, the chemical instability (hydrolysis and oxidation) of drugs and compounds is also relevant in the detoxification process of drugs (Skopp 2004).

Putrefaction of the human body, i.e. decomposition and eventual liquefaction of tissues, will start after death depending on the ambient temperature, other environmental factors and the state of the body (Pélissier-Alicot 2003, Drummer 2004). Bacteria from the gastrointestinal tract enter the blood and lymph vessels after death and transmigrate throughout the body. This migration may occur within the first few hours postmortem, especially at ambient temperature. These bacteria could metabolize and/or produce many compounds in the postmortem blood. The bacteria most likely involved is this process are those originating from the gastrointestinal tract such as *Bacillus* spp., *Escherichia coli, Proteus miribalis, Clostridium perfringens, Staphylococcus aureus, Staphylococcus epidermis, Streptococcus faecalis* and *Bacteroides fragilis* (Robertson 1995).

Some drugs, such as nitrobenzodiazepines, are known to undergo postmortem degradation by bacteria. Nitrobenzodiazepines are converted by anaerobic bacteria to 7-amino-metabolites (Drummer, 2004, Robertson 1995). Increase of temperature increased the rate of nitrobenzodiazepine bioconversion, whereas addition of sodium fluoride with or without potassium oxalate reduced the rate of conversion in blood (Robertson 1995).

Compounds that are known to be produced after death by bacteria are volatiles, mainly ethanol but also n-propanol, isopropanol, n-butanol, sec-butanol and possibly methanol. In the presence of glucidic substrates, such as glucose or ribose, and amino acids from protein breakdown, bacteria and yeasts can produce alcohol (Boumba 2008). Furthermore, postmortem formation of indole and amines such as phenylethylamine, tyramine and tryptamine has been described (Oliver 1977, Skopp 2004), relating to endogenous compounds.

A.3.2.3 Postmortem degradation of CWAs and organophosphorus pesticides

Similar to drugs (as described in the previous paragraph), postmortem degradation of CWAs and OP pesticides occurs by continued metabolism, chemical degradation and putrefaction.

OP nerve agents are unstable in whole blood (Flanagan 2005). According to Moriya et al. (1999b), OPs are degraded more rapidly by esterase activities than by chemical mechanisms. Sodium fluoride, which is often used as a preservative for biological fluids, can accelerate the chemical degradation of OP chemicals. Moreover, fluoride should also be avoided in blood specimens taken after a sarin intoxication because it will regenerate sarin from inhibited esterases in blood and erythrocytes (Baselt 2008).

Temperature has a great effect on the decomposition of OP pesticides in blood (Ageda 2006). However, within the OP compounds there was also a large variation in degradation rates. OP pesticides with a methylphosphate degraded most rapidly, followed by methyl thiophosphate and methyl dithiophosphate types (Ageda 2006). There seems to be many factors responsible for the decomposition of OP pesticides in blood. Besides the phosphates and esterases, it has been reported that albumin also hydrolyses OP pesticides (Ageda 2006).

Considering the above findings, it is possible that during postmortem interval degradation of OP pesticides might occur in the body (Elsirafy 2000, Kupfermann 2004). This has been shown for instance for diazinon (Elsirafy 2000). Diazinon was recovered from the stomach, intestine and liver of rats who had been given a large oral dose of diazinon. The diazinon concentrations decreased in the stomach and intestine during a postmortem interval of 8 weeks. After 8 weeks, there was still a detectable amount present. The degradation of diazinon might be due to chemical instability and bacterial metabolism. In the liver, the diazinon concentration increased substantially during the first five days of the postmortem interval and then began to decrease gradually. The rise in concentration might be due to postmortem redistribution from the stomach to the liver (see A.3.1).

Similar to OP pesticides, nerve agents (i.e. sarin, soman, VX) are believed to undergo rapid hydrolysis by blood esterases in animals and human beings (Baselt 2008). In vitro, the degradation of nerve agents is also fast, i.e. in whole blood more than 90% of the sarin and soman concentrations were lost within 30 minutes at room temperature. VX seems to be more stable in vitro, i.e. in plasma VX has a half-life of 2 hours at 37ºC (Baselt 2008).
The metabolism of drugs continues several hours after death (Pélissier-Alicot 2003). Similarly, enzymatic hydrolysis and oxidation of CWAs is expected to continue for some time after death, but possibly at a lower rate due to the decreasing body temperature. Spontaneous hydrolysis is likely to continue.

Only a few reports in literature describe the determination of CWAs in deceased human victims who were exposed to CWA. Intact sulfur mustard has been measured in postmortem tissue samples from a Iranian victim (Table 4; Drasch 1987).

Intact sarin and metabolites (isopropylmethylphosphonic acid and methylphosphonic acid) could not be detected in deceased victims who were exposed to sarin. Nagao et al (1997) have described the indirect analysis of sarin metabolites bound to acetylcholinesterase. Thus, sarin is (also) detoxified by scavenging to esterases. Furthermore, Leikin et al. (2003) also mentioned the instability of sarin during storage of specimens. Storage at -20ºC and avoiding addition of fluoride as a conservative in the containing tubes is advised.

It is unlikely and not expected that CWAs will be metabolized into yet unknown, toxic reaction products under postmortem conditions, other than those already reported for the in vivo situation (see Van der Schans, 2008 for a few examples related to VX). Such degradation processes have not been described (or investigated) for drugs, pesticides or similar compounds.

A.3.2.4 Postmortem stability ChE activity and its use for screening

Factors such as time before postmortem samples can be collected, type of carrier and time before samples can be analyzed suggest that postmortem blood samples will be hemolyzed when they can be analyzed (Klette et al, 1992). Therefore, it is best to use whole blood samples.

Ante mortem RBC (red blood cell), plasma and whole blood cholinesterase activity measured at day one and day seven at 4°C and 25°C. More than 80% of the originally measured enzyme activity remained after one week. Cholinesterase activity in postmortem whole blood samples (collected by dry syringe or scooped from the pericardial sac) which were stored at room temperature decreased less than 10% over a week period. Therefore, whole blood postmortem enzyme activities can be used to screen for nerve agent exposure (Klette et al, 1992).

Battlefield specimens from regions with considerably higher temperatures than 25°C suggested that the uninhibited cholinesterase enzyme was stable over a period of time up to two weeks.
Analytical method of cholinesterase activity: measuring of the change in pH per unit of time, monitoring the formation of acetic acid from added acetylcholine. For plasma and whole blood cholinesterase, the manual plasma method was used (Klette et al, 1992).

A.4. Concluding remarks

In this study, the biological fate of CWA's and closely related compounds has been addressed, in particular the postmortem changes that may effect the detoxification of these compounds. The extent of natural detoxification and redistribution is of influence on the hazard of the agent for personnel handling contaminated human remains.

Data was collected on postmortem tissue distribution of CWAs and OP pesticides. Only one case was described for the distribution of sulfur mustard. The agent appeared to accumulate in the lipid compartments of the body (highest concentration in fat tissue). Also, some data was available on the skin absorption of sulfur mustard. No data was available on the tissue distribution of nerve agents in humans, but some data was available on skin absorption of VX and distribution of soman. Many cases were reported on the distribution of different OP pesticides after fatal intoxication. As with drugs, large variation in concentrations was observed in various tissues. Similar to sulfur mustard, there was a tendency to accumulate OP pesticides in fat tissue in the majority of cases. It is concluded that sulfur mustard and OP pesticides are susceptible to postmortem redistribution, and it is likely that this is also valid for nerve agents.

Postmortem detoxification may take place by scavenging, metabolism, chemical degradation and putrefaction. Scavenging by ChE's plays a limited role after a fatal nerve agent intoxication. It was shown that fatal OP pesticide poisoning led to ChE activity levels of nearly zero. Other potential scavengers are also expected to play an insignificant role in detoxification. Degradation by continued metabolism (at least for some hours after death) and chemical degradation will contribute to the detoxification of the agent, although these processes could be slow and depend on various factors. For example, it was shown that postmortem breakdown of the OP diazinon was still incomplete after 8 weeks. On the other hand, nerve agents are rapidly hydrolyzed and sarin could not be detected in the blood of deceased victims.

It can be concluded that postmortem detoxification is complex and depends highly on the nature of the involved CWA, the exposure route, the time between exposure and death and between death and autopsy, the amount of agent and the temperature of the body. From the data obtained on the postmortem tissue concentration of sulfur mustard and OP pesticides, it is clear that these compounds are not detoxified completely and that redistribution leads to accumulation in organs and fat tissue. Postmortem formation of toxic metabolites has not been described, but cannot be ruled out completely.

Intact agent and/or their toxic metabolites that have accumulated in organs or fat could present a hazard to personnel, especially pathologists performing autopsy. Research in this area is necessary to estimate the risks and develop procedures for handling contaminated human remains.

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