ZRÍNYI MIKLÓS
NATIONAL DEFENSE UNIVERSITY
Doctoral Council

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A microdialysis model for studying the percutaneous penetration of sulfur mustard *in vivo*

Theses and official reviews

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Supervisor:

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Introduction

Weapons of mass destruction present the most effective means of exterminating human population and the environment. These devices, especially nuclear and chemical weapons, were stockpiled by the armed forces of several states between 1914 and 1990 and have been used on numerous occasions. In spite of signed non-proliferation treaties and global disarmament efforts the existence of these agents, especially in view of emerging terrorism, present a military as well as a serious environmental threat. These threats pose challenges for the countries of the Euro-Atlantic region including Hungary [1].

Sulfur mustard (2,2'-dichlorodiethylsulfide, SM) is a highly toxic vesicant and a chemical warfare agent of outstanding military importance. Having been in the possession of many states, thousands of tons of SM stockpiles are known or suspected to exist. SM is also the chemical warfare agent deployed in the largest amounts throughout the 20th century. Nevertheless, its physico-chemical properties and toxicity continue to render SM a potent weapon of asymmetric warfare [2].

From a military point, SM exerts its important biological effects on the skin, eyes and respiratory tract. Irreversible molecular phenomena which lead to clinical changes are completed within minutes following exposure. Nevertheless, it is common that many hours pass before the first symptoms of intoxication present which results in the development of severe pathological changes often affecting considerable areas of epithelia. Neither medical prophylaxis or therapy of these changes is resolved in any of the commonly affected tissues. Guidelines are available for decontaminating liquid SM on the skin and for clinical care including life support and the alleviation of local and systemic symptoms. There are no recommendations, however, for prophylaxis, early diagnosis of exposure, antidotes and targeted therapy of the injured [3].

Severe dermal symptoms are the most common signs of exposure to SM. The underlying events include the massive necrosis of epidermal and dermal cells as well as the degradation of extracellular proteins. An inflammatory response is triggered in return, eventually leading to vesication, ulceration and the erodation of the epidermis. Secondary infections often develop. Although SM reaches the systemic circulation rapidly, causing disturbance in various systemic organs such as the red bone marrow, local symptoms are severe enough to result in prolonged hospitalization [4].

Countering the threat presented by CBRN weapons requires complex measures, many of which are in the field of military medicine. Efficient prophylaxis is of outstanding

importance due to the severity and prolonged healing of injuries caused by SM. Several fluids, gels and powders have been developed recently which, at least in animal studies, have proved to significantly inhibit the penetration of SM as well as the intensity of dermal response when used as a topical protectant. These preparations could be administered at the joints of the protective garment or to cover a large area of skin when no protective suit is available, but also when excluding physical and psychic stress or maintaining the coordination of movement is essential.

In order to promote operations in a contaminated environment by protecting personnel, the efficiency and safe use of topical protectants need to be established. This requires the development of biological models for testing and comparing candidate preparations. Both *in vitro* and *in vivo* models have been elaborated for this purpose.

Most *in vivo* models are based on the evaluation of biological phenomena such as edema or erythema given in response to SM exposure. While these models are suitable for the characterization of the severity of pathological changes and the healing process, they are difficult to validate and do not yield data eligible for comparison or extrapolation. Moreover, large numbers of animals are commonly used in these studies which raises ethical and financial issues. Nevertheless, *in vivo* models are essential for characterizing the interaction between SM and the human body. This raises the necessity of having a protocol which allows the rapid and specific chemical detection of the dermal penetration of SM as well as the characterization and comparison of the efficacy of protective preparations and measures.

In the present thesis an *in vivo* model is described which has been elaborated with the purpose of meeting these aims. The underlying idea of the model is that the penetration of SM results in changes in the composition of the extracellular fluid in the vicinity of the site of exposure. Detecting these changes allows for the characterization of the penetrating amount of SM. This is feased by the continuous sampling of the subcutaneous extracellular space with microdialysis and the evaluation of the levels of thiodiglycol (2,2'-thiodiethanol; TDG), a hydrolysis product of SM, in collected fractions.

Aims

Aim 1: To develop an in vivo experimental model which allows for the characterization of the percutaneous penetration of chemical warfare agents as well as the efficacy of countermeasures against dermal SM exposure. The model was required to include:

Aim 1a: a procedure for exposing skin to highly toxic agents while inhibiting their evaporation;

Aim 1b: means of monitoring the concentration of permeants and their metabolites.

Aim 2: To adapt, characterize and optimize microdialysis as a sampling technique *in vitro* and *in vivo* as part of the model.

Aim 3: To identify an analyte which could serve as a specific marker of the percutaneous penetration of the model chemical SM. Further, to develop a quantitative analytical procedure for evaluating the concentration of the selected analyte in aqueous microsamples (volume below 80 µl).

Aim 4: To test the suitability of the *in vivo* model by:

Aim 4a: characterizating SM penetration as a function of its applied dose;

Aim 4b: characterizating the efficacy of protective preparations found to inhibit SM penetration in earlier studies;

Aim 4c: testing a preparation not used earlier for the prevention of SM penetration.

Methods

The analysis of thiodiglycol (2,2'-thiodiethanol, TDG) was performed using a gas chromatograph coupled with a pulsed flame photometric detector. A surrogate standard (3,3'-thiodipropanol, TDP) was employed throughout sample preparation and analysis. Both TDG and TDP were assayed following derivatization with heptafluorobutyric anhydride.

Male Wistar rats were used in the experiments. The animals were put to anaesthesia by the intramuscular administration of ketamin and xylazin. Liquid SM was applied on the shaved abdominal skin. 2 sites of exposure and sampling were established in each animal. Exposure was carried on for 360 minutes under occluded conditions. In the model development phase the applied SM dose was 1,0; 2,0; 3,0 or 5,0 μL while in the model testing phase 2,0 μL SM volumes were administered. Applied protective preparations included a gel containing nanoparticles and antioxidants, a perfluoropolyether product Fomblin Y45® and the pharmaceutical preparation Pasta Zinci Oxydati. All preparations were applied as topical prophylactics.

Continuous microdialysis sampling was performed in the subcutaneous adipose tissue directly beneath the site of exposure for 360 minutes. 20-minute fractions were collected. Sampling conditions which affected tissue monitoring were investigated *in vitro* and *in vivo*. The *in vivo* recovery ratio of TDG was assessed with the retrodialysis method.

The exposed skin was excised in full thickness on 24 hours postexposure and was examined under a light microscope for pathological changes after staining with the hematoxylin eosin method.

The penetration of SM was characterized by evaluating noncompartmental toxicokinetic values obtained from subcutaneous TDG concentration profiles. Mean peak concentration ($\underline{c}_{max}$), the mean area under the 60-minute-curve ($\underline{AUC}_{0\text{-}60}$) and the mean area under the 360-minute-curve ($\underline{AUC}$) were employed for evaluating the efficacy of protective preparations.

Results

Gas chromatography coupled with pulsed flame photometric detection proved to be a useful approach for the qualitative and quantitative analysis of TDG in microdialysates. TDP was used as a surrogate standard. A sample preparation procedure was applied to all samples which yielded a clear and fortified benzene solution of TDG-HFBA and TDP-HFBA. Derivatization of TDG and TDP was complete within 10 minutes. After removing interfering matrix components with liquid-liquid extraction the chemicals of interest could be separated to baseline and were the only peaks to appear in the relevant section of the chromatogram. The limit of detection for TDG was 0,200 nmol ml^{-1}, while the limit of quantitation was 0,364 nmol ml^{-1}. A linear relationship was identified between the c_{TDG}/c_{TDP} ratio and the square root of TDG and TDP peak heights in the range of 0,41-3,69 nmol ml^{-1} (50,0-450,2 ng ml^{-1}), as proved by running 3 independent standard sample series. Quantitative analysis was subsequently performed in this concentration range.

The accuracy of the analytical method was determined by measuring 3 solutions containing different concentrations of TDG. Measured concentrations differed by 1,64-7,26% from the nominal concentrations. The precision was assessed by running 5 solutions containing the same amounts of TDG. Concentrations found differed by 1,09-7-01% from the nominal value with a relative standard deviation of 2,75%.

Recovery of thiodiglycol with microdialysis

As a first step, microdialysis of an aqueous TDG solution was performed at flow rates between 1,0 and 20 µl min^{-1}. The results of these experiments confirmed that there is no interaction between the analyte and the materials of the microdialysis probe. Increasing perfusate flow rate resulted in an exponential drop in the recovery ratio (RR) of TDG. A 55,50±5,49% recovery was identified at a flow rate of 2,0 µl min^{-1} which was used in the rest of the studies being an acceptable trade-off between fraction collection frequency and sample volume.

Time and dose dependence of the dermal penetration of SM

Subcutaneous 360-minute TDG concentration profiles were obtained following exposure to 1.0-5.0 μL SM. TDG was detected in the first sample in 50%, 86%, 100% and 100% of cases when 1.0, 2.0, 3.0 and 5.0 μL SM, respectively, was applied. The analyte was present in the dialysates collected in the rest of the exposure period irrespective of the administered SM volume.

Relationship between the applied dose of the agent and the subcutaneous presence of TDG was identified by evaluating the following toxicokinetic values obtained from 360-minute TDG concentration profiles: mean 0-to-20-minute ($\underline{c}_{0\text{-}20}$) and mean peak concentration ($\underline{c}_{max}$), mean time to reach peak level ($\underline{t}_{max;0\text{-}360}$) mean area under the 60-minute ($\underline{AUC}_{0\text{-}60}$) and mean area under the 360-minute ($\underline{AUC}$) curve. In the range of 1.0-3.0 μL, higher SM doses yielded increases in these values. In addition, a linear relationship was identified between dose and TDG $\underline{c}_{max}$, $\underline{AUC}_{0\text{-}60}$ and $\underline{AUC}$. TDG levels, and, consequently, calculated toxicokinetic values did not differ significantly after applying 3.0 and 5.0 μL SM (*Figure 1*).

Dermal histological changes caused by the percutaneous penetration of SM

The histological evaluation of skin exposed to SM showed remarkable morphological changes following contact with SM. The application of 1.0 μL resulted in dermal and subcutaneous edema. Coalescence of the dermis and the adipose tissue was also apparent. The same changes were observed with higher intensity after exposure to 2.0 μL SM, with the subcutaneous fascia being affected as well. Following exposure to 3.0 μL and 5.0 μL SM the dermis and the adipose tissue underwent liquefaction necrosis. The fascia disappeared or was highly segmented and the response involved the peritoneal muscle. The affected area of the integument was the largest when 5.0 μL SM was applied. None of the changes described were observed in unexposed skin.

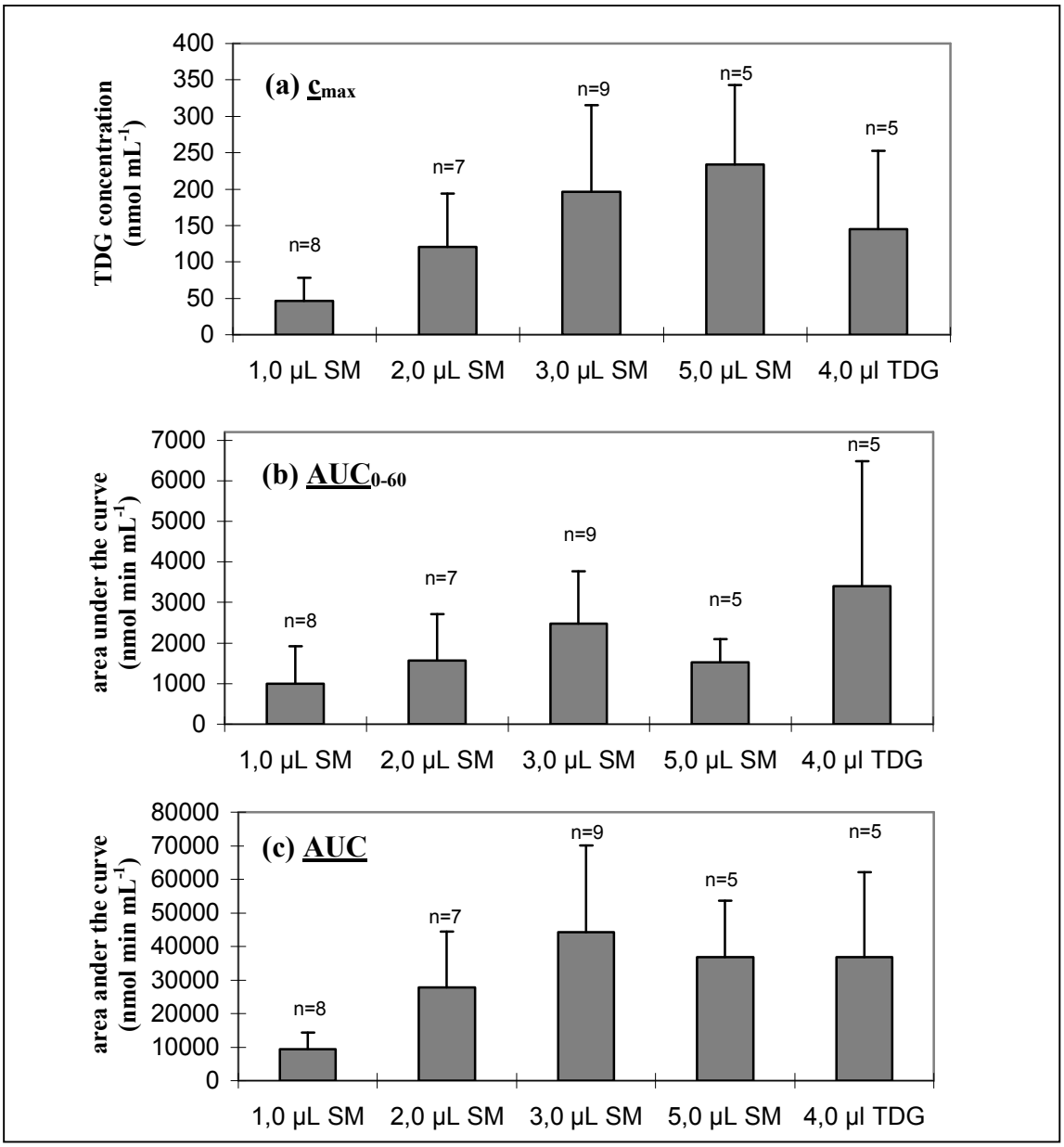

Figure 1. Toxicokinetic values calculated from 360-minute TDG concentration profiles following exposure to 1.0-5.0 µL SM or 4.0 µL TDG. (a) $\underline{c}_{max}$, (b) $\underline{AUC}_{0\text{-}60}$ and (c) $\underline{AUC}$.

Efficacy of prophylactic topical preparations

The suitability of the model was tested by studying the efficacy of three prophyactic topical preparations against SM. After the application of HP01, TDG was found in 3 0-to-20-minute samples out of 5 and all dialysates collected afterwards. When HP02 was applied, TDG did not appear before 20 minutes in 80% of experiments. In addition, the increase in TDG levels showed linearity with time betwen 60 and 360 minutes. The application of HP03 did not delay the appearance of the agent.

When HP03 was applied, $\underline{c}_{max}$, $\underline{AUC}_{0\text{-}60}$ and $\underline{AUC}$ values agreed with those obtained without pretreatment. All of these values were significantly lower, however, after the application of HP01 and HP02. $\underline{AUC}_{0\text{-}60}$ found after pretreatment with HP02 was significantly lower even than that calculated when 1.0 µL SM was applied (*Figure 2*).

Percutaneous penetration of thiodiglycol

TDG was detected in all microdialysates in a 360-minute period collected after its topical application. Concentrations showed a rapid increase in the early period and reached a plateau in 60%, increased constantly in 20% and decreased after reaching a peak in 20% of experiments. No histological changes from normal morphology were seen in exposed regions.

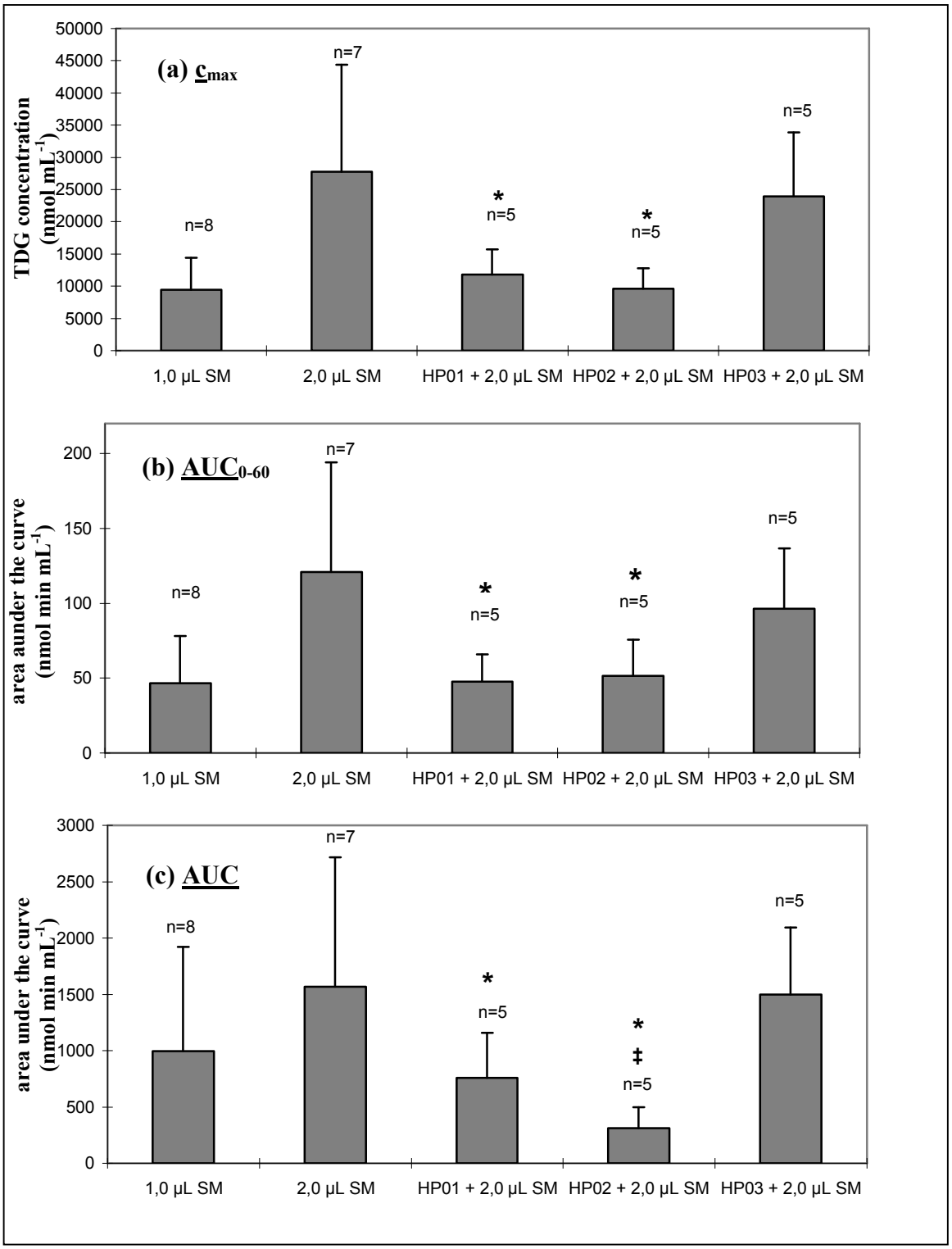

Figure 2. Toxicokinetic values obtained from 360-minute TDG profiles after ptretreatment with topical preparations. An asterisk indicates significant difference from the value obtained after the application of 2.0 uL SM ($p<0,05$). ‡ indicates significant difference from values obtained after the application of 1.0 µL SM ($p<0,05$). Values calculated after the application of 1.0 and 2.0 uL SM without pretreatment is displayed to promote comparison. (a) $\underline{c}_{max}$, (b) $\underline{AUC}_{0\text{-}60}$, (c) $\underline{AUC}$.

Conclusions

1. Gas chromatography coupled with pulsed flame photometric detection allows for the sensitive detection of thiodiglycol after derivatization with heptafluorobutyric anhydride. In addition, this approach can be employed for the quantitative analysis of thiodiglycol in *in vivo* microdialysates using 3,3'-thiodipropanol as surrogate standard. Both sample preparation and analysis are rapid, reproducible and cost-effective.

2. The presence of thiodiglycol in the subcutaneous extracellular fluid is a specific and imminent evidence of sulfur mustard exposure.

3. There is a relationship between the amount of contacted sulfur mustard and (1) the peak subcutaneous concentration of thiodiglycol as well as (2) the 0-60 minute and (3) the 0-360 minute area under the thiodiglycol concentration curve.

4. The presented model allows for the verification of cutaneous exposure to SM as well as for the evaluation of the efficacy of protective topical preparations and related countermeasures.

5. The percutaneous penetration of SM can be effectively inhibited by (1) topical preparations containing nanoparticles and antioxidants, and (2) Fomblin Y45®, when used as a prophylactic.

Summary of scientific results

- The microdialysis technique has been adapted and optimized by peforming *in vitro* and *in vivo* experiments for studying the composition of the subcutaneous extracellular fluid in rats.

- A microanalytical approach has been elaborated for the quantitative analysis of thiodiglycol, a specific marker of SM exposure, in aqueous matrices to allow the characterization of the percutaneous penetration of SM.

- The dermal consequences of exposure to SM have been evaluated in the skin of rats by histological characterization.

- The percutaneous penetration of various amounts of SM was characterized in anaesthetized rats *in vivo*. As a result, a semi-quantitative relationship was identified between the applied SM dose and toxicokinetic values derived from subcutaneous TDG concentration profiles. The suitability of the elaborated model for assessing the efficacy of prophylactic topical protectants was subsequently established.

- A topical protectant containing nanoparticles and antioxidants was found to be effective against the percutaneous penetration of SM, possibly as a result of chemical interaction.

Propositions for the practical implication of findings

I propose the following applications of the presented analytical method:

- quantitative assessment of thiodiglycol concentrations in small volume (>15 µl) biological samples (microdialysates, blood, urine, saliva, interstitial fluid) with the purpose of the verification of sulfur mustard exposure;

- quantitative assessment of hydrolysis products of mustard-like vesicant chemicals in small volume (>15 µl) biological samples (microdialysates, blood, urine, saliva, interstitial fluid) with the purpose of verification of exposure to a vesicant.

I propose the following applications of the presented *in vivo* model:

- evaluation and comparison of the efficacy of topical protectants with regards of the inhibition of the percutaneous penetration of sulfur mustard in *in vivo* experiments;

- the model may be included in complex *in vivo* approaches which allow for the characterization of sulfur mustard dermal penetration characteristics as well as the efficacy of topical protectants by combining local chemical detection with the evaluation of microscopic and macroscopic pathological changes;

- investigation of the percutaneous penetration of mustard-like vesicant chemicals and the efficacy of topical protectants against them by monitoring the extracellular concentrations of their hydrolysis products in the hypodermis;

- investigation of the percutaneous penetration of other chemical warfare agents and the efficacy of topical protectants against them by monitoring the extracellular concentrations of a related chemical in the hypodermis;

- the model may be included in complex *in vivo* approaches which allow the investigation of the percutaneous penetration of toxicants by combining local chemical detection with the evaluation of microscopic and macroscopic pathological changes.

Communications related to the topic of the thesis

1. **Karvaly G**, Halász L, Fűrész J, Solymosi J: [Chemical weapons of Saddam Hussein]. *Hadtudomány* **13** (3-4) (2003); 36-45. (article in Hungarian)

2. **Karvaly G**, Gachályi A, Boldis O, Mátyus M, Kocsis Gy, Némethné Karpova N, Fűrész J: [Toxicological and bioanalytical properties of toxins of military importance]. *Honvédorvos* **56** (2003); 62-77. (article in Hungarian)

3. **Karvaly G**, Gachályi A, Boldis O, Mátyus M, Kocsis Gy, Némethné Karpova N, Fűrész J: [The biological fate of sulfur mustard]. *Honvédorvos* **56** (2003); 78-95. (article in Hungarian)

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10. **Karvaly G**, Jäckel M, Némethné Karpova N, Gachályi A, Fűrész J: [A complex *in vivo* model for studying the dermal penetration of sulfur mustard using microdialysis and histological evaluation]. Oral presentation. Symposium of the Surgical Scientific Board, Hungarian Defence Forces, Budapest, 14 June 2006.

11. **Karvaly G**, Némethné Karpova N, Gachályi A, Fűrész J: [An *in vivo* model for studying the percutaneous penetration of sulfur mustard using microdialysis]. Oral presentation. Tox'2006 Symposium, Society of Hungarian Toxicologists, Galyatető, 6 October 2006.

12. **Karvaly G**, Gachályi A, Fűrész J: Application of *in vivo* microdialysis for studying the efficacy of protective preparations against sulfur mustard penetrating the skin. *J Appl Toxicol* **28** (2008); 21-6.

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Curriculum vitae

1. Personal data

Name, rank:	Karvaly, Gellért Balázs, captain
Place and date of birth:	Szeged, 1978. augusztus 24.

2. Education

a. High School and undergraduate studies

High school:	Deák Ferenc High School, Szeged
Graduation:	Faculty of Pharmacy, University of Szeged
Profession:	Pharmacist (MPharm), English-Hungarian Medical Translator

b. Other trainings

- OPCW Course on Analytical Skills Development (2004)
- Quality assurance fellow (2006)
- Senior quality assurance fellow (2006)

3. Languages spoken

English (professional)

German (intermediate)

4. Grants:

Republic Grant of the Ministry of Education	1999-2001.
Education Grant of the Ministry of Defence	1999-2001.

5. Positions held:

Department of Budgeting and Controlling, Medical Training Center	pharmacist	2001-2002.
Department of Chemical and Environmental Security, Zrínyi Miklós National Defense University	graduate student	2002-2003.
Department of Toxicology, Institute of Health Protection	medical officer	2003-2007.
Department of Toxicology, Scientific Institute, Dr. György Radó Military Medical Center	medical officer	2007.
Central Department of Laboratory Diagnostics, State Health Center	pharmacist	2007-

6. Scientific background:

Undergraduate research program, Institute of Pharmaceutical Chemistry, University of Szeged, Szeged, Hungary	1998-2001.
demonstrator, Institute of Pharmaceutical Chemistry, University of Szeged, Szeged, Hungary	1999-2001.