TNO report
PML 1997-A48

Toxicokinetics of sulphur mustard and its DNA-adducts in the hairless guinea pig - DNA-adducts as a measure for epithelial damage. Midterm report

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July 1998

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Classification		
Classified by	:	Dr. J.P. Langenberg
Classification date	:	24 June 1998 (This classification will not change)
Title	:	Ongerubriceerd
Managementuittreksel	:	Ongerubriceerd
Abstract	:	Ongerubriceerd
Report text	:	Ongerubriceerd

Copy no.	:	11	
No. of copies	:	22	
No. of pages	:	55	(excl. RDP & distribution list)
No. of annexes	:	-	

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Managementuittreksel

Titel	:	Toxicokinetics of sulphur mustard and its DNA-adducts in the hairless guinea pig - DNA-adducts as a measure for epithelial damage. Midterm report
Auteur(s)	:	Dr. J.P. Langenberg, Dr. ir. H.P. Benschop en Dr. G.P. van der Schans
Datum	:	juli 1998
Opdrachtnr.	:	A94M444
Rapportnr.	:	PML 1997-A48

De behandeling in Europese ziekenhuizen van mosterdgasslachtoffers van het Iran-Irak conflict werd bemoeilijkt door het ontbreken van een specifieke en causale therapie voor vergiftigingen door dit strijdmiddel. De behandeling beperkte zich tot het ondersteunen van de vitale functies van de patiënt, bevordering van de genezing van huidlesies en het voorkomen van secundaire infecties. Na de Tweede Wereldoorlog is het onderzoek naar de toxicologie van mosterdgas nagenoeg verwaarloosd omdat de dreiging van de toen recent ontwikkelde zenuwgassen groter werd geacht. Het grootschalige gebruik van mosterdgas in het Iran-Irak conflict heeft echter de actuele dreiging van deze blaartrekker aangetoond.
Teneinde een kwantitatieve basis te bieden voor de ontwikkeling van strategieën voor voorbehandeling en behandeling van vergiftigingen door mosterdgas wordt in het kader van opdracht A94M444 de toxicokinetiek van dit strijdmiddel bestudeerd. Deze studie wordt mede ondersteund door Cooperative Agreement DAMD17-94-V-4009 van het U.S. Army Medical Research and Materiel Command.
De toxicokinetiek van mosterdgas wordt bestudeerd voor de meest relevante blootstellingsroutes, zijnde respiratoir en percutaan. Daarbij wordt de intraveneuze toxicokinetiek bestudeerd als referentie voor voornoemde routes. Als diermodel is de haarloze cavia gekozen, waarvan de huid vergelijkbaar is met die van de mens. Naast de toxicokinetiek van het intacte mosterdgas in bloed en diverse weefsels wordt ook de toxicokinetiek van het voornaamste adduct van mosterdgas aan DNA bestudeerd. Het DNA-adduct vormt een maat voor de weefselschade.
In het kader van dit project zal tevens de effectiviteit van voorbehandeling met scavengers tegen de respiratoire toxiciteit van mosterdgas worden bestudeerd. Mochten de scavengers bescherming bieden, dan zal de invloed van de scavengers op de intraveneuze en respiratoire toxicokinetiek worden vastgesteld.
Daarnaast zal de beschermende werking van Topical Skin Protectants tegen de huideffecten van mosterdgas in vloeibare en dampvorm worden bestudeerd.
In dit 'midterm' rapport worden de resultaten verkregen in de eerste anderhalf jaar van de driejarige studie beschreven. Een gevoelige methode voor de analyse van mosterdgas in bloed en weefsels is ontwikkeld, gebaseerd op gaschromatografie met geautomatiseerde thermodesorptie-injectie en massaspectrometrische detectie. Gedeutereerd mosterdgas wordt hierbij gebruikt als interne standaard. Mosterdgas

Gedeutereerd mosterdgas wordt hierbij gebruikt als interne standaard. Mosterdgas en de gedeutereerde interne standaard kunnen met een rendement van ongeveer 90% met ethylacetaat uit biologische monsters worden geëxtraheerd. Dankzij de injectie van een groot volume van het extract in de gaschromatograaf en de hoge gevoeligheid en selectiviteit van de detector kan een detectielimiet van 5 pg/ml voor mosterdgas in bloed routinematig worden gerealiseerd.
DNA-adducten worden gemeten met een eerder ontwikkelde immuno-slot-blot methode, welke een detectielimiet heeft van 1 adduct op $2*10^7$ ongemodificeerde nucleotiden.
De intraveneuze LD50 (96 uur) in de haarloze cavia bleek 8,2 mg/kg te zijn, hetgeen hoger is dan de in de literatuur gerapporteerde waarden voor de rat en het konijn, maar vergelijkbaar met de LD50 van mosterdgas in de muis.
De toxicokinetiek van mosterdgas in de haarloze cavia na intraveneuze toediening van een dosis corresponderend met 1 LD50 wordt gekarakteriseerd door een zeer snelle distributiefase en een langzame eliminatiefase met halfwaardetijden van respectievelijk 0,77 en 107 minuten. Op 3 minuten na intraveneuze toediening van deze dosis mosterdgas zijn DNA-adducten aantoonbaar in bloed, long, milt, beenmerg, lever en dunne darm. Verreweg de hoogste concentratie DNA-adduct is in de long gevonden, welke één tot twee orden hoger is dan in het bloed. Hoewel de adducten voor een belangrijk deel verdwijnen binnen 6 uur zijn nog steeds significante concentraties adduct aantoonbaar in de weefsels op 48 uur na toediening van mosterdgas. De concentraties intact mosterdgas in de diverse weefsels zijn nog niet gemeten.
Volledigheidshalve zij nog vermeld dat in het kader van opdracht A95M052 de toxicokinetiek van mosterdgas en diens DNA-adducten zal worden bestudeerd in de marmoset voor de intraveneuze en respiratoire route. Hierdoor wordt vergelijking van de toxicokinetiek van mosterdgas tussen de twee species mogelijk en kunnen op termijn voorspellingen van het toxicokinetisch profiel in de mens worden gedaan met behulp van fysiologisch-gebaseerde modelering.

Contents

1 Introduction

The treatment of sulphur mustard (SM) casualties from the Iran-Iraq war has provided ample evidence that a specific and causal therapy for the local and systemic effects of this vesicant is not available (Willems, 1989). In fact, treatment had to be restricted to sustaining the vital functions of the patient, enhancing the healing process of the lesions and at prevention of secondary infections. Evidently, research on the toxicology of SM has been neglected since the end of World War II, when the threat of the newly developed nerve agents became apparent. However, the use of SM in the Iran-Iraq war has reconfirmed the threat of vesicants on the battlefield. Since several countries have provided themselves with stocks of this agent, great efforts have to be made in order to develop a better treatment of SM intoxication. This is true for the local effects on skin, eyes and respiratory tract, as well as for the systemic effects (Papirmeister *et al.*, 1991). In general, it may be stated that the local effects of SM are responsible for turning a soldier into a casualty, whereas the duration of his incapacitation, and of his burden on the medical staff, is determined by the systemic effects of the agent (Stade, 1964). Efforts in the field of diagnosis and dosimetry of SM exposure (Benschop, 1991) and with regard to treatment of its effects on skin are well under way (Papirmeister *et al.*, 1991). However, relatively little effort has gone into the prophylaxis and/or therapy of systemic intoxication with the agent. In virtue of its large penetrative capacity and extensive biochemical activity, SM is transported rapidly by skin absorption and inhalation to various organs, where it exerts its effects. It affects, amongst other things, the bone marrow, liver, kidneys, stomach and intestinal tract, as well as the central nervous system and the metabolic pathways in general. A large number of symptoms appear, such as general malaise, apathy and deep depression. These symptoms are, to some extent, even more insufferable than the vesicant effects. Moreover, it has been well established that victims suffer from a variety of chronic and delayed effects for the rest of their lifetime, such as asthma, bronchitis, premature ageing, loss of libido and potency, central nervous system effects and cancers of the respiratory tract (SIPRI, 1975). The only positive results to prevent or diminish the effects of systemic intoxication with SM have been obtained with scavengers with a high reactivity for the episulfonium derivative of the agent, e.g., thiosulphate, which is active in the extracellular space, or cysteine and other thiol derivatives, which may penetrate the cell (Connors, 1966; Callaway and Pearce, 1958). High doses of thiosulphate or cysteine given shortly before or after intoxication afford some protection against the lethality of SM in experimental animals, but the effects of thiosulphate on non-lethal damage have hardly been investigated (Vojvodic *et al.*, 1985). Recent investigations concentrate on the scavenger activity of N-acetyl cysteine (Trouiller and Lainee, 1992; Anari *et al.*, 1988) and on cysteine alkyl esters which are enzymatically hydrolyzed within the cell (Upshall and Lawston, 1991).
Efforts to develop a causal treatment of systemic intoxication with SM, or with any toxic agent in general, require an intimate knowledge of the toxicokinetics of

the intact agent as well as insight into the etiology of the lesions which are of major importance in the development of systemic intoxication. In the case of intoxication with nerve agents, our detailed toxicokinetic investigations of the agents sarin and soman in several species including primates have provided a quantitative basis for further toxicological research of this agent and for development of strategies for treatment or pretreatment of intoxication (Benschop and De Jong, 1990, 1991; Langenberg *et al.*, 1989). By analogy, we expect that similar toxicokinetic studies of intact SM will provide a quantitative basis for causal treatment of systemic intoxication, in particular with scavengers. Toxicokinetic studies of SM including distribution of the agent in various tissues will also provide a starting point for the development of a physiologically based toxicokinetic model, which enables interspecies scaling and, eventually, extrapolation to man. Such a model is currently being developed for soman toxicokinetics (Langenberg *et al.*, 1993).

In current theories on the etiology of local and systemic damage due to exposure to SM, it has been hypothesized that alkylation of DNA is often the starting point for a chain of reactions leading to the observed damage (Papirmeister *et al.*, 1991), although current interest is focused on the effects of direct alkylation of proteins. Therefore, the amount of SM adducts of DNA and proteins in a tissue can be regarded as a measure of the amount of damage in that tissue. Since we have developed several methods to measure the amount of DNA-adducts of SM in our investigations on diagnosis and dosimetry of exposure to SM (Benschop, 1991), we propose to combine the toxicokinetic investigations of intact SM in blood and selected organs with the analysis of DNA-adducts in these tissues, in order to obtain insight into the relationship between the challenge and the damage due to that challenge.

Until recently, studies on the *in vivo* distribution of SM were performed mostly with radiolabelled SM, which leaves in doubt whether results pertain to intact agent or to hydrolyzed and metabolized derivatives (Black *et al.*, 1992). Moreover, these studies involved mostly intravenous administration, whereas absorption via the respiratory tract and the skin are the military relevant portes d'entrée for systemic intoxication. Investigations with intravenous administration of ^{35}S-SM in mice (Clemedson *et al.*, 1963) and in rabbits (Boursnell *et al.*, 1946) indicate a rapid and rather even distribution over the body with some accumulation appearing in the nasal region, kidneys, liver, lungs and intestines. Upon percutaneous administration of liquid SM to mice (Clemedson *et al.*, 1963), a slower clearance was observed, as expected from gradual uptake through the skin. Assessment of the damage in the respiratory tract and in the lungs after inhalation of SM suggests that most of the agent is absorbed before it reaches the lungs (Cameron *et al.*, 1946).

Recently, two groups have reported results on the toxicokinetics of intact SM. Zhang and Wu (1987) measured blood levels of SM after intravenous, subcutaneous, and percutaneous administration of SM to piglets. Using gas chromatography on packed columns and flame-photometric detection, they were able to follow the blood levels of intact SM for 20 min after intravenous administration of 10 mg

SM/kg and for 90 min after subcutaneous administration of 200 mg SM/kg. Intact SM could not be observed after percutaneous application of 200 mg liquid SM/kg on a 5 cm^2 skin area. However, their apparent lower detection limit is in the range of 200 ng SM/ml blood, which should be considered approximately. 3 orders of magnitude too high for toxicologically relevant analysis of such highly reactive agents. Maisonneuve *et al.* (1993) measured blood levels of intact SM in rats after i.v. administration of 10 mg SM/kg, corresponding with approximately 3 LD50. Using gas chromatography with capillary columns and flame ionisation detection, they obtained a detection limit of approximately 10 ng SM/ml blood, which allowed them to follow blood levels of intact SM for 8 h. These authors, as well as Zhang and Wu (1987), observed a rapid distribution of the agent with a half-life of approximately 5.6 min, followed by slow elimination with a half-life of 3.6 h. The observed blood levels in piglets and rats after administration of multiple lethal doses of SM cast extra doubt on the colorimetrically measured levels of intact SM in blood (1.1 µg/ml) and tissues of an Iranian patient who died 7 days after exposure to SM (Drasch *et al.*, 1987).
In summary, these preliminary investigations on the toxicokinetics of SM show that, as in the case of nerve agents, intact SM has a much longer *in vivo* lifetime than assumed previously on the basis of rapid hydrolysis of SM in aqueous solution. When this persistence is confirmed in our proposed investigations, these results will have to be integrated into future investigations on the toxicology of SM and on treatment of intoxications with the agent.

On the basis of the considerations mentioned above, we proposed investigations for a 3-year Cooperative Agreement on the toxicokinetics of SM in the hairless guinea pig. In the following, the various aspects of the proposed research are further elucidated.

a Stabilization, work-up and analysis of SM in biological samples

Our experience with the analysis of nerve agents in biological samples (*cf.* Benschop *et al.*, 1985) suggests that immediate stabilization of SM in blood and tissue samples is crucial, in view of the high reactivity of the agent towards the biological matrix. The influence of parameters such as pH, temperature and concentration of chloride ions will be studied. The analysis of known concentrations of SM spiked into stabilized blood samples will show whether the stabilization procedure is adequate. It will be investigated whether solid-liquid extraction with C_{18}- or other cartridges will improve the work-up of samples in terms of preconcentration for gas chromatographic analysis, removal of contaminants, and convenience in comparison with previously applied liquid-liquid extraction.
For the final analysis of SM, we will rely on the gas chromatographic configuration which has provided excellent results with nerve agent analysis, i.e. thermal desorption (from Tenax)/cold trap (TCT) injection and two-dimensional chromatography (Benschop and De Jong, 1990). For detection we intend to use a flamephotometric, mass spectrometric, chemoluminescence or electron capture detector. We will attempt to use fully deuterated SM, i.e. D_8-SM as an internal

standard. This two-dimensional configuration assures superior 'on-line work-up' and selectivity. Also, it will be attempted to automate the TCT-injection in order to increase sample throughput. With this analytical ensemble, we anticipate a detection limit of approximately 5 pg SM/ml blood.

b The hairless guinea pig as selected species

The hairless guinea pig is used extensively in studies on skin damage by SM and of skin protectants, since the structural effects of SM on the cells in the basal layer of skin are nearly identical to those in human skin (Papirmeister *et al.*, 1991). The epidermis in the hairless guinea pig is much thicker than in other laboratory animals, i.e. more similar to that in humans (Mershon *et al.*, 1990). Therefore, this species allows highly relevant investigations on skin damage, skin protection, and systemic absorption upon exposure to SM vapour.
The hairless guinea pig will also be used for toxicokinetic investigations following intravenous administration and respiratory exposure.

c Doses and routes of administration of SM

Experience with the use of SM on the battlefield has taught us that this agent should be characterized as a physical incapacitating agent rather than as a lethal agent. In World War I, only about 2% of SM casualties died, often due to secondary infections resulting from damage to the respiratory tract (Papirmeister *et al.*, 1991). Therefore, we propose to use doses of SM which are equivalent to 1.0 LD50 or 1.0 LCt50 as the highest dose. In the several cases where the influence of dose on the toxicokinetics is investigated we propose to use an additional dose corresponding to 0.3 LD50 or LCt50. However, if the latter dose does not lead to measurable blood levels of SM, it will be replaced by a tenfold higher dose.

- *Intravenous administration of SM*
  In order to obtain insight into the basic aspects of the toxicokinetics of SM in hairless guinea pigs, the investigations are initiated by measuring the blood levels of SM after intravenous administration of the agent at doses corresponding to 1.0 and 0.3 LD50. This will provide data on the blood levels of intact SM that can be expected in further experiments, on linearity of the toxicokinetics with dose, as well as on the time scale in which detectable concentrations can be measured.
- *Respiratory exposure to SM*
  It is generally accepted that absorption of SM vapour in the respiratory tract and via the skin are the military relevant portes d'entrée for this agent. Therefore, further toxicokinetic experiments will pertain to these routes of exposure. A new apparatus will be designed for respiratory exposure of hairless guinea pigs, using the experiences obtained in our inhalation toxicokinetic studies on the nerve agents soman and sarin (Benschop and Van Helden, 1993). We will measure the blood levels of SM in hairless guinea pigs after 'head-only' exposure for 5 min to a dose of SM corresponding to 1.0 LCt50. In order to measure the degree of linearity of the toxicokinetics with exposure dose, the same experiment will be performed at a dose corresponding to 0.3 LCt50.

- *Percutaneous exposure to SM*
  Exposure of the skin to SM vapour leads to skin damage as well as to systemic intoxication. Blood levels of SM will be measured during and after a 35-min whole body (except the head) exposure of hairless guinea pigs to the estimated percutaneous LCt50 of SM in various species, i.e. 10.000 mg.min.m^{-3} (Papirmeister *et al.*, 1991).

d Quantitative analysis of SM-DNA adducts in blood and various tissues

As mentioned above, it is assumed that the concentration of SM-DNA adducts in a tissue is a reasonable measure of the damage due to SM in that tissue. We have developed an ELISA based on a monoclonal antibody against the major SM-adduct with DNA, i.e. the monoadduct at the 7-position of guanine (7-SM-guanine). This ELISA detects one such adduct amongst $2*10^7$ nucleotides in DNA, i.e. ≥ 0.15 pmol of adduct/g tissue (Benschop and Van der Schans, 1995). We propose to combine our toxicokinetic investigations of intact SM in blood with measurements of the formation of 7-SM-guanine in blood and in selected tissues, concomittantly with the analysis of SM in these tissues. In this way, insight is obtained into the relationship between the concentrations of SM in blood and these tissues and the concentration of the resulting DNA adducts. Also, the repair of the adducts with time will be followed. Similar integrated investigations on toxicokinetics of direct or metabolically formed alkylating agents and of resulting DNA-adducts have been rarely performed (see for example Ginsberg and Atherholt, 1990) and may have general usefulness for studies on carcinogenesis.
The selection of cell types and tissues for analysis of SM-DNA adducts is not straightforward, since SM is rather unselective in its systemic toxicity. The following cell types and tissues are proposed for initial measurement of SM-DNA adducts:

- *white blood cells*, being primary targets and indicators of exposure to SM, no matter how the agent reaches the general circulation;
- *bone marrow* and *spleen*, since in all systemic intoxications with SM, damage to the bone marrow and the haematopoietic system in general are the most striking effects (Needham *et al.*, 1947). Leucopenia is the main result of the damage; the red cell system is much less affected;
- *(small) intestine*, since the so-called radiomimetic effects of SM relate primarily to damage to the small intestine;
- *lungs*, being often invoked as damaged tissues in systemic intoxications with SM;
- *liver*, because of its crucial role in general metabolism.

We propose to investigate in some detail the formation and repair of 7-SM-guanine adducts in DNA of the above-mentioned tissues upon intravenous administration of 1 LD50 of SM to hairless guinea pigs. It will be investigated on which time scale the maximal concentrations of adducts are formed. Based on the results of Ginsberg and Atherholt (1990), it is assumed that the adducts are formed within

the first half hour after intravenous administration or respiratory exposure. In addition, the time scale of formation of the adducts will be investigated separately upon percutaneous exposure, since we anticipate that the adducts will be formed more slowly in this case. In view of our data on the *in vivo* repair of SM-DNA adducts in skin (Benschop and Van der Schans, 1995), the repair of SM-DNA adducts in the above-mentioned tissues will be investigated 24 and 48 h after intravenous administration.
Based on the exploratory results after intravenous administration and percutaneous exposure, the time points for analysis of formation and repair of 7-SM-guanine, as well as of SM, will be determined for the remaining toxicokinetic experiments. The adduct will be measured in white blood cells, bone marrow and spleen. In addition, 7-SM-guanine will be measured in either the lungs, liver, or small intestines, whichever of these tissues accumulates the highest concentration of adduct upon intravenous administration of SM.

e Absorption of SM in the nasal-pharyngeal airway and respiratory tract

Cameron *et al.* (1946) mention the following concise observation: 'When animals inhale lethal concentrations of mustard gas [or nitrogen-mustard] vapour, death results from direct damage to the respiratory tract with or without systemic poisoning. With certain small species, however (rabbit, guinea pig and rat), death from systemic absorption is frequently observed with little or no damage in the respiratory tract, apart from severe inflammation of the nose, which is always present. ... It appears therefore that a lethal dose of certain vapours may be absorbed through the mucous membrane of the nose'. These authors confirmed in their experiments that rabbits absorb about 80% of inhaled SM vapour in the nose. Our proposed experiments dealing with the inhalation toxicokinetics of SM at a dose corresponding with 1.0 LCt50 in guinea pigs (*cf.* c(ii)), and our ability to measure the major DNA-adduct of SM in various tissues (d), offer a unique possibility to combine the investigation of systemic absorption with the measurement of DNA adducts in various parts of the nasal-pharyngeal airway and the further respiratory tract. Assuming that the concentration of DNA-adducts in a certain area of the respiratory system is proportional to the concentration of SM vapour that is encountered (Casanova *et al.*, 1991), these adducts will be measured in the mucous membranes of the middle turbinates, anterior lateral walls and septum, nasopharynx, maxillary sinuses, larynx-trachea-carina, major interpulmonary airways, and lung. In combination with a histopathological investigation of the damage in the respiratory tract, detailed information will be obtained on the sites of absorption in the respiratory tract, in conjunction with observed damage.

f Quantification of skin damage based on SM-DNA adducts in the epidermis

In order to assess skin damage due to exposure to SM, the so-called Draize test is often used. This test depends on the evaluation of the degree of skin erythema by way of a rather subjective visual assessment (Papirmeister *et al.*, 1991). A priori,

the procedures that we developed for analysis of 7-SM-guanine in DNA of blood and various tissues (*cf.* item d) can be used to assess skin damage in a far more quantitative and objective way, taking also into account that the amount of DNA adducts is proportional to the amount of protein adducts (Skipper *et al.*, 1994). Based on this reasoning we propose the following experiments in order to develop an objective test for skin damage due to exposure to SM.
Using whole body exposure, hairless guinea pigs will be exposed for a 10 min period to various concentrations of SM vapour in air to yield Ct values of SM ranging between 10 and 3000 mg.min.m^{-3}. These Ct values will cause very slight erythema at the lower Ct range up, to very severe skin damage at the higher Ct range (Papirmeister *et al.*, 1991). The amount of 7-SM-guanine will be analyzed immediately after exposure according to the procedure mentioned under item (d) in the epidermal layer from skin biopts.
The skin damage will also be assessed according to the Draize procedure 24 h after exposure, using appropriately protected skin sites as controls, in order to correlate results with the new procedure.

g Quantification of eye damage based on SM-DNA adducts in corneal epithelium

It is well known that the human eye is extremely sensitive to exposure to SM in vapour or liquid form. A Ct value of 10 mg.min.m^{-3} will already cause reddening of the eye. Exposure to higher Ct values leads to conjunctivitis, photophobia, corneal oedema and clouding (Papirmeister *et al.*, 1991; Grant, 1986; Duke-Elder, 1954). Tests used to estimate damage due to SM exposure of the eye are very indirect, by way of measurement of loosening of corneal epithelium (Hermann and Hickman, 1948). We propose to develop a procedure to test eye damage which is analogous to that proposed for the quantification of skin damage. Eyes from the same hairless guinea pigs as used for the exposures described in item (f) will be enucleated and the amount of 7-SM-guanine will be determined in corneal epithelium. Hence, data will become available which allow a straightforward, sensitive and objective test for eye damage. Moreover, a comparison of the concentrations of SM-DNA adduct in epidermis and in corneal epithelium from the same animal will allow a comparison of the relative susceptibility of these two epithelial tissues to damage caused by exposure to SM. Similar experiments have been performed with regard to UV-damage in these two types of tissue (Freeman *et al.*, 1988; Ley *et al.*, 1988).

h Quantification of the protective efficacy of topical skin protectants based on SM-DNA adducts in the epidermis

Topical skin protectants (TSPs) are being developed for use in cases where military personnel will have to perform tasks in an environment contaminated by sulphur mustard, without being fully protected by means of protective clothing. In such special circumstances, contamination of the skin by liquid as well as vapour of SM might be a realistic threat. Therefore, we propose to test the protective efficacy of two TSPs (to be selected by the U.S. Army Medical Research and

Materiel Command), as measured by the reduction in the concentration of 7-SM-guanine in the epidermis due to the application of the TSP.
According to the procedure of Mershon *et al.* (1990), skin exposure sites (approximately 1 cm^2) on hairless guinea pigs will be covered with a topical skin protectant. Seven minutes after application of 1 µl of liquid SM on these covered sites, the site will be decontaminated and the amount of 7-SM-guanine in the epidermis of the exposed site will analyzed, two hours after exposure. On the same animal, comparable unprotected and covered sites will be challenged for the same period of time with the same amount of liquid SM. After subsequent decontamination and analysis of adduct in the epidermis, a comparison of the amount of adduct in the protected and unprotected sites will provide a protective ratio for the TSP.
Although TSPs are primarily being developed for protection against liquid SM, these protectants will also be tested for their efficacy against SM vapour. Skin sites on hairless guinea pigs protected by TSP as described above will be challenged for 10 min with a Ct of SM vapour that would yield severe skin damage (Draize score approximately 4-5; Draize *et al.*, 1944; Draize, 1965), according to the results of the experiments described under item (f). Subsequently, the amount of 7-SM-guanine in the epidermis of the protected skin site will be determined as mentioned above. A comparison with the results obtained in experiments described under item (f) will provide a protective ratio for the TSP against SM vapour.

i Influence of scavengers on the intravenous and inhalation toxicokinetics of SM and SM-DNA adducts

As mentioned by Papirmeister *et al.* (1991), administration of sodium thiosulphate at high doses has consistently been found to provide some protection from the systemic toxicity of SM. This effect has been related to the high competition factor of thiosulphate relative to water in its reaction with the episulfonium derivative of SM (Ogston *et al.*, 1948). For example, Vojvodic *et al.* (1985) showed that sodium thiosulphate, administered intraperitoneally at a dose of 3 g/kg 30 min after subcutaneous poisoning with SM in rats (i) decreased lethality resulting in a protective ratio of 1.7, (ii) prolonged survival time, (iii) antagonized decrease in body weight, and (iv) lessened the degree of histopathologic damage to various tissues, e.g. spleen and liver.
In view of the extremely high doses of thiosulphate which are needed to provide a protective effect, the use of this scavenger as a (pre)treatment agent is questionable. Moreover, it is known that thiosulphate does not penetrate into the intracellular space. Therefore, recent investigations on potential scavengers have concentrated on thiol derivatives which can penetrate the cell and are active at lower doses than thiosulphate. Iranian investigators (Anari *et al.*, 1988) have published a paper on the efficacy of N-acetyl cysteine in experimental SM poisoning. Their results have been confirmed (Trouiller and Lainée, 1992). This compound enters the cell and has proven its value in a variety of intoxications in which highly electrophilic species such as the episulfonium ion should be scavenged. For example, N-acetyl cysteine is used frequently, at an intravenous dose of approximately 300 mg/kg, in paracetamol intoxications (Prescott *et al.*, 1979). Very re-

cently, Upshall and Lawston (1991) have filed a patent application dealing with cysteine esters as scavengers for electrophilic compounds, e.g. perfluoroisobutylene and SM. These compounds also enter the cell, where they are hydrolyzed enzymatically to cysteine, especially in the lungs.

In view of the expected scavenging effect of N-acetyl cysteine and of cysteine esters, it should be expected that administration of these scavengers shortly before or after systemic intoxication with SM, should decrease the area under the curve for the blood levels of SM, as well as the concentrations of SM-DNA adducts in blood and in various tissues. In order to investigate the scavenging effect on the blood levels of SM, we propose to investigate the protective efficacy of intraperitoneal administration of N-acetyl cysteine and of a cysteine ester 1 min before inhalatory exposure of hairless guinea pigs for 5 min to various concentrations of SM vapour.
If these scavengers show a promising antidotal efficacy against SM, we propose to:

i analyze the concentrations of SM, 7-SM-guanine, and of N-acetyl cysteine in blood and in several tissues at various time points after intravenous administration of SM to hairless guinea pigs at a dose corresponding to 1 LD50, with intraperitoneal administration of N-acetyl cysteine at 1 min before intoxication;
ii perform similar analyses as described in (i) upon inhalatory exposure of hairless guinea pigs for 5 min to SM vapour to yield a Ct corresponding to 1 LCt50, with intraperitoneal administration of N-acetyl cysteine 1 min before exposure to SM vapour;
iii perform experiments and analyses similar to those described in (ii) (except for scavenger levels) using a cysteine alkyl ester as scavenger, which will be administered 30 min before exposure to SM;
iv attempt a description of the effect of N-acetyl cysteine on the blood levels of SM in a toxicokinetic model (*cf.* Fast and Sorbo, 1973).

The above-mentioned investigations serve as a starting point for quantitative investigations on the effect of scavengers on systemic intoxication with SM. If modelling is successful, this will allow the prediction of the effect of reactivity, affinity of antibodies (Lieske *et al.*, 1992), doses, and methods of administration (e.g. continuous infusion, *cf.* Hatea, 1986) on the efficacy of scavengers.

In this midterm report, the experiments performed and the results obtained in the first six quarters of the study are described.

2 Materials and methods

2.1 Materials

Technical grade sulphur mustard (SM) was purified by fractional distillation in a cracking tube column (Fischer, Meckenheim, Germany) to a gas chromatographic purity exceeding 99.5%. The internal standard, D_8-sulphur mustard (D_8-SM) was obtained as described elsewhere (Benschop and Van der Schans, 1995). Ethyl acetate ('zur Rückstandsanalyse') was procured from Merck (Darmstadt, Germany) and was distilled over a column packed with Dixon rings (plate number 80; NGW, Wertheim, Germany) before use. Isopropanol (purity > 99.5%) was purchased from Fluka (Buchs, Switzerland).
The following products were obtained commercially and were used without further purification: heparin (Vitrum, Stockholm, 5000 IU/ml), ketamine hydrochloride (Vetalar®, Parke-Davis, Morris Plains, NJ, USA), buprenorfine hydrochloride (Temgesic®, Schering-Plough, Amstelveen, The Netherlands), HPLC-grade water (Fisons, Loughborough, UK), Tenax TA, 60-80 mesh (Chrompack, Middelburg, The Netherlands). Disodium edetate (EDTA), Triton® X-100, sodium chloride, potassium chloride, disodium hydrogen phosphate dihydrate, potassium dihydrogen phosphate, phenol, phosphoric acid (85%), chloroform and ethanol were obtained from Merck (Darmstadt, Germany); sodium dodecyl sulphate (SDS), Tris.HCl from J.T. Baker (Phillipsburg, NJ, USA), and isoamyl alcohol from UCB (Brussels, Belgium); calf thymus DNA, proteinase K (EC 3.4.21.14, activity approximately 20 units/mg protein) and RNase T1 (EC3.1.27.5, activity approximately 40 units/mg protein) from Boehringer (Mannheim, Germany); RNase A, and Tween 20 from Sigma Chemical Co. (St. Louis, MO, USA); and skimmed milk powder, less than 1% fat from Campina (Eindhoven, The Netherlands).

2.2 Gas chromatography

The gas chromatographic configurations used or developed for our investigations are described below. They will be referred to by number in this report.

2.2.1 GLC configuration 1 (GC-FID)

This configuration was used to study the extraction recovery of SM from blood. A Carlo Erba (Milano, Italy) HRGC 5160 Mega series gas chromatograph was equipped with an AS550 autosampler and a flame ionization detector. The analytical column was CPSil 5 CB (length, 30 m; i.d., 0.32 mm; film thickness, 1 µm), connected to the injector via a retention gap of uncoated deactivated fused silica (medium polarity; length, 1 m; i.d., 0.53 mm). Carrier gas (helium) pressure was set at 100 kPa. The oven temperature was programmed from 87 °C to 135 °C at a

rate of 10 °C/min. The temperature was kept at 135 °C for 5 min, after which the oven was cooled to 87 °C for the next run. The temperatures of the injector and detector bases were both set at 250 °C. The pressures of air and hydrogen for the FID were set at 100 and 70 kPa, respectively.

2.2.2 GLC configuration 2 (LV-GC-GC-ECD, cf. Figure 1, subsection 3.1.3)

This configuration was equipped for automated analysis of SM in extracts from blood, using large volume on-column injection, two-dimensional chromatography and electron capture detection.

A Carlo Erba 5300 Mega series gas chromatograph was equipped with an electron capture detector (ECD) and a flame ionization detector (FID), a Waters WISP 590B (Millipore, Milford, MA, USA) HPLC autosampler and a Waters HPLC 590 programmable pump for micro flow pumping, a Valco (Schenkon, Switzerland) 8-port injection valve with electrical actuator and a Chrompack (Middelburg, The Netherlands) MUSIC (**Mu**ltiple **S**witching **I**ntelligent **C**ontroller) system. Flow rates of air and hydrogen through the FID were 350 and 35 ml/min, respectively. Nitrogen was used as make-up gas for the ECD, at a flow rate of approximately 40 ml/min. The temperature of the detector bases was set at 250 °C.

A small piece of deactivated uncoated fused silica (length, 50 cm; i.d., 0.10 mm) was used for transferring the analytes from the 8-port injection valve to the injector. The retention gap was an uncoated deactivated (medium polarity) fused silica column (length, 15 m; i.d., 0.53 mm). The retaining precolumn (length, 4m; i.d., 0.53 mm) was coated with chemically bonded CPSil 8 CB (film thickness, 5 µm), connected via a Y-shaped splitter to the 8-port valve. The precolumn in the MUSIC system (length, 10 m; i.d., 0.53 mm) was coated with chemically bonded CPSil 8 CB (film thickness, 5 µm). The analytical column for the analysis of SM and D_8-SM was a fused silica column (length, 33 m; i.d., 0.32 mm) coated with chemically bonded CPSil 19 CB (film thickness, 1µm). Small pieces of deactivated uncoated fused silica (i.d., 0.25 mm) were used as connecting material and trap intermediary in the MUSIC. A piece (length, 0.30 m; i.d., 0.25 mm) of deactivated uncoated fused silica column was mounted to the 8-port injection valve and used as a restriction capillary. All columns were purchased from Chrompack (Middelburg, The Netherlands). Connections between the various columns were made with glass press-fit connectors.

The HPLC pump operated at an ethyl acetate flow-rate of 185 µl/min. The injection volume of the autosampler was set at 400 µl. The dead volume between the autosampler and the end of the injection capillary was 57 µl. At a flow-rate of 185 µl/min, this dead volume was passed in 0.31 min. During this time period the solvent passed through the 8-port valve to the 'waste'. After this period the valve was switched to the injection position, upon which the waste was closed and the 'early solvent vapour exit' (ESVE) was opened. During injection, the ESVE was kept at 100 °C, to prevent condensation of the solvent in the valve. The solvent (ethyl acetate) evaporated and was vented, while the analytes were concentrated on the retaining precolumn. The time period for ESVE equalled the injection time of

2.26 min. A shorter time period could result in insufficient removal of the solvent, whereas a longer time period could result in loss of analyte. After the 2.26-min injection time, the valve switched back, opening the waste, and closing the ESVE. The HPLC pump stopped at time 3 min, awaiting the next injection.
After the ESVE step, the analytes were injected on the precolumn. The temperature programme of the GC was as follows: 70 °C for 8 min, heating to 140 °C at 10 °C/min, 140 °C for 15 min, cooling to 70 °C at 'infinite' rate. The pressure of the carrier gas was set at 184 kPa. The fraction containing SM and the deuterated internal standard were trapped in the cold trap at -70 °C. The time at which trapping had to start and end, which was operated by the MUSIC controller, was determined by evaluating the precolumn chromatogram for which a flame ionization detector (FID) was connected to the precolumn. Ideally, SM and D_8-SM are not resolved on the precolumn, enabling trapping of a narrow fraction of the precolumn chromatogram.
Next, the trapped analytes were reinjected onto the analytical column, by flash-heating of the cold trap. Both the trap and trap-base temperature were set at 180 °C. Carrier gas pressure for the analytical column was set at 150 kPa.
The detector base temperature was set at 300 °C, whereas the detector itself was kept at 310 °C. The nitrogen pressure was set at 110 kPa. The reference current was 1 mA, the pulse voltage was 5 V.

2.2.3 GLC configuration 3 (GC-MS)

The GC-MS configuration consisted of a Carlo Erba HRGC 5300 gas chromatograph equipped with an AS 550 autosampler and connected to a quadruple mass-spectrometer (Automass 150, Unicam).
MS detection was performed by positive electron impact ionization under full-scan conditions at m/z 50-200 for samples containing relatively high concentrations of SM, and under semi-single ion monitoring conditions, at m/z 109 for SM and 115 for D_8-SM for samples containing relatively low concentrations of SM.
The analytical column was CPSil 5 CBMS (length, 30 m; i.d., 0.25 mm; film thickness, 0.25 µm), connected to the injector via a retention gap of uncoated deactived fused silica (medium polarity; length, 2 m, i.d., 0.53 mm). Carrier gas (helium) pressure was set at 130 kPa. The oven temperature was programmed from 87 to 135 °C at a rate of 25 °C/min. The temperature was kept at 135 °C for 3 min, after which the oven was cooled to 87 °C for the next run. The interface temperature and the source temperature were set at 120 °C.

2.2.4 GLC configuration 4 (TDAS-GC-MS)

This configuration resembles GLC configuration 3, albeit that the AS550 autosampler was replaced with a thermodesorption autosampler (TDAS, Carlo Erba). The maximum capacity of the autosampler tray is 30 desorption tubes. The desorption tubes (length, 10 cm; i.d., 3 mm) were partly (about 30%) filled with Tenax. A glass-wool plug was firmly pushed on the top of the Tenax material and was fixed with a metal clamp. The tubes were preconditioned by heating under a stream of helium at 280 °C for at least 4 h. The sample (up to 500 µl), dissolved in

ethyl acetate, was brought onto the Tenax material in portions of 100 µl, using a 100-µl syringe. After each portion, the solvent was partially removed by leading a nitrogen flow of 80 ml/min through the tube for 1 min. After applying the last portion, the nitrogen flow was led through for 5 min. The tubes were placed in the autosampler tray. Residual ethyl acetate was removed just before desorption, by purging with helium for 999 s (when 500 µl sample was applied). Next, the sample was desorbed from Tenax by heating for 2 min at 210 °C. Meanwhile, the cold trap of the injection system (deactivated CPSil 8 CB; length, 1 m; i.d., 0.53 mm; film thickness, 5.25 µm) was kept at -60 °C with liquid nitrogen. Cooling was started before positioning of the tube which had to be desorbed. The temperatures of the TDAS valve and the interface between this valve and the desorption tube were kept at 140 °C. The analytes were reinjected from the cold trap via a temperature increase from -60 to 160 °C at infinite rate. This flash heating signal started the programme of the gas chromatograph.
After desorption, the tube was reconditioned for 1 min at 210 °C in the TDAS, and subsequently as described above.

2.3 Extraction of SM from blood

A known volume of blood was pipetted into a glass tube containing ethyl acetate to which a known concentration of D_8-SM was already added. This tube was kept in melting ice. Blood volumes of 0.1-2 ml were extracted with 1 ml of ethyl acetate, whereas 5-ml blood samples were extracted with 3 ml of ethyl acetate. The phases were mixed on a whirlmixer for 10 s, and then placed in an ultrasonic bath for 10 min. After that, the phases were separated by centrifugation. The ethyl acetate phases were transferred into glass vials and placed in the autosampler of either GLC configuration 1, 2 or 3, or were transferred onto Tenax for analysis with GLC configuration 4.

2.4 Calibration curves

Calibration curves were constructed relating the peak height of SM to that of the internal standard, D_8-SM. Separate curves were constructed for the SM concentration range from near the detection limit (approximately 10 pg/ml) up to 100 ng/ml, and for the range from 100 ng/ml up to 5 µg/ml.

2.5 Animal experiments

2.5.1 Animals

Male hairless guinea pigs [400-500 g; species identification Crl:IAF(HA)BR] were purchased from Charles River Wiga GmbH (Sulzfeld, Germany). The animals

were allowed to eat and drink *ad libitum*. They were allowed to acclimatize to their new environment for at least 1 week before they were used in any experiment. The protocols for the animal experiments were approved by the TNO Committee on Animal Care and Use.

2.5.2 Determination of the 96-h LD50 of SM after intravenous bolus injection

Five groups of 6 hairless guinea pigs were anesthetized with racemic ketamine (Vetalar®, 80 mg/kg i.m.). A small incision was made in the skin and some tissue was spliced in order to gain access to the jugular vein. Just before i.v. administration, a solution of SM in isopropanol (50 mg/ml) was diluted with saline in such a way that injection of 1 ml/kg body weight of this solution resulted in the required SM dose. After administration, the wound was closed with a few stitches. Survival times were assessed by housing the animals individually in a cage equipped with an ultrasonic device which detects respiratory movements. Twice a day for 4 days after SM administration, the animals were treated with the long-lasting analgesic buprenorfine (Temgesic®, 0.02 mg/kg s.c.). The animals had access to food and water *ad libitum*. The number of dead animals in each dose group was determined 96 h after SM administration, after which the LD50 was calculated via probit analysis (Litchfield and Wilcoxon, 1949).

2.5.3 Toxicokinetics of 1 LD50 SM (i.v.) in the anesthetized hairless guinea pig

Hairless guinea pigs were anesthetized as described above. A cannula was inserted into the carotid artery. The jugular vein was made accessible. A blood sample was taken from the carotid artery, after which a corresponding volume of saline was administered via the same cannula. A dose of SM corresponding to 1 LD50 (96-h, i.v.) was injected into the jugular vein (injection volume 1 ml/kg). Blood samples were taken at several time-points up to 6 h after administration. Throughout the experiment, the animals were kept under anesthesia. After taking the final blood sample, the animals were sacrificed with an overdose of Nembutal®, and various tissues were sampled for analysis of SM and its major DNA-adduct.

2.6 Curve-fitting of toxicokinetic data

Curve-fitting of the measured concentration-time courses was performed by non-linear regression with the BMDP-3R program (University of California, Los Angeles, CA, USA) on a personal computer, as decribed previously (Benschop and De Jong, 1990).
The data were fitted to a bi- and a triexponential equation:

$$[SM]_t = A*e^{-\alpha t} + B*e^{-\beta t} \quad (1)$$

$$[SM]_t = A*e^{-\alpha t} + B*e^{-\beta t} + C*e^{-\gamma t} \quad (2)$$

by calculation of the parameters A, B, C, α, β, and γ. In these equations, $[SM]_t$ is the blood concentration of SM at time t. The F-ratio test (Boxenbaum *et al.* 1974) was used to determine whether the data were significantly better described with a triexponential equation than with a biexponential equation.
Several toxicokinetic parameters were calculated.

The area under the curve (AUC)

$$AUC = A/\alpha + B/\beta \quad (3)$$

The total body clearance (Cl)

$$Cl = Dose/AUC \quad (4)$$

The rate constant of transport from compartment 2 to compartment 1 ($k_{2,1}$)

$$k_{2,1} = (A*\beta + B*\alpha)/(A + B) \quad (5)$$

The rate constant of total body clearance (k_{el})

$$k_{el} = \alpha*\beta/k_{2,1} \quad (6)$$

The rate constant of transport from compartment 1 to compartment 2 ($k_{1,2}$)

$$k_{1,2} = \alpha + \beta - k_{2,1} - k_{el} \quad (7)$$

The blood concentration at time 0 (C_0)

$$C_0 = A + B \quad (8)$$

The half-life of distribution ($t_{½,dis}$)

$$t_{½,dis} = ln\ 2/\alpha \quad (9)$$

The terminal half-life ($t_{½,el}$)

$$t_{½,el} = ln\ 2/\beta \quad (10)$$

The volume of the central compartment (V_1)

$$V_1 = Dose/C_0 \quad (11)$$

The distribution volume at steady state (V_{dss})

$$V_{dss} = V_1(1 + k_{1,2}/k_{2,1}) \quad (12)$$

The mean residence time (MRT)

$$MRT = (A*(1/\alpha)^2 + B*(1/\beta)^2)/AUC \quad (13)$$

2.7 Isolation of DNA from various tissues

The following protocol concerns the processing of 1 g of liver tissue.

Frozen liver was homogenized with a Potter homogenizer in 0.25 M sucrose, 0.1 M EDTA, pH 7.4. Nuclei were isolated by centrifugation for 10 min at 3,000 rpm. The nuclear pellet was washed with the above-mentioned buffer and was resuspended in 5 ml sucrose, 25 mM EDTA, 1% Triton, pH 7.4, and incubated for 40 min at 4 °C. The chromatin was isolated by centrifugation for 10 min at 3,000 rpm and washed three times with 10 mM Tris.HCl, pH 7.4. Next, the pellet was resuspended in 2.5 ml TEN buffer (20 mM Tris.HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl).
The protein components of the chromatin were degraded by addition of 2.5 ml TEN-buffer, containing 1% (w/v) sodium dodecylsulphate (SDS) and 100 µg Proteinase K/ml, and overnight incubation at 37 °C. Next, protein was removed by three successive extractions with 5 ml of phenol (saturated with Tris.HCl, pH 8.0), phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v), and chloroform/isoamyl alcohol (24:1, v/v), respectively. The nucleic acids in the aqueous phase were precipitated by addition of 2.5 volumes of cold absolute ethanol (-20 °C). The precipitate was washed twice with 70% ethanol, dried *in vacuo*, and finally dissolved in 5 ml of 10 mM Tris.HCl, pH 7.4, 1 mM EDTA (TE-buffer).
RNA was degraded by addition of RNAse A (75 µg/ml) and RNAse T1 (75 U/ml) and incubation at 37 °C for 2 h. After this incubation, 500 µl of a 10 times more concentrated TEN-buffer and 500 µl of 10% SDS were added. Next, the same series of extractions as described above was repeated in order to remove enzymes and degraded RNA. The purified DNA was precipitated, washed with 70% ethanol, dried, and dissolved in 1 ml TE-buffer.
The DNA concentration was determined from the absorbance at 260 nm, on the assumption that the A_{260} of a 1 mg/ml solution of native DNA is 20. The overall purity of DNA was verified by determination of the A_{260}/A_{280} and the A_{260}/A_{230} ratios of the DNA solution.
Isolation of DNA from other organs and epithelial tissues was carried out according to the procedure described above, except that the separate isolation of cell nuclei was omitted. After homogenization of the tissue, the isolation procedure is started with Proteinase K and SDS treatments (*vide supra*).
The yield of DNA was usually 1-1.5 mg per gram tissue.

2.8 Immuno-slot-blot assay of sulphur mustard adducts to DNA

In the immunoslotblot assay (ISB, Nehls *et al.*, 1984), the DNA solution of 50 µg/ml, was first heated at 52 °C for 15 min in TE-buffer containing 4.1% formamide and 0.1% formaldehyde and subsequently 10-fold diluted in PBS (0.14 M sodium chloride, 2.6 mM potassium chloride, 8.1 mM disodium hydrogen phosphate and 15 mM potassium dihydrogen phosphate, pH 7.4). Then, one hundred µl of this DNA solution were spotted on a nitrocellulose filter (pore size 0.1 mm; Schleicher and Schuell). After washing with PBS, the DNA was adsorbed to the filter by drying for 15 min at 37 °C. Next, the filter was treated with 5% milk powder (Campina, Eindhoven, The Netherlands; less than 1% fat) in PBS contain-

ing 0.1% Tween 20, under continuous shaking at room temperature for 30 min in order to prevent nonspecific antibody binding. After washing, the filters were treated and incubated with N7-SM-guanine monoadduct-specific monoclonal (2F8) antibody (1:500 dilution of culture supernatant in PBS containing 0.1% Tween 20 and 0.5% milk powder) at 4 °C overnight. After washing with PBS containing 0.1% Tween 20, the filters were incubated with conjugated second antibody (rabbit-anti-mouse-Ig-horse radish peroxidase, diluted 1:1000 in PBS containing 0.1% Tween 20 and 0.5% milkpowder). The filters were incubated for 2 h at room temperature. After a final washing step, peroxidase activity was revealed with a Boehringer enhanced chemiluminescence blotting detection system. Peroxidase labelled antibodies catalyze the oxidation of luminol resulting in emission of light in the presence of H_2O_2 (two solutions supplied by the manufacturer, added in a mixture 100:1). After 1 min, the filters were transferred to cassettes with photographic film (Hyperfilm ECL). After 5 min, the films were exposed to the filters for 10 -120 s. The signal was quantified by scanning the developed film with an Ultroscan XL densitometer (Pharmacia, Uppsala, Sweden).
The lower detection limit of the ISB assay was reached when 0.5 µg DNA was spotted on the filter (DNA exposed in its double-stranded form in solution for 1 h to 1 nM SM at 37 °C, and subsequently made single-stranded according to the abovementioned procedure). The adduct level at the detection limit is 1 modified guanine amongst $2x10^7$ nucleotides (based on calibration with HPLC with electrochemical detection (Benschop and Van der Schans, 1995)). This corresponds to 0.08 fmol of adduct per spot of 0.5 µg DNA. Since 1 g tissue yields 1-1.5 mg DNA, the lower detection limit can also be expressed as 0.1-0.15 pmol adduct/g tissue.

3 Results and discussion

3.1 Gas chromatographic analysis of sulphur mustard in biological materials

3.1.1 Extraction of sulphur mustard from blood and tissue samples

Based on our experience with the gas chromatographic trace analysis of nerve agents in biological materials and on the preliminary reports of Maisonneuve *et al.* (1993) on the gas chromatographic analysis of SM in rat blood, it was checked whether SM could be extracted with ethyl acetate from guinea pig blood samples stabilized with 18% aqueous sodium chloride solution. Extraction recoveries from blood should be as high as 90%, whereas the limit of detection with gas chromatography with FID should be approximately 3 ng/ml blood (Maisonneuve *et al.* 1993).

Once these results were reproduced, the extraction procedure needed to be optimized with respect to several aspects. First of all, the stabilization procedure for SM in blood is essential to obtain valid data. Due to the high reactivity of SM towards the biological matrix, this aspect required special attention. The influence of parameters such as pH, temperature, concentration of chloride ions and incubation time were studied. Furthermore, the extraction of SM from various tissues, i.e. fat, bone marrow, spleen, liver, lung and dorsal skin, was studied using the same procedure as for blood, for which some adjustments to the procedure might be necessary.

Since the reported recovery from liquid-liquid extraction is high, no significant increase in sensitivity could be gained by further optimizing this step in the analysis. It remained worthwhile, however, to study whether solid-liquid extraction with C_{18}- or other cartridges would be more convenient, with respect to the removal of contaminants from the samples or time consumption, for example.

The extraction of sulphur mustard (SM) was studied using gas chromatographic configuration 1. On this configuration, SM and deuterated SM (D_8-SM), which was used as the internal standard in the extraction procedure, were completely resolved. The absolute detection limit for SM appeared to be approximately 400 pg. The calibration curve of SM appeared to be linear up to a concentration of 3.5 µg/ml. The within-day variability for this system, based on SM peak height, appeared to be 4.8%, the between-day variability 11%.

A long-term study of the stability of SM and D_8-SM in ethyl acetate was started in January 1995. Solutions in ethyl acetate containing respectively 171 ng D_8-SM and 250 ng SM per ml, 16 µg D_8-SM and 25 µg SM per ml, and 34.2 ng D_8-SM/ml were prepared. One portion of each of these solutions was kept at room temperature, another portion at -20 °C. Once every two weeks the concentration of each of the solutions was determined with GLC configuration 1. Some variation in the

concentration was observed with time, but there are no indications of the degradation of SM or D_8-SM in any of the solutions. However, in a solution of SM in saline of 100 µg/ml, SM appeared to degrade at room temperature (ca. 22 °C) with a half-life of 26 min. In melting ice (approximately 4 °C), degradation proceeds about ten times more slowly. Therefore, it was decided to perform extractions from aqueous solutions such as saline and blood at 4 °C.

SM and D_8-SM (ca. 250 µg/ml) were spiked into saline and extracted with cold (4 °C) ethyl acetate in 1:1 volume ratio. The phases were mixed on a whirlmixer for 10 s and then placed in an ultrasonic bath for 10 min. Next, the phases were separated by centrifugation. Ultrasonication appeared to improve the extraction recovery. With a 1-min ultrasonication, the absolute recovery of SM was approximately 60%, which increased up to 91% for a 10-min ultrasonication. Extending the duration of the ultrasonication beyond the 10-min period did not improve the extraction recovery. In fact, the temperature of the sample increases with the ultrasonication duration, which may accelerate degradation of SM in the sample.

The extraction recovery was not significantly improved by increasing the saline:ethyl acetate phase ratio from 1:1 to 1:5. However, decreasing the saline:ethyl acetate ratio down to 5:1 had a detrimental effect on the recovery. The lowest phase ratio which can be used for an acceptable recovery is 3:2. Under all extraction conditions, the recovery relative to D_8-SM was 99%, which indicates that D_8-SM is indeed a suitable internal standard for SM.

Solid phase extraction of SM from saline by elution with ethyl acetate from a SepPak C_{18} cartridge appeared to lead to a recovery of approximately 70%. Of the recovered amount of SM, 97% was eluted in the first ml of ethyl acetate. Again, the recovery relative to D_8-SM was near 100%.

Extraction of SM from guinea pig blood was performed by mixing on a whirlmixer followed by ultrasonication and centrifugation as described above. The absolute recovery of SM was 86 ± 4% (n=6) at the 500-ng SM/ml blood level, and 91 ± 4% (n=6) at the 1-ng/ml level, whereas the recovery relative to D_8-SM was 99 ± 3%.

Validation of the analytical procedure in blood samples was performed in the concentration range from approximately 10 pg/ml up to 5 µg/ml. However, thorough validation at the lowest concentrations was hampered by problems with GLC configuration 3. A complete validation report for the concentration range of 10 pg/ml up to 5 µg/ml for n=6 will be presented in the Final Report.

With the SepPak C_{18} solid phase extraction the recovery of SM was in the range of 50-60%. From the appearance of the chromatograms it was concluded that solid phase extraction does not provide a higher selectivity than liquid-liquid extraction. Furthermore, the solid phase extraction procedure is more labour intensive, and we therefore preferred to continue with the liquid-liquid extraction procedure.

We intend to perform the extraction in the actual toxicokinetic experiments by pipetting the blood sample into a tube containing ethyl acetate to which D_8-SM is already added. We verified that the final concentration of D_8-SM in the ethyl acetate phase after this procedure was the same as when D_8-SM was first added to the blood sample and subsequently extracted.

After ultrasonication, the samples do not need to be centrifuged immediately. When the samples were kept in melting ice, no degradation of SM and/or D_8-SM was observed up to 1 h. This is a useful finding, since it will be more convenient to gather several samples for centrifugation when performing a toxicokinetic experiment. Usually, the sonicated samples will be centrifuged within 30 min.
Under these extraction conditions, the concentration of chloride ions, in the range of 0.17 up to 3.4 M, did not significantly influence the recovery of SM, which was spiked at a concentration of 156 ng/ml blood. This seems to be in contradiction with findings of Maisonneuve *et al.* (1993) and others, but one must realize that our method is based on immediate extraction of the blood sample. Likewise, we are confident that we will be able to extract the blood sample immediately after drawing it from the animal in the toxicokinetic experiments.
The influence of the pH on the extraction recovery from blood was studied with a concentration of 156 ng SM/ml blood in the pH range of 5 to 7.5. In this pH range, the absolute extraction recovery remained unaltered at 92 ± 7%, whereas at lower or higher pH the recovery appeared to decrease, most likely due to degradation of SM. Since the physiological pH is approximately 7.4, no problems with respect to the stability are anticipated with our extraction method.
After addition to homogenates of lung, liver, fat, spleen and bone marrow at a concentration of approximately 0.5 µg/g homogenate, SM was extracted with recoveries (± s.d., n=3) of 87 ± 5, 95 ± 4, 101 ± 4, 98 ± 4, and 98 ± 9%, respectively. These recoveries are comparable to the value found for extraction from blood. Obviously the tissues containing SM have to be homogenized prior to extraction in the actual experiments, which will take some time since they cannot all be processed at the same time. Depending on how long processing will take, loss of SM due to hydrolysis or binding in the tissue can be expected. Freeze-drying of the tissue samples appeared to be unsuitable, as this procedure leads to a considerable loss of SM. The most promising approach seems to be to freeze the tissue samples in liquid nitrogen until processing, which we intend to perform by homogenizing directly with ethyl acetate. Whether this is actually feasible remains to be studied.
Furthermore, we have confirmed that SM is not formed from thiodiglycol under the extraction conditions used.

3.1.2 Comparison of gas chromatographic detectors for sulphur mustard

Only a few papers have been published concerning the analysis of intact SM in biological matrices. In nearly all reported methods, gas chromatography is used to analyze SM. This is a logical choice in view of the volatility of SM, the versatility of gas chromatography in general, and because of the combination of selectivity with superior detection limits, especially in the case of two-dimensional chromatography. The reported detection systems are flame-ionization detection (FID; Maisonneuve *et al.*, 1993), flame photometric detection (FPD; Zhang and Wu, 1987), electron capture detection (ECD; Heyndrickx *et al.*, 1984) and mass-spectrometric detection (MS; Vycudilik, 1985; Vycudilik, 1987, Drasch *et al.*, 1987).

For detection of sulphur-containing compounds, a flame photometric detector (FPD) is mostly first choice, as it combines ease of operation with a high selectivity and a detection limit of approximately $5*10^{-12}$ g S/s, corresponding with a detection limit of approximately 45 pg of SM at a peak width at half the peak height of 2 s (Degenhardt, 1992). With this configuration, in combination with the high extraction efficiencies, we anticipated a detection limit of 45 pg SM/ml blood or even somewhat better. However, preliminary experiments with electron capture detection (ECD) suggested that a detection limit of 5 pg SM/ml blood or g tissue could be obtained (Degenhardt, 1992). Although ECD tends to be less stable and reliable than FPD in routine analysis of biological samples due to contamination by matrix components, we were confident that such problems can be avoided by using two-dimensional chromatography. In such a configuration, the fraction of the sample that is injected onto the analytical column and actually reaches the detector is practically devoid of contaminants.

The limit of detection of SM with ECD appeared to be approximately 10 pg, which was somewhat disappointing. A calibration curve in the range of 10 ng/ml up to 150 µg/ml could be described with a second order polynoma. Increasing the reference current of the ECD from 1 to 5 mA in steps of 1 mA resulted in an increase of the signal offset which reduced the sensitivity of detection. Therefore, the reference current was set at 1 mA. The same effect was observed upon lowering the pulse voltage from 5 V to 1 V. Increasing the nitrogen flow of the ECD reduced the offset, but did not improve the sensitivity.

The sensitivity of ECD was compared with sulphur chemiluminescence detection (SCD), FPD and mass spectrometric detection (MS).

The comparison between ECD and SCD was performed at the Technical University of Eindhoven on a straightforward GLC configuration with a CPSil 5 column. A solution of SM in hexane was injected into the system. The SCD was a Sievers Model 350 B. On the basis of the manufacturer's specification a detection limit of 15 pg of sulphur was calculated, which corresponds with approximately 75 pg of SM. The actually measured detection limit appeared to be 78 pg of SM (S/N=3), which is almost equal to the specified value. The detection limit with ECD was 17 pg under comparable conditions. Therefore, SCD is not a suitable alternative for ECD in terms of sensitivity. However, SCD appeared to offer a better selectivity than ECD. Obviously, since only standard solutions of SM were injected, the difference in selectivity towards biological samples is unknown.

FPD appeared to be more selective, but at least one order of magnitude less sensitive than ECD. Therefore, FPD is not an attractive substitute for ECD. Recently, the pulsed flame photometric detector (PFPD) has been introduced (Amirav and Jing, 1995). The detection limit for sulphur with this new detector is reported to be 180 fg/s, which can even be improved to 30 fg/s with a sulphur doping method. The PFPD is reported to be more selective than FPD. Furthermore, the linear dynamic range of the PFPD is increased up to one order of magnitude in comparison with FPD. The PFPD is now commercially available. We intend to test this detector with respect to selectivity and sensitivity for SM in an extract from blood.

Next, the detection limit of SM was studied with GC-MS. The stationary phase was DB5-MS (length, 30 m; i.d., 0.25 mm; film thickness, 0.25 µm). The oven was heated from 80 °C to 135 °C with a rate of 10 °C/min. The temperature of the interface between the GC and the MS was set at 280 °C and the source temperature at 120 °C. MS-detection was performed by positive electron impact ionization under semi-single ion monitoring conditions, at m/z 109 for SM and 115 for D_8-SM (detector gain 0.78 kV, filament current 0.25 mA, pressure 0.43 Torr). The signal-to-noise ratio of injection of 3.9 pg of SM was 20/1, which corresponds to a detection limit of approximately 700 fg at S/N=3. Therefore, MS is the most sensitive detection principle tested so far.

3.1.3 Two-dimensional gas chromatography with large volume injection onto an on-column interface

Originally, we intended to use Thermal Cold Trap (TCT) injection as a means of large volume injection into the chromatographic system, since this proved to be a convenient and reliable technique in our previous studies on the toxicokinetics of soman and sarin (Benschop and Van Helden, 1993). In the course of those studies, we purchased a TCT autosampler which reduced the labour-intensity of the method in comparison with manual TCT injection. Nevertheless, the extraction solvent had to be transferred onto the Tenax adsorbent. Furthermore, in the nerve agent studies, a volume of approximately 5 ml of ethyl acetate had to be reduced to approximately 500 µl by evaporation, which was about the maximum volume that could be applied onto the Tenax. Therefore, the method remained rather time-consuming. We tried to circumvent this problem at an earlier stage by using automated large volume on-column injection into the two-dimensional chromatographic system. Unfortunately, this technique appeared to be most unreliable and led to unreproducible results, and was abandoned in favour of TCT injection (Benschop and Van Helden, 1993). The problems with the large volume on-column injection were the result of the high sensitivity of the injection to small variations in gas flow-rates and pressures in the system which were inevitable with that configuration. However, we had been able to solve these problems to such an extent that we were confident that this technique could be applied routinely for the purposes of the current study.

The newly developed configuration is shown in Figure 1. It comprises an HPLC autosampler, an HPLC pump and a gas chromatograph arranged for two-dimensional chromatography. Between the 'LC' and 'GC' parts of the configuration, an 8-port valve is interconnected. The system is fully automated and is controlled by the autosampler and HPLC pump. At the moment of injection by the autosampler, the time programme of the HPLC pump is started, which in its turn controls the position of the valve as well as the time programme of the gas chromatograph. The flow from the HPLC pump transports the sample via the valve into the retention gap in the GC. This interface is set-up for Early Solvent Vapor Exit (ESVE), which is meant to vent the bulk of the solvent while the compounds of interest are being concentrated on a so-called retaining pre-column. Next, the analytes enter the precolumn of the two-dimensional system, after which the

fraction of interest is trapped and subsequently reinjected onto the analytical column. Initially, detection was performed with a flame ionization detector (FID). The various steps in the process were subsequently optimized.

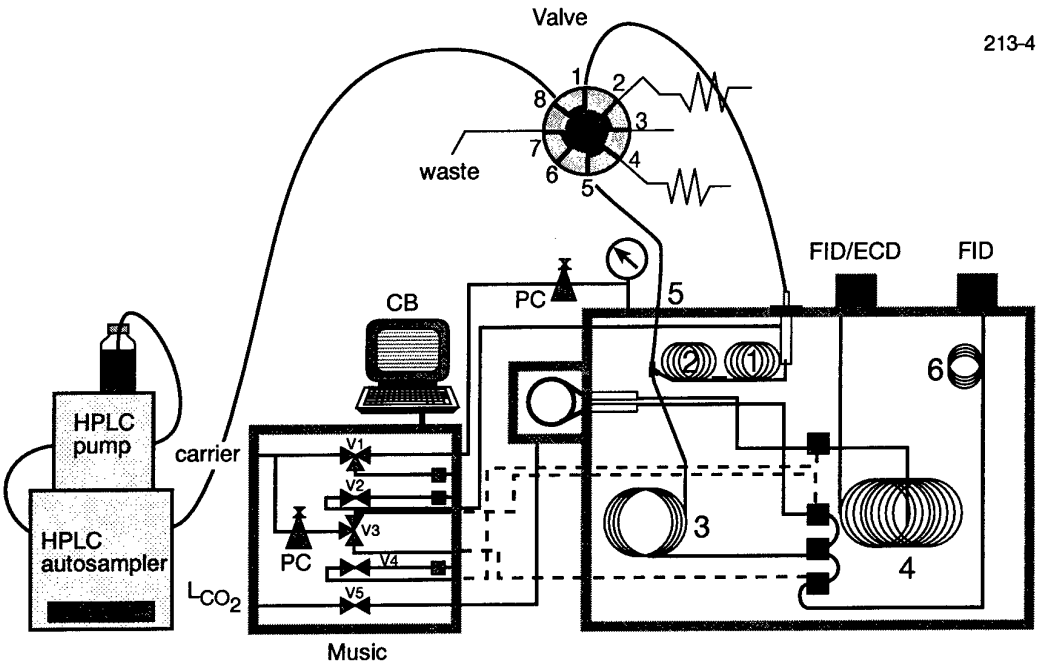


Figure 1: *Schematic representation of the automated large volume GC-GC system (GLC configuration 2). (Valve), 8-port injection valve; (MUSIC), Multiple Switching Intelligent Controller; (PC), constant pressure valve; (V1-V5), valves; (CB), control box of the MUSIC; (1), retention gap; (2), retaining pre-column; (3), pre-column; (4), analytical column; (5), early solvent vapour exit; and (6), restriction capillary. FID and ECD were used as detectors. The FID connected to the restriction capillary served as a monitor detector.*

Optimization of the MUSIC system

The original MUSIC system was equipped with a flow regulator for the injection onto the wide bore pre-column, and a pressure regulator for the analytical column. The interdependence of the carrier gases of such a combined column system is evident. For our newly developed system, we exchanged the flow regulator for a pressure regulator, which allows a rapidly responding carrier adjustment during injection. An additional valve had to be mounted just before this regulator so that the back-flush capabilities of the MUSIC system remained available. Next, a retention gap was connected in between the pre-column and the injector to prepare the system for large volume injection. The system was tested with on-column injection of a standard solution of SM and D_8-SM in ethyl acetate to study the retention behaviour of the analytes on the combined pre-column combination as well as on the analytical column, see Figure 2. Optimum conditions were reached when the injection pressure (p_I) was set to 120 kPa and the pressure on the analytical column (p_A) to 78 kPa. The oven temperature was kept at 87 °C for 1 min, subsequently heated at a rate of 20 °C/min up to 158 °C, and kept constant at this

temperature for 15 min. The fraction eluting between 5.2 and 5.7 min was trapped at -70 °C in the cold trap and after 1.3 min reinjected onto the analytical column by flash-heating the cold trap to 180 °C. Detection was performed with a FID. Figure 2 shows the pre-column and analytical column chromatograms for these conditions. A considerable tailing of the solvent peak was observed, which is probably due to the 'dead' volume in the connecting materials and the dimensions of the injector, such as the internal diameter.

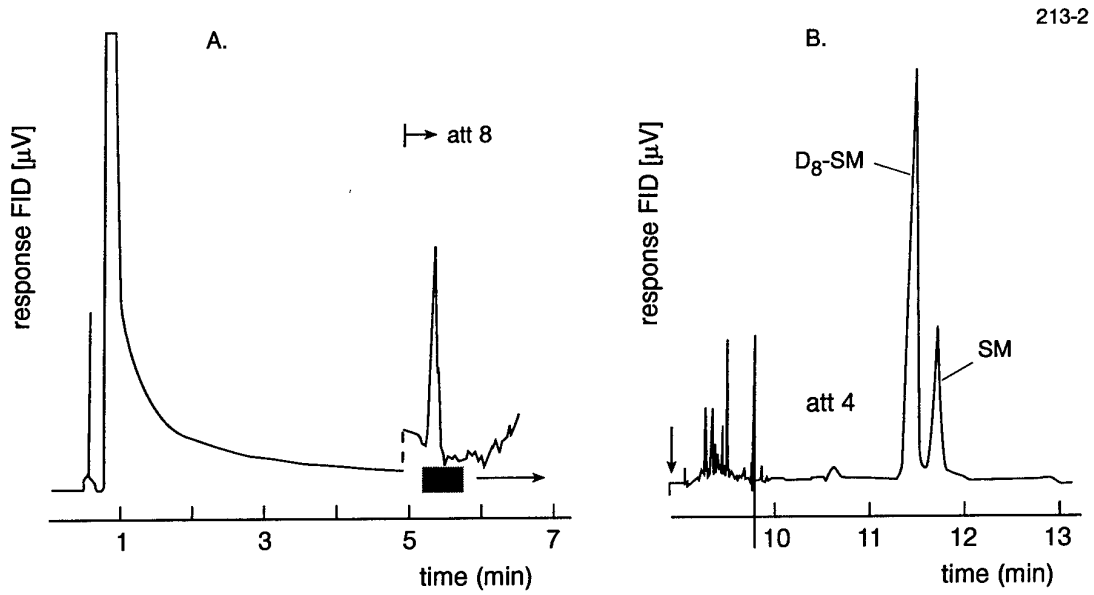


Figure 2: *Pre-column (A) and analytical column (B) chromatograms after an on-column injection of 1 µl of a standard solution of sulphur mustard (SM) and deuterated sulphur mustard (D_8-SM) in ethyl acetate (0.75 and 1.0 µg/ml, respectively) on GLC configuration 2.*

Optimization of the large volume injection

Large volume on-column injection onto a gas chromatographic column has been described extensively (Grob et al., 1984, 1985; Munari et al., 1985; Vreuls et al., 1990). However, large volume injection onto a two-dimensional gas chromatographic system is not a common feature. Mol et al. (1993) showed the possibilities of the PTV injector as an interface. Chappel et al. (1993) described a system in which an on-column interface (OCI) was used for connecting an HPLC to a multidimensional gas chromatographic system. The columns were placed in series. The analytes were cryofocused in a cold trap after monitoring the first column by using a split interface and a related detector.

We investigated the possibilities for large volume injection using the OCI in combination with the MUSIC system for the analysis of SM and D_8-SM.

SM and D_8-SM are medium boiling compounds in comparison with the solvent, therefore partially concurrent solvent evaporation (PCSE) conditions, as described by Munari et al. (1985), are most suitable for large volume injection. Furthermore, the interdependence of the column flows would not allow fully concurrent solvent evaporation. The large amount of evaporated solvent leads to 'backshooting' of the

introduced compounds into the carrier tubing. Under PCSE conditions, the large volume injection leads to a reconcentration of the analytes in the remaining solvent, which forms a liquid layer in the retention gap. A number of parameters had to be optimized, such as injection pressure on the column, injection speed, evaporation speed, and injection temperature.
The injection pressure was set to 122 kPa and the oven temperature was kept constant at 70 °C during injection. Under these conditions, the 'flooded' zone of the retention gap was estimated to be 10 - 11 cm/µl and the evaporation speed was calculated to be 27 µl/min when injecting at a speed of 40 µl/min for 10 min. After injection, the temperature of the oven was increased to 80 °C for 16 min, in which time period the compounds elute from the retention gap and the pre-column. The fraction between 24.4 and 25.4 min was trapped at -70 °C in the cold trap, and after heating the oven to 158 °C, the trap was subsequently flash heated to 180 °C upon which the analytes were reinjected (p_A = 78 kPa) onto the analytical column. Detection was performed with an FID, which resulted in a detection limit of 2 ng/ml of SM. Figure 3 shows the chromatograms obtained under these conditions.

Optimization of the Early Solvent Vapor Exit (ESVE)
Due to tailing of the solvent peak on the pre-column, a large amount of solvent was trapped in the cold trap of the MUSIC system. This resulted in the introduction of a large amount of solvent on the analytical column, which had an adverse effect when using a sensitive detector such as an electron capture detector or a mass spectrometric detector. If a non-chemically bonded column should be used, the result may even be disastrous, by stripping the phase from the column. The ESVE (Grob et al., 1989) in combination with a retaining pre-column is intended to remove most of the solvent before entering the analytical column, without loss of analyte. After installing the ESVE in our MUSIC sytem (see Figure 4), the optimum flow conditions changed dramatically in comparison with the conditions as described above.
Due to the pressure balance in the system, a steady-state situation was established. However, by opening the ESVE valve, this balance was severely distorted. The pressure drop between the top of the injector and the point to which the ESVE was connected, was increased from approximately 20 kPa to more than 100 kPa. Under these conditions, maintaining a constant evaporation speed and a liquid layer in the retention gap for the solvent effect is rather difficult. The conditions for injecting a sample of 400 µl were optimized. In order to maintain a flooded zone, which is necessary for partially concurrent solvent evaporation, the injection speed was increased from 40 to 185 µl/min. Under these conditions, the evaporation speed was estimated to be 175 µl/min. The injection pressure for the pre-column was 182 kPa and the injection pressure for the analytical column 150 kPa; the oven temperature was set to 70 °C for 8 min and subsequently heated at a rate of 10 °C/min to 140 °C and kept constant for 12 min. The ESVE valve was opened during the 10 min of injection and subsequently closed after introduction of the last amount of sample. Obviously, the evaporation speed changes after closing this

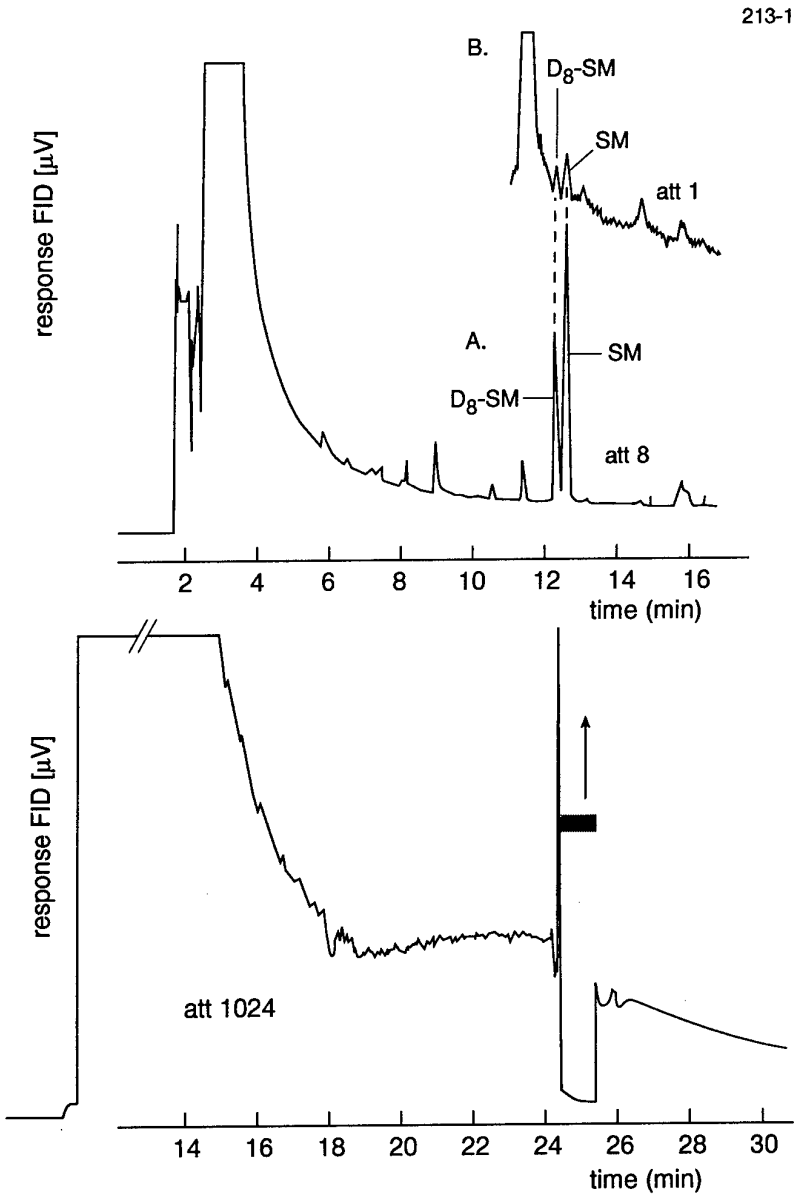


Figure 3: *Chromatograms of a 400-µl on-column injection of a standard solution of SM and D_8-SM on GLC configuration 2. At the bottom, the pre-column chromatogram is shown. The shaded area designates the fraction which was trapped and reinjected onto the analytical column. The top chromatograms show (A) a standard of SM and D_8-SM and insert (B), concentrations of SM and D_8-SM near the detection limit.*

valve. Due to the back pressure of the MUSIC system (p_A = 150 kPa), the injection speed setting is critical. Next, the 22-m retention gap was reduced to 10 m. The fraction between 17.3 and 18.5 min was trapped in the MUSIC cold trap at -70 °C and after flow adjusting, flash-heated to 180 °C for reinjection onto the analytical column. Next, this configuration was combined with electron capture detection

(ECD), in order to attain the required sensitivity. In this configuration, we anticipated that the ECD could function routinely, since the sample reaching the detector is relatively clean due to ESVE and the two-dimensional chromatography. The detection limit for SM was 10 pg for an injection of a 400-µl sample. Consequently, the limit of detection corresponds with 25 pg SM/ml blood without concentrating the ethyl acetate extract, which is a factor of 5 higher than the limit we aimed at. However, this might be sufficient for the purpose of our study. The within-day variability of the LV-GC-GC-ECD system appeared to be 2.8% for SM (based on peak height), 6.5% for D_8-SM, and 4% for the SM/D_8-SM peak height ratio.

A cause for concern was the lack of reliability of the LV-GC-GC-ECD configuration in routine use. For no apparent reason, no SM could be detected in some standard samples. Furthermore, the selectivity of the ECD in this configuration was not adequate: many small peaks were observed in the chromatograms, which made detection of SM at very low concentrations very difficult, in particular since the retention times of SM and D_8-SM were not very reproducible. A considerable effort was needed to keep the configuration going, which left hardly any time for analysis of samples. Therefore, it was decided not to continue along this line and to try another approach.

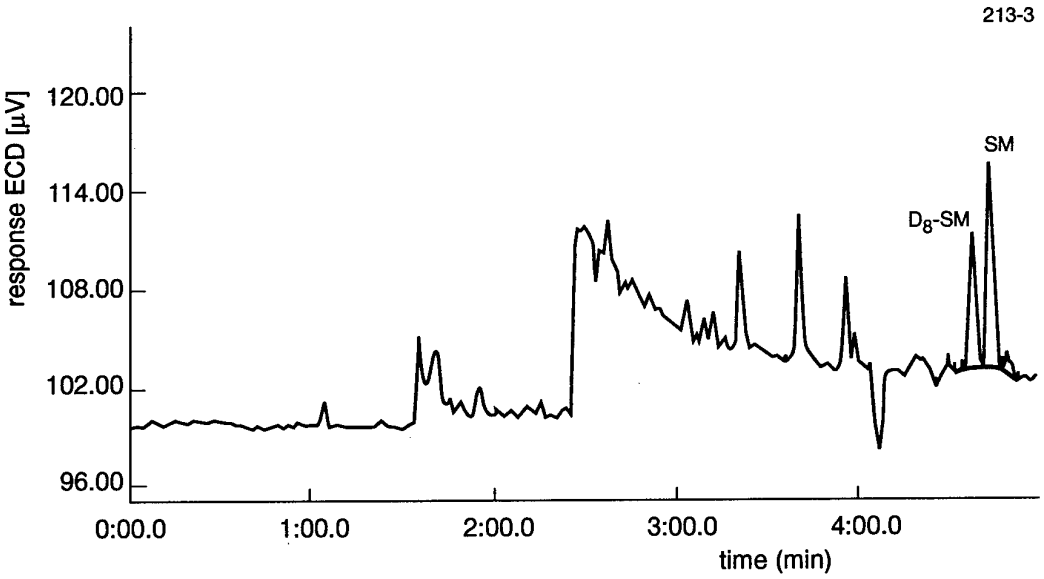


Figure 4: Detection of SM and D_8-SM in a 3-ml extracted blood sample, by on-column injection of 400 µl of ethyl acetate extract on GLC configuration 2. Notice the absence of a solvent peak.

3.1.4 Gas chromatography with mass spectrometric detection

In view of the high sensitivity of mass spectrometric detection for SM, GC-MS seemed a promising approach for bioanalysis of SM. We chose not to make the configuration too complex in order to avoid technical problems as much as possible. By using an on-column autosampler, the daily sample throughput is increased

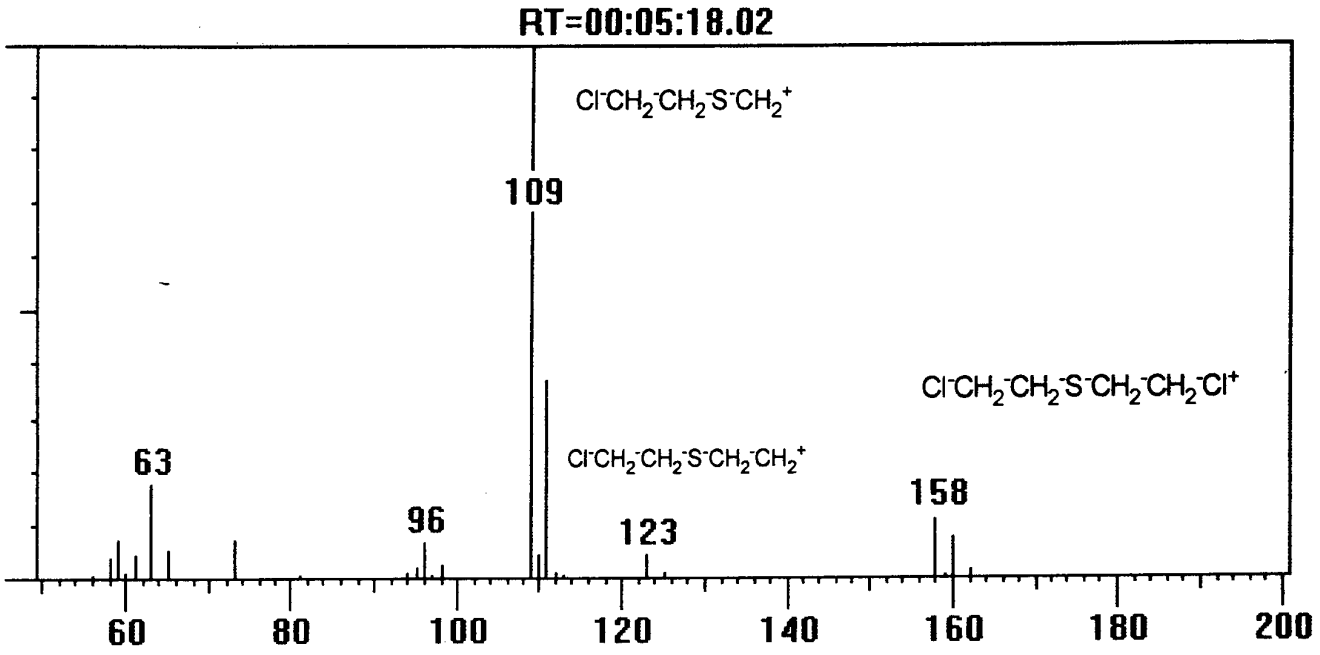
considerably. This autosampler has been used extensively in previous studies on the elimination pathways of C(±)P(±)-soman (Benschop and De Jong, 1990), and appears to be most reliable. Only 1-3 µl of sample can be injected in this way. This may be considered a drawback of the method, since the extract is used in a very uneconomical way. However, the high sensitivity of the detection compensates for the small volume injection to such an extent that the required detection limit of approximately 5 pg SM/ml blood is within reach. Since we did not have experience with multidimensional chromatography in combination with MS detection, we decided not to spend time on developing such a combination. Due to the high selectivity of MS detection, multi-dimensional chromatography is not deemed necessary. However, there is a considerable risk of contamination of the system without multi-dimensional chromatography.

Mass spectra of SM and D_8-SM are shown in Figures 5a and b. It is obvious that mass-fragment 109.1, which is $ClCH_2CH_2SCH_2^+$, is the most abundant fragment and therefore allows the most sensitive analysis.

An example of a GC-MS chromatogram of an extract of blood is shown in Figure 6. This figure shows that under these chromatographic conditions SM and D_8-SM are resolved, which is not necessary for MS detection. Furthermore, the figure demonstrates the high selectivity of the MS detection. The high selectivity in the TIC-chromatogram is the result of a rather narrow mass range of m/z 50-200. This range is scanned in 300 ms. Total analysis time is 4.5 min, which allows a high daily sample throughput. At 1.1 min after injection of the sample, the filament is switched on. The bulk of the solvent has passed the detector by that time. In this way contamination of the detector is limited. Detection is started at 1.5 min after injection, which explains that there is no real injection peak in the chromatograms shown in Figure 6. When the concentrations of the analytes are too low for a full-scan of the aforementioned m/z range, detection of the major mass fragments 109 and 115 is chosen.

With some additional tuning of the mass spectrometric detection, the absolute detection limit was improved further from 700 fg down to approximately 100 fg. For a 3-µl injection, this corresponds to a detection limit of 33 pg SM/ml. The desired detection limit for SM in blood samples of 5 pg/ml can only be reached with this method, if the ethyl acetate extract is concentrated approximately sixfold under reduced pressure.

On the basis of the toxicokinetic pilot experiments, it was anticipated that for most of the samples obtained in the toxicokinetic experiments, such a low detection limit would not be required, since at 4 h after intravenous administration of a dose of SM corresponding to 1 LD50, the concentration of SM in blood was still around 1 ng/ml. However, volume reduction of the ethyl acetate extract will be necessary for samples taken at time points later than 4 h, or if lower doses of SM are administered.



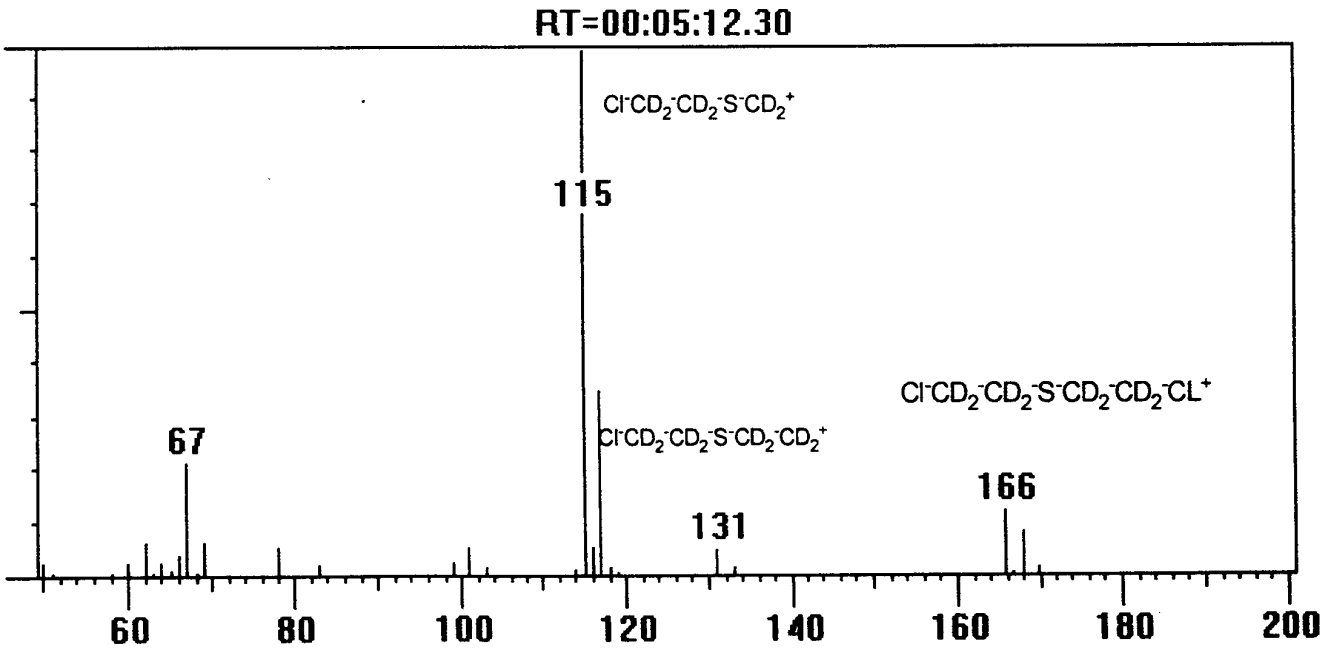


Figure 5: Mass spectra (ei) of (a) sulphur mustard, and (b) D_8-sulphur mustard.

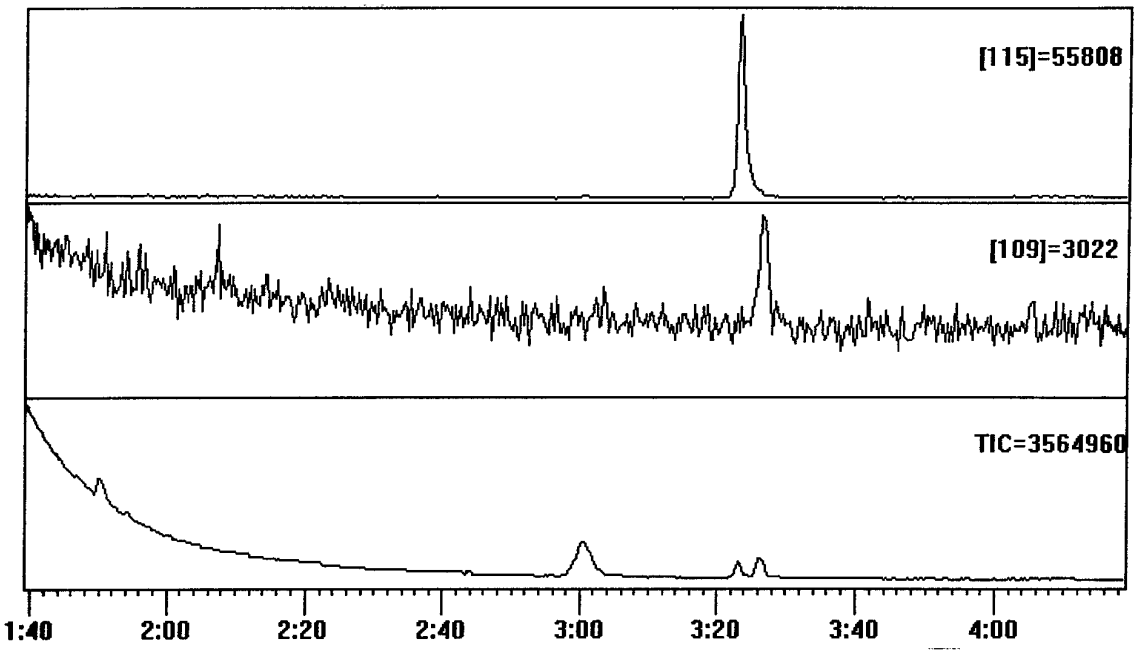


Figure 6: GC-MS chromatograms of an ethyl acetate extract of a blood sample drawn from a hairless guinea pig at 40 min after intravenous administration of 8.2 mg SM/kg. The peak detected at m/z 109 (middle chromatogram) is SM (12 ng/ml blood), the peak detected at m/z 115 (upper chromatogram) is D_8-SM (525 ng/ml blood). The lower chromatogram shows the total ion current (TIC).

Unfortunately, problems arose during the application of this configuration in the toxicokinetic studies. The linearity between the injected amount and peak height was lost in the range of 1-100 pg, resulting in a detection limit of approximately 10 pg and irreproducible results. These problems appeared to be mainly of a chromatographic nature. The retention gap was heavily contaminated, with droplets visible in the capillary. Furthermore, the performance of the analytical column had deteriorated. After renewal of the retention gap and analytical column, and some fine-tuning of the MS, linearity and sensitivity were restored. Due to the high selectivity, one tends to forget that the samples are not as clean as they seem to be by the appearance of the chromatograms. Lipids will be extracted from the sample with ethyl acetate, which amounts to about 5 mg/ml blood. As a consequence, approximately 5 µg of lipids are injected upon each 1-µl injection into the GC-system, which form a 'stationary phase' in the retention gap and subsequently also in the analytical column. This leads to deterioration of chromatographic performance. A procedure for preventive maintainance was established, based on replacement of the retention gap after injection of 40 biological samples. Unfortunately, this procedure appeared to be insufficient.

In order to reduce the contamination of the chromatographic system, two approaches were chosen. Firstly, additional clean-up of the extract using solid phase extraction cartridges was studied. For a high recovery, this additional step should be as simple as possible. It was attempted to get rid of the lipids by leading the

ethyl acetate extract over a Florisil® cartridge, anticipating that SM and D_8-SM would not be retained on the cartridge.
Ethyl acetate extracts of guinea pig blood were led over a SepPak Florisil® cartridge. The slightly yellow extract was decolorized by this action. Furthermore, upon evaporation of the ethyl acetate eluate from the cartridge, the mass of the residue was reduced by about 50% in comparison with the ethyl acetate extract not led over Florisil®. By spiking SM and D_8-SM into ethyl acetate extracts from blood, it was shown that they were not retained on the Florisil® cartridge. Therefore, this additional clean-up of the ethyl acetate extract from blood appeared to be a promising approach, and was incorporated into our analytical procedure. However, it was anticipated that this additional clean-up would not be sufficient to prevent serious contamination of the chromatographic system when analyzing large numbers of samples.
As a second approach, it was attempted to reduce the contamination of the chromatographic system by using thermal cold trap (TCT) injection. This injection technique was successfully applied in previous toxicokinetic studies of the nerve agents soman and sarin (Benschop and Van Helden, 1993). TCT injection has two important advantages: it allows large volume injection and on-line sample clean-up by choosing appropriate desorption conditions. Furthermore, TCT injection can be automated by using a thermodesorption autosampler (TDAS), which we have at our disposal. Conditions for TDAS-GC analysis of SM were already known in our laboratory from other applications and were adapted for our specific situation. Conditions for desorption from Tenax, trapping onto the cold-trap and injection into the retention gap were optimized using an FID, at relatively high concentrations of SM, i.e. in the ng-range. Next, the analytical column was connected with the MS. The sensitivity of the TDAS-GC-MS configuration appeared to be inadequate, with a detection limit as high as approximately 1 ng. Upon on-column injection of SM however, the detection limit was about 1 pg, demonstrating that the MS was sufficiently sensitive. It appeared that the TDAS was leaking at various locations, most likely leading to loss of analyte in the injection system. After a thorough check-up, the TDAS worked fine again, and is now functioning well in routine analysis. Since up to 500 µl of the ethyl acetate extract from blood can be injected via Tenax, the required detection limit can easily be reached.

3.2 The 96-h LD50 (i.v. bolus) of sulphur mustard in the hairless guinea pig

3.2.1 Pilot experiments

Hardly any LD50 values for SM are documented. In an early article by Anslow *et al.* (1948), values for i.v., s.c., and percutaneous 15-day LD50s are mentioned for mice, rats and rabbits. As far as the i.v. LD50 was concerned, the indicated values for mice, rats and rabbits were 8.6, 0.7 and 2.7 mg/kg, respectively. Maisonneuve *et al.* (1993) reported a 14-day LD50 for SM in rats of 3.83 mg/kg.

Usually, LD50 values are based on 24-h lethality scores. However, in view of the quiescent period of 3-12 h after SM intoxication, we anticipated that a 24-h period will be too short to obtain a realistic value for the LD50. Therefore, we proposed to use 96-h lethality scores to determine the LD50 value. During this period, the animals had access to water and food *ad libitum*. To reduce the suffering of the animals during this long time period, an analgesic was used. Approximate times of death of the animals were registered, which would allow calculation of LD50 values for other periods than 96 h, in case the latter time period would prove to be too long.

Because of the divergent values reported in literature for the various species and complete lack of toxicity data on the hairless guinea pig, some pilot experiments for dose-range finding purposes were necessary. In a first pilot experiment, doses of 1, 2, 3, 4, or 5 mg/kg were i.v. injected in anesthetized animals (2 animals per dose) using a stock solution of 50 mg/ml of SM in isopropyl alcohol. Just before administration, an adequate portion of this solution was diluted with saline, in such a way that administration of 1 ml/kg body weight of this solution resulted in the doses mentioned above. After administration of SM, survival times were assessed by keeping them one to a cage and recording (breathing) movements using an ultrasonic detection device. Twice a day for the next 5 days, the animals were treated by the long-lasting analgesic Temgesic®. During this 5-day observation period the animals had access to food and water *ad libitum*. Within the 96-h time period there was no mortality except for one animal in the 3 mg/kg group which died shortly after the administration of SM. After 96 h, one animal in the 4 mg/kg group died. The data of this first pilot experiment are gathered in Table 1.

In the second pilot experiment, doses in the range of 7-15 mg/kg were administered to the animals.

The results are shown in Table 2.

Table 1: Weight changes and mortality in the first pilot experiment for the determination of the 96-h i.v. LD50 of SM in the male hairless guinea pig.

Guinea pig #	SM dose (mg/kg, i.v.)	Day 0 Weight (g)	%	Day 2 Weight (g)	%	Day 5 Weight (g)	%
1	1	447	100	433	96.9	459	102.7
2	1	491	100	470	95.7	476	96.9
3	2	458	100	451	98.5	442	96.5
4	2	543	100	532	98.0	537	98.9
5	3	507	100	†			
6	3	534	100	498	93.3	490	91.8
7	4	497	100	472	95.0	†	
8	4	513	100	474	92.4	430	83.8
9	5	528	100	500	94.7	376	71.2
10	5	487	100	456	93.6	433	88.9

† = deceased

Table 2: Weight changes and mortality in the second pilot experiment for the determination of the 96-h i.v. LD50 of SM in the male hairless guinea pig.

Guinea pig #	Dose SM (mg/kg)	Time 0 h Weight (g)	%	Time 24 h Weight (g)	%	Time 48 h Weight (g)	%	Time 72 h Weight (g)	%	Time 96 h Weight (g)	%	Survival time (h)
11	7	398	100	346	86.9	342	85.9	324	81.42	294	73.9	> 96
12	7	424	100	382	90.1	369	87	355	83.7	320	75.5	>96
13	9	382	100	332	86.9	†						36.8
14	9	348	100	301	86.5	277	79.6	283	81.3	252	72.4	> 96
15	11	374	100	†								5.7
16	11	411	100	†								0.75
17	13	420	100	†								20.2
18	13	368	100	†								6.9
19	15	398	100	†								6.9
20	15	426	100									3.3

† = deceased

3.2.2 The 96-h LD50 of sulphur mustard

In view of the results of the pilot experiments, the doses chosen for the actual LD50 experiment were 7.5, 8, 8.5, 9, and 9.5 mg/kg. Each dose was administered to 6 hairless guinea pigs. The results are presented in Table 3.
The 96-h LD50 was calculated from the mortality data by probit analysis (Litchfield and Wilcoxon, 1949). The results of the probit analysis are presented in Table 4 and Figure 7. From these data, the intravenous bolus 96-h LD50 of SM in the hairless guinea pig was calculated to be 8.2 mg/kg (95% confidence limits 7.1-8.8 mg/kg). This value is relatively high in comparison with rats and rabbits but is comparable to the value reported for mice.

Table 3: Number and percentage of dead animals per dosing group, 96 h after i.v. bolus administration of SM to anesthetized hairless guinea pigs.

Dose of SM (mg/kg, i.v.)	Number of dead animals at 96 h/total number	Percentage deaths
7.5	1/6	16.6
8.0	4/6	66.6
8.5	3/6	50
9.0	4/6	66.6
9.5	6/6	100

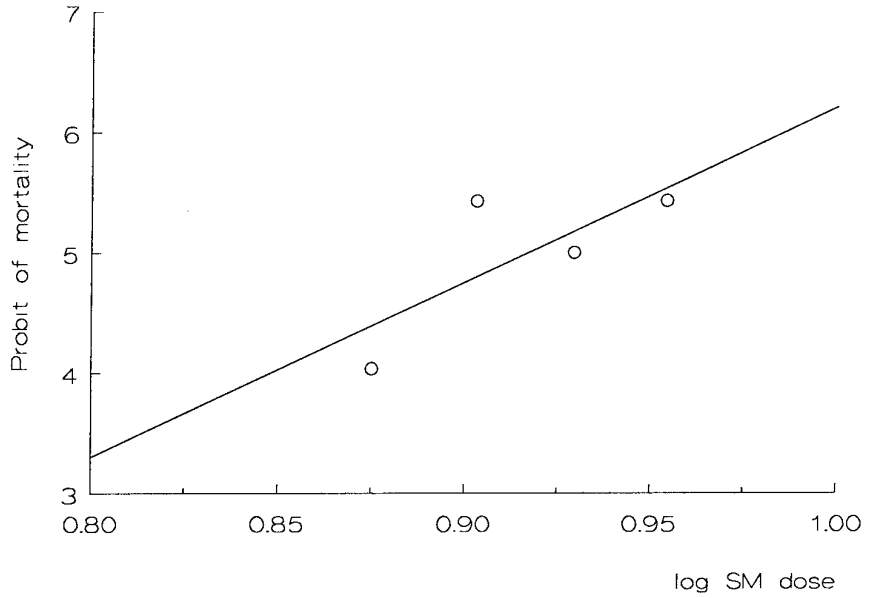


Figure 7: *Probit of mortality of hairless guinea pigs, 96 h after intravenous bolus administration of SM to the anaesthetized animals, versus the administered dose of SM.*

Table 4: *LD10, LD30, LD50, LD70 and LD90 (96-h) values with 95% confidence limits, for i.v. bolus administration of SM to anaesthetized hairless guinea pigs, calculated via probit analysis[a].*

LD	mg/kg	95% confidence limits (mg/kg)
10	7.0	3.6 - 7.7
30	7.7	5.4 - 8.2
50	8.2	7.1 - 8.8
70	8.7	8.2 - 10.6
90	9.5	8.8 - 15.8

a Probit equation: probit = 19.6*log (dose sulphur mustard) - 12.9

3.3 Toxicokinetics of sulphur mustard in anaesthetized hairless guinea pigs after intravenous bolus administration of a dose corresponding with 1 LD50

So far, six toxicokinetic experiments have been performed, with two hairless guinea pigs each, following intravenous administration (vena jugularis) of 8.2 mg/kg of SM, which corresponds to 1 (96-h) LD50. Blood samples were drawn via a carotid artery just before administration of the toxicant (time 0) and at 0.5, 1, 2, 4, 10, 15, 20, 30, 40, 60, 90, 120, 180, 240, 300, 360 min after administration. The size of the samples ranged from 0.1 ml in the first samples up to 3 ml for the

final two sampling times. In order to avoid too much strain on the individual animals, the samples were taken from each of the two animals alternately. The samples were extracted with ethyl acetate and analyzed in duplicate with GLC configuration 3. Some samples were re-analyzed with GLC configuration 4.
The mean concentrations measured in the blood samples are presented in Table 5. The mean concentration-time course of SM is shown in Figure 8. Preliminary toxicokinetic parameters derived from this concentration-time course are listed in Table 6. Additional toxicokinetic experiments are needed to attain n=6 for each time point.

Table 5: *Mean concentration in blood (ng/ml ± s.e.m.) of SM in anaesthetized hairless guinea pigs at various time points after i.v. administration of 1 LD50 (8.2 mg/kg) of SM.*

Time (min)	Concentration of SM (ng/ml blood ± s.e.m.)	
0	n.d.	(n=12)
0.5	4100 ± 650	(n=6)
1	2600 ± 600	(n=5)
2	850 ± 250	(n=6)
4	180 ± 70	(n=6)
10	7.0 ± 3.9	(n=2)
15	5.7 ± 3.6	(n=2)
20	2.2 ± 1.8	(n=3)
30	1.6 ± 0.8	(n=3)
40	1.9 ± 0.8	(n=4)
60	0.8 ± 0.3	(n=5)
90	1.6 ± 0.8	(n=4)
120	1.2 ± 0.2	(n=4)
180	1.0 ± 0.3	(n=3)
240	1.0 ± 0.6	(n=4)
300	0.22 ± 0.15	(n=2)
360	0.13 ± 0.05	(n=3)

n.d. = not detectable (< 5 pg/ml)

The concentrations of SM in abdominal fat, bone marrow, spleen, liver and lung have not yet been determined. The tissues are kept at -70 °C until analysis.
The concentration-time course could not be fitted adequately to a triexponential equation with our non-linear regression program. This may be due to the fact that in the data obtained so far, the elimination seems to proceed faster at the latest time points of the curve. Probably, we will be able to try both a bi- and a triexponential fit of the data, when n=6 for all time points. Meanwhile, the concentration-time course has been described with a biexponential equation.
It is clear from Figure 8 and the parameters in Table 6 that the toxicokinetics of SM in the hairless guinea pig are characterized by a very rapid initial phase, which can be designated as the distribution phase, and a very rapid terminal phase, which can be designated as the elimination phase, with half-lives of 0.77 and 107 min, respectively. From the terminal half-life, it can be calculated that the preferred

detection limit of 5 pg SM/ml blood will be reached approximately 15 h after administration of the toxicant, which will be verified in animal experiments.

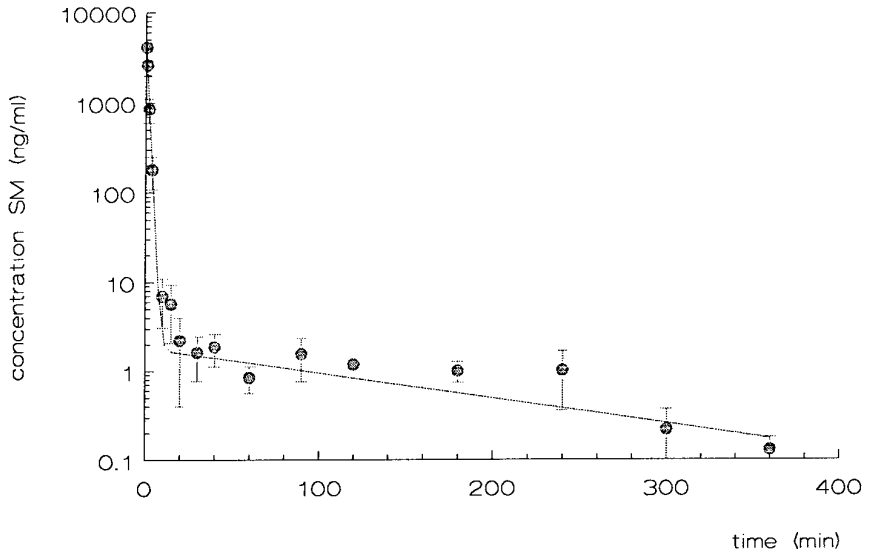


Figure 8: *Mean concentration-time course of SM in the blood of anesthetized hairless guinea pigs after i.v. administration of 8.2 mg SM/kg, which corresponds with 1 LD50 (96-h).*

One of the conclusions we would like to draw from the toxicokinetic studies is at which time point after administration of SM the concentration in blood and tissues will be below the toxicologically relevant level, which has important consequences for the strategy for pretreatment and/or therapeutic intervention. For the nerve agent soman, the toxicologically relevant level was defined as the AUC which would be able to inhibit the minimum essential concentration of acetylcholinesterase with a half-life of 1 h (Benschop and De Jong, 1990). The blood concentration of C(±)P(-)-soman which corresponded with this treshold value was calculated to be 150 pM (approximately 30 pg/ml). For soman the calculation of lowest concentration of toxicological relevance is relatively simple, since the toxic effect is well defined, which is not the case for SM. Reduction of the viability of cells within 24 h of exposure can be used as a criterion for defining the lowest concentration of toxicological relevance. Exposure of cells to 75 µM (approximately 12 µg/ml) of SM reduces cell viability within 24 h, whereas 50 µM has no apparent effect (Mol, 1996). This concentration is two-fold higher than the retrapolated blood concentration of SM at time 0 after i.v. administration of 8.2 mg/kg SM. Since this dose corresponds with 1 LD50, it seems unlikely that 75 µM is a realistic value for the lowest level with toxicological relevance. It is also known that after exposure of white blood cells to 10 nM (approximately 1.6 ng/ml) SM for 1 h DNA-adducts can be detected (see also Chapter II, Detection of DNA-sulphur mustard adducts by use of the immuno-slot-blot assay). Although somerepair may occur, and it is not clear how this adduct formation correlates with cytotoxicity,

this adduct formation can be considered to be an adverse effect. A blood concentration of 1.6 ng/ml will already be reached at approximately 18 min after i.v. administration of 1 LD50 SM. However, it may be that concentrations in tissues lag behind the concentration in blood. Obviously, the concentration-time course of SM in tissues needs to be determined and compared with the concentration-time course in blood before the time point can be established after which the SM concentration in blood is below the toxicologically relevant level. Whether 10 nM is a realistic estimation of the lowest level of toxicological relevance will be a topic for further discussion.

Since SM is designated as a primary carcinogenic compound, one could also advocate to define the toxicologically relevant concentration as the lowest concentration which is carcinogenic. However, such a concentration is unknown for SM. The most conservative approaches to defining carcinogenic concentrations are based on the one-hit model, assuming that even 1 molecule of a carcinogenic compound can cause cancer. Obviously, our method is not suitable for the detection of 1 molecule SM in the body of the hairless guinea pig.

Table 6 also contains toxicokinetic parameters for SM in the rat after i.v. administration of a dose corresponding with 3 LD50. These parameters were calculated from the coefficients and exponents of the biexponential equation as reported by Maisonneuve *et al.* (1993). In the rat, also a very rapid and a very slow phase are observed in the toxicokinetics, albeit that the distribution half-life is 7.5 times longer than that in the hairless guinea pig, whereas the elimination half-life is 2 times longer in the rat than in the hairless guinea pig. These longer half-lives lead to a three-fold higher AUC value in the rat in comparison with the hairless guinea pig, after correction for the difference in dose. Since the AUC and the clearance (Cl) are related parameters, the total body clearance of SM in the hairless guinea pig is approximately three-fold higher than in the rat.

The rate constants of transfer between compartments 1 and 2, $k_{2,1}$ and $k_{1,2}$, are comparable for the two species, whereas the elimination rate constant, k_{el}, is 15-fold higher in the hairless guinea pig than in the rat. Marked differences are observed between the values calculated for the volume of the central compartment (V_1) and the volume of distribution under steady-state (V_{dss}) for the two species. Furthermore, there is a 25-fold difference in the mean residence time (MRT). This parameter represents the mean lifetime expectancy of each SM molecule in the body.

In addition, Table 6 contains toxicokinetic parameters for C(-)P(-)-soman after i.v. administration of a dose corresponding with 0.8 LD50 C(±)P(±)-soman in the atropinized guinea pig. These parameters were calculated from the coefficients and exponents of the biexponential equation as reported elsewhere (Benschop and Van Helden, 1993). The distribution half-lives of SM and C(-)P(-)-soman are nearly the same, whereas the elimination half-life of C(-)P(-)-soman is 18.5 times shorter than that of SM. The AUC of C(-)P(-)-soman is nearly 700-fold lower than that of SM, which can be explained by the 1600-fold lower dose of C(-)P(-)-soman in comparison with SM. The transfer rate constants between compartments 1 and 2 are higher for C(-)P(-)-soman than for SM , whereas the elimination rate constants

are of the same order of magnitude. The volumes of the central compartment are nearly the same for SM and C(-)P(-)-soman, whereas the steady-state distribution volume of SM is nearly 3-fold larger than that of C(-)P(-)-soman, despite the fact that the n-octanol/water partition coefficient for SM (log P_{ow} 1.37) is lower than that of C(±)P(±)-soman (log P_{ow} 1.78).
The total body clearance of SM is about 3-fold higher than that of C(-)P(-)-soman. The mean residence times are comparable for both compounds.

Table 6: Preliminary toxicokinetic parameters[a] for SM derived from the data presented in Table 5. Toxicokinetic parameters for SM in the rat and for C(-)P(-)-soman in the guinea pig are presented for comparison.

		SM 1 LD50 i.v. hairless guinea pig	SM 3 LD50 i.v. rat[b]	C(-)P(-)-soman 0.8 LD50 i.v. guinea pig[c]
Number of exponents		2	2	2
A	($ng.ml^{-1}$)	6154	1293	3.8
B	($ng.ml^{-1}$)	1.8	45.8	0.80
α	(min^{-1})	0.90	0.12	0.95
β	(min^{-1})	0.0065	0.0032	0.12
AUC	($ng.min.ml^{-1}$)	7136	25088	10.6
C_0	($ng.ml^{-1}$)	6156	1339	4.6
$k_{2,1}$	(min^{-1})	0.0068	0.0072	0.26
k_{el}	(min^{-1})	0.86	0.053	0.44
$k_{1,2}$	(min^{-1})	0.035	0.063	0.37
$t_{½,dis}$	(min)	0.77	5.8	0.73
$t_{½,el}$	(min)	107	216	5.78
V_1	($l.kg^{-1}$)	1.33	7.47	1.08
V_{dss}	($l.kg^{-1}$)	8.16	72.8	2.62
Cl	($l.min^{-1}.kg^{-1}$)	1.15	0.40	0.47
MRT	(min)	7.04	181	5.6

a The concentration of sulphur mustard at time t is described by: $[SM] = A*e^{-\alpha t} + B*e^{-\beta t}$.

b Data calculated from Maisonneuve *et al.*, 1993.

c Data calculated from Benschop and Van Helden, 1993.

Abbreviations used: AUC, area under the curve; C_0, retrapolated concentration in the central compartment at time 0; $k_{2,1}$, rate constant of transfer from compartment 2 to compartment 1; k_{el}, rate constant of elimination; $k_{1,2}$, rate constant of transfer from compartment 1 to compartment 2; $t_{½,dis}$, distribution half-life; $t_{½,el}$, terminal half-life; V_1, volume of the central compartment; V_{dss}, volume of distribution under steady-state; Cl, total body clearance; MRT, mean residence time.

3.4 Toxicokinetics of the major DNA-adduct of sulphur mustard in anaesthetized hairless guinea pigs after intravenous bolus administration of a dose corresponding with 1 LD50

From the hairless guinea pigs receiving 1 LD50 SM (i.v., 8.2 mg/kg) in the toxicokinetic experiments, tissues were sampled at 3 time points after administration, i.e. 3, 5, and 6 h after administration. Separate experiments were performed in which the samples were taken at 3 min, 10 min, 24 h and 48 h after administration.

The tissues samples were blood, lung, spleen, bone marrow, liver and small intestine. In these tissues the concentration of the major adduct of SM to DNA, i.e., 7-SM-guanine, was determined via ISB. The concentration was expressed as a number of adducts per 10^6 nucleotides. The results are presented in Table 7 and Figures 9-11.

The data presented in Table 7 and Figures 9, 10 and 11 clearly show that adducts can be detected in all selected tissues after intravenous administration of SM at a dose corresponding with 1 LD50 SM. The results for blood are shown in each of the three figures for comparison.

There appeared to be large differences in adduct levels between the various tissues. The highest level was found in lung tissue. The lung samples even had to be diluted and re-analyzed, as the photographic film was overilluminated. As is clear from Figure 9, the adduct level in lung is already high 3 min after administration and remains high up to 48 h after administration. It is remarkable that the adduct level in blood, as early as 3 min after intravenous administration, is almost 200-fold lower than in the lung. This is still the case 48 h after administration, when the adduct level in blood is approximately 1 per 10^7 nucleotides, i.e. still more than 20-fold lower than in the lung.

Table 7: *Concentration of 7-SM-guanine in tissues of the hairless guinea pig, expressed as the number of adducts per 10^6 nucleotides ± s.e.m., at various time points after i.v. administration of 8.2 mg SM/kg, which corresponds with 1 LD50.*

Time (h)	n	Concentration of 7-SM-guanine per 10^6 nucleotides ± s.e.m.					
		Blood	Lung	Spleen	Bone marrow	Liver	Small intestine
0.05	3	0.42 ± 0.18	21 ± 7	0.54 ± 0.44	0.13 ± 0.10	0.13 ± 0.06	0.18 ± 0.09
0.17	3	0.22 ± 0.13	38 ± 10	1.1 ± 0.8	1.5 ± 0.9	0.11 ± 0.04	1.1 ± 0.4
3	3	n.d.[a]	6.1 ± 1.7	3.8 ± 0.6	1.21 ± 0.01[b]	1.0 ± 0.3	0.01 ± 0.01[d]
5	4	n.d.[a]	2.4 ± 0.5	2.1 ± 0.4	0.65 ± 0.15	0.54 ± 0.23	n.d.[a]
6	7	n.d.[a]	3.3 ± 1.1	1.7 ± 0.3	1.2 ± 0.5	0.33 ± 0.11[c]	0.35 ± 0.09
24	1	0.09 ± 0.01	1.8 ± 0.6	0.21 ± 0.02	0 ± 0	0.04 ± 0.03	0.40 ± 0.05
48	1	0.12 ± 0.01	3.1 ± 0.9	0.05 ± 0.01	0.05 ± 0.04	0.02 ± 0.02	0.35 ± 0.04

a n.d. = not determined

b n = 2

c n = 6

d n = 1, in this case the s.e.m. represents the s.e.m. of 2-4 determinations, in duplicate, of the same DNA sample.

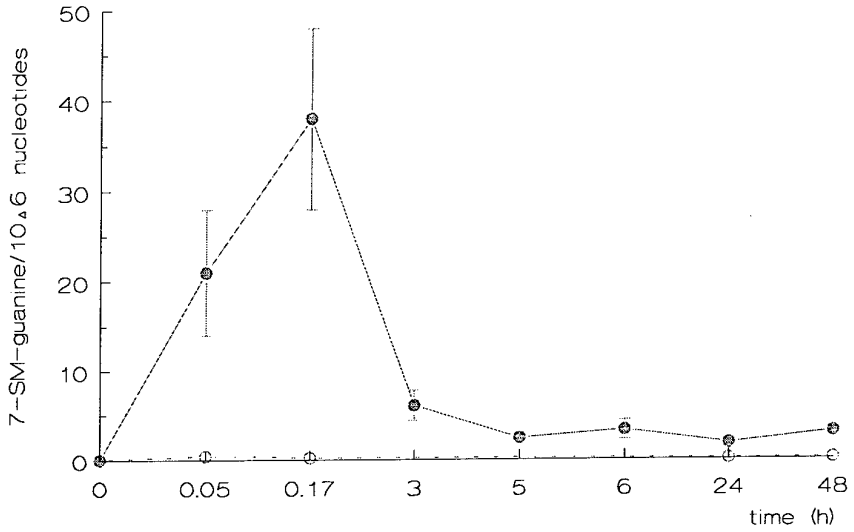


Figure 9: *Concentration of 7-SM-guanine, expressed as the number of adducts per 10^6 nucleotides in DNA ± s.e.m., in blood (○) and lung (●) of hairless guinea pigs at various time points after intravenous administration of 8.2 mg SM/kg, which corresponds to 1 LD50 (96-h). The labels along the X-axis represent the time points (in h) after administration.*

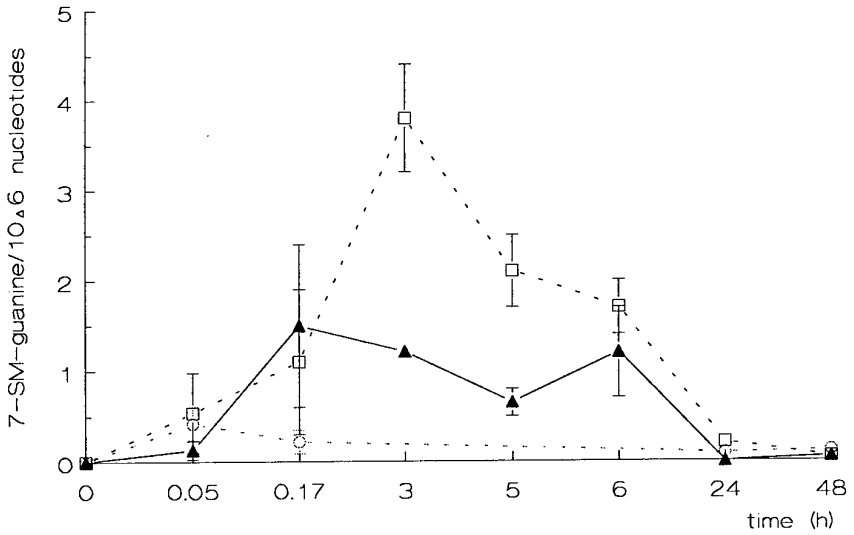


Figure 10: *Concentration of 7-SM-guanine, expressed as the number of adducts per 10^6 nucleotides in DNA ± s.e.m., in blood (○), spleen (□) and bone marrow (▲) of hairless guinea pigs at various time points after intravenous administration of 8.2 mg SM/kg, which corresponds with 1 LD50 (96-h). The labels along the X-axis represent the time points (in h) after administration.*

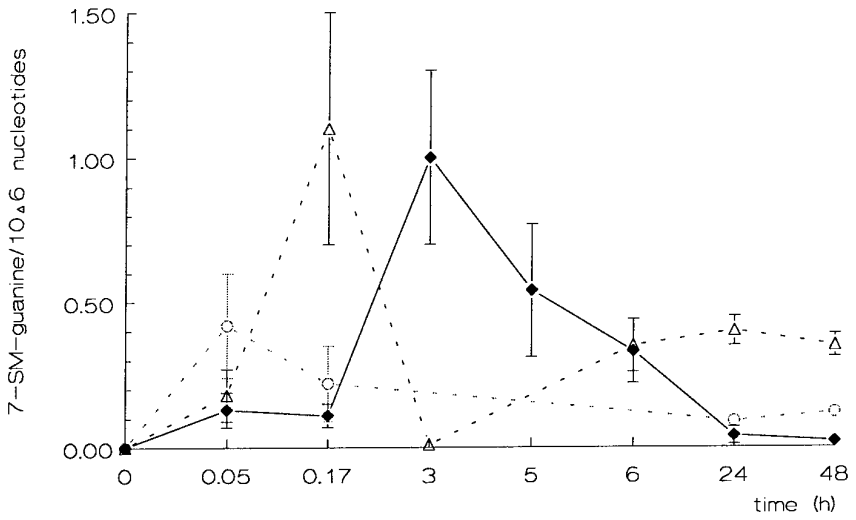


Figure 11: Concentration of 7-SM-guanine, expressed as the number of adducts per 10^6 nucleotides in DNA ± s.e.m., in blood (○), liver (♦) and small intestine (Δ) of hairless guinea pigs at various time points after intravenous administration of 8.2 mg SM/kg, which corresponds with 1 LD50 (96-h). The labels along the X-axis represent the time points (in h) after administration.

In the spleen (Figure 10), the strongest accumulation of adducts occurred during the period up to 3 h after administration. After 24 h, the number of adducts has decreased to approximately 2 per 10^7 nucleotides. In bone marrow (Figure 10), the adduct level showed large variations between the animals during the first 6 h, whereas at 24 h, all adducts seem to have disappeared.
The adduct level in the liver (Figure 11) appeared to be maximal 3 h after administration with a rather insignificant adduct formation in the first 10 min. The level had decreased at 6 h, whereas the adducts had nearly disappeared at 24 h. In the small intestine (Figure 11), a fast accumulation of adducts during the first 10 min after administration was observed, which subsequently decreased, but remained at a level of about 4 adducts per 10^7 nucleotides for up to 48 h. Obviously, more experiments need to be performed with sampling at 24 and 48 h, in order to obtain more solid data on the adduct levels at these time points. Furthermore, blood samples at time points 3, 5 and 6 h will be taken for analysis of 7-SM-guanine in the remaining toxicokinetic experiments, in order to obtain the data for blood at these time points.

4 Conclusions

SM can be extracted from blood by liquid-liquid extraction with ethyl acetate, by mixing on a whirlmixer followed by ultrasonication. The absolute recovery of SM is approximately 90%, whereas the recovery relative to the internal standard D_8-SM is almost 100%. The extraction is performed at 4 °C. The pH does not influence the recovery of SM in the 5-7.5 range. The concentration of chloride ions (1.7-34 M) does not significantly influence the recovery either.

Mass spectrometric (MS) detection via positive electron impact ionization under semi-single ion monitoring is the most sensitive detection principle for SM. Absolute detection limits of approximately 100 fg can be attained. Furthermore, MS detection is highly selective.

Our newly developed gas chromatographic (GC) configuration, based on automated on-column large volume injection, two-dimensional chromatography, and electron capture detection increases the daily sample throughput, whereas the large volume injection and sensitive detection mode allow for minimum detectable concentrations of SM in blood samples of approximately 25 pg/ml. In this configuration, SM and D_8-SM are completely resolved. This complex and delicate configuration needs further optimization before it can be used in routine bioanalysis.

SM can be analyzed in blood samples by GC-MS. By using an on-column autosampler, the daily sample throughput is increased. The detection limit of 33 pg/ml blood is adequate to study the toxicokinetics of SM after i.v. administration of a dose corresponding with 1 LD50. However, the system is contaminated very rapidly in routine bioanalysis, resulting in deterioration of chromatographic performance and loss of sensitivity. Therefore, additional clean-up of the extracts is necessary.

Leading the ethyl acetate extract from blood over a SepPak Florisil® cartridge reduces the amount of contaminants, without loss of SM. This clean-up step is not sufficient to prevent serious contamination of the GC-MS configuration in routine bioanalysis.

Replacement of the on-column autosampler of the GC-MS configuration with a thermodesorption autosampler (TDAS) enables large volume injection and additional on-line sample clean-up by selecting appropriate desorption conditions. A detection limit for SM in blood below 5 pg/ml can be reached on a routine basis with this configuration.

The 96-h intravenous (i.v.) LD50 in hairless guinea pigs is 8.2 mg/kg (95% confidence interval 7.1-8.8 mg/kg).

The i.v. toxicokinetics of SM for a dose corresponding with 1 LD50 are characterized by a very rapid distribution phase and a very slow elimination phase, with half-lives of 0.8 and 107 min, respectively. The detection limit of the bioanalysis is well below the estimated lowest level of toxicological relevance of approximately 1.6 ng SM/ml blood. This estimated lowest level of toxicological relevance is derived from the lowest concentration of SM which forms DNA adducts in white

blood cells. This level is already reached at approximately 18 min after i.v. administration of 1 LD50 SM.
The calculated toxicokinetic parameters for SM are in reasonable agreement with the scarce data reported in literature.
Already at 3 min after i.v. administration of SM at a dose corresponding to 1 LD50, 7-SM-guanine was present in blood, lung, spleen, bone marrow, liver and small intestine, as determined with an immuno-slot-blot assay. The highest initial adduct levels are observed in the lung, which are much higher than in blood. Most of the adducts disappear within 6 h after SM administration. However, 48 h after administration, significant adduct levels are still present in most tissues.

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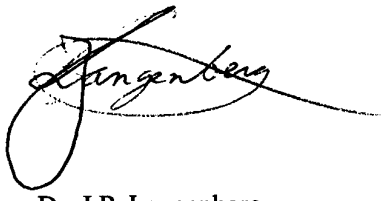
6 Acknowledgements

The authors are grateful to Helma E.T. Spruit, who developed the analytical procedures and measured the concentrations of sulphur mustard in the biological samples; to Willem C. Kuijpers, who performed the animal experiments; to Henk C. Trap for his contribution to the development of the gas chromatographic configurations; to John P. Oostdijk for development of the large volume gas chromatographic configuration; to Roos H. Mars-Groenendijk for the analysis of 7-SM-guanine; to Herman P.M. van Helden for supervising the animal experiments; to Cees van Hooidonk for the quality assurance within the project; to Herma J. van der Wiel for her advice with respect to the animal experiments and processing of tissues; to Marijke M.A. Mol for her advice with respect to the toxicologically relevant concentrations of sulphur mustard; to Harry A. Versteegh for technical support; and to Martin C. Neeleman and Priscilla Vink for taking good care of the hairless guinea pigs.

7 Authentication



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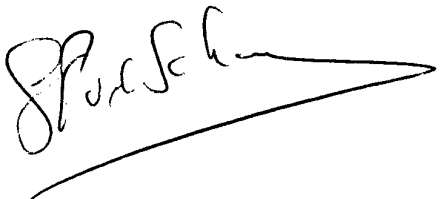

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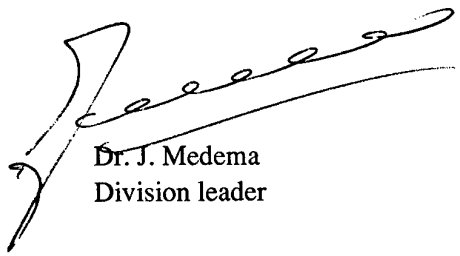

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REPORT DOCUMENTATION PAGE
(MOD-NL)

1. DEFENCE REPORT NO. (MOD-NL)	2. RECIPIENT'S ACCESSION NO.	3. PERFORMING ORGANIZATION REPORT NO.
TD97-0048		PML 1997-A48
4. PROJECT/TASK/WORK UNIT NO.	**5. CONTRACT NO.**	**6. REPORT DATE**
213497110	A94M444	July 1998
7. NUMBER OF PAGES	**8. NUMBER OF REFERENCES**	**9. TYPE OF REPORT AND DATES COVERED**
55 (excl. RDP & distribution list)	59	Final

10. TITLE AND SUBTITLE

Toxicokinetics of sulphur mustard and its DNA-adducts in the hairless guinea pig - DNA-adducts as a measure for epithelial damage. Midterm report

11. AUTHOR(S)

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12. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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Lange Kleiweg 137, Rijswijk, The Netherlands

13. SPONSORING AGENCY NAME(S) AND ADDRESS(ES)

DMGB, P.O. Box 20701, 2500 ES The Hague, The Netherlands

14. SUPPLEMENTARY NOTES

The classification designation Ongerubriceerd is equivalent to Unclassified.

15. ABSTRACT (MAXIMUM 200 WORDS (1044 BYTE))

In order to provide a quantitative basis for pretreatment and therapy of intoxication with sulphur mustard (SM) the toxicokinetics of this agent as well as its major DNA-adduct, 7-SM-guanine, are studied in male hairless guinea pigs for the intravenous (i.v.), respiratory and percutaneous routes. A highly sensitive method for bio-analysis of the intact agent in blood and tissues was developed, involving gas chromatography with mass-spectrometric detection. Deuterated sulphur mustard (D_8-SM) is used as the internal standard. 7-SM-guanine is measured with an immuno-slot-blot assay. In this midterm report, the first results on the i.v. toxicokinetics of SM and 7-SM-guanine in hairless guinea pigs are presented. The 96-h i.v. LD50 appeared to be 8.2 mg/kg (95% confidence interval 7.1-8.8 mg/kg). The i.v. toxicokinetics of SM for a dose corresponding with 1 LD50 are characterized by a very rapid distribution phase and very slow elimination phase, with half-lives of 0.77 and 107 min, respectively. Three mins after i.v. administration of 1 LD50 SM, 7-SM-guanine was already present in blood, lung, spleen, bone marrow, liver and small intestine. The highest initial adduct levels were found in lung. Whereas most of the adducts had disappeared within 6 h of SM administration, significant adduct levels were still present in most tissues 48 h after administration.

16. DESCRIPTORS

Toxicokinetics	Adducts	Gas chromatography
Mustard agents	Guinea pigs	Mass spectroscopy
DNA	Therapy	Chemical agent detection

17a. SECURITY CLASSIFICATION (OF REPORT)	17b. SECURITY CLASSIFICATION (OF PAGE)	17c. SECURITY CLASSIFICATION (OF ABSTRACT)
Ongerubriceerd	Ongerubriceerd	Ongerubriceerd

18. DISTRIBUTION AVAILABILITY STATEMENT

Unlimited Distribution

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