TOXICOKINETICS OF NERVE AGENTS

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Summary

Toxicokinetic studies of nerve agents deal with the in vivo absorption, distribution, and elimination of these agents as a function of animal species, route of administration, dose and time after administration. Such studies provide a quantitative basis for toxicodynamic studies of nerve agents, which should result in causal treatment of intoxications with these agents. While toxicodynamic studies of nerve agents have been intensively pursued since WW II, toxicokinetic studies were only initiated in the last two decennia of the twentieth century. The late start of the latter type of research was caused by the long held but uncorrect assumption that nerve agents were so-called "hit-and-run" poisons, with extremely short in vivo persistence. On the other hand, such in vivo analyses had to wait for the development, in that period of time, of trace analytical methods which were needed to determine the extremely low (ng to low pg range/ml plasma) but toxicologically significant in vivo levels of the various stereoisomers of nerve agents.

This review will deal first with the development of analytical procedures for chiral trace analysis of the stereoisomers of nerve agents, as well as with the toxicology and routes of in vivo elimination of the stereoisomers. Next, results of toxicokinetic studies will be given, mainly dealing with soman but some results for other nerve agents will also be given. The effect of dose, route of adminstration (iv, inhalation, pc) and animal species will be dealt with, emphasizing the toxicokinetics after low level respiratory exposure. Finally, consequences for prophylactic and therapeutic approaches as well as future directions of research are discussed.

Introduction

Toxicokinetic studies of nerve agents deal with the *in vivo* absorption, distribution and elimination of these agents as a function of animal species, route of administration, dose, and time after administration. Such studies are essential to provide a quantitative basis for the toxicology of nerve agents and, in combination with toxicodynamic studies, are the starting point for development of causal treatment of intoxications with these agents. Toxicodynamic studies of nerve have led to, e.g., the development of prophylaxis of intoxication based on partial inhibition of cholinesterase activity with carbamates and therapy of intoxication through administration of the muscarinic cholinergic antagonist atropine, reactivation of phosphylated cholinesterases with oximes, often in combination with administration of a central nervous depressant in order to suppress convulsions and other central effects.

Toxicokinetic studies of nerve agents were initiated not before the last two decennia of the twentieth century. The reasons for this late development were twofold. Firstly, it was often assumed that nerve agents, especially at supralethal doses, act so quickly and are so rapidly degraded *in vivo* that toxicokinetic studies were not relevant for treatment of intoxications, i.e., nerve agents should be regarded as so-called "hit and run poisons." Secondly, it was intuitively assumed that *in vivo* concentrations of the extremely toxic nerve agents are too low for bioanalysis. However, Wolthuis *et al* showed in 1981 that rats surviving initially a challenge with a supralethal dose of soman by immediate treatment with atropine and the oxime HI-6 became fatally re-intoxicated 4–6 h later. Hence, soman appeared to be far more persistent than previously assumed. This suggested that toxicokinetic investigations of nerve agents are toxicologically relevant, especially in view of the refractoriness of intoxication with these agents towards treatment. Moreover, the development of analytical techniques, especially for gas chromatographic analyses of the rather volatile nerve agents had evolved to a level that detection limits of a few picograms (10^{-12} g) of these agents became feasible. Finally, the development of chiral gas chromatography opened up the possibility to analyze the separate stereoisomers of nerve agents, which is needed for toxicological interpretation of toxicokinetic studies of nerve agents.

In the following, after expanding on the importance of stereoisomerism in the toxicology of nerve agents, the techniques needed for stabilization, isolation and trace analysis of nerve agent stereoiso-

mers will be treated. In view of the recent publication of an extensive review on the toxicokinetics of nerve agents which deals almost exclusively with soman (Benschop and de Jong, 2000), this paper will deal mainly with the comparitive toxicokinetics upon iv, respiratory and percutaneous adminstration of soman, sarin and VX. For the latter two agents, recent results have been published (Spruit *et al*, 2000; van der Schans *et al*, 2000). Future directions of toxicokinetic investigations will be indicated.

Chiral analysis, isolation and toxicology of nerve agent stereoisomers

Interpretation and understanding of the toxicokinetics of nerve agents would not be possible without taking into consideration that these agents consist of mixtures of stereoisomers, which are often extremely different in their toxicokinetic and toxicodynamic properties. A common feature of soman, sarin, and VX is the presence of chirality (asymmetry) around the phosphorus atom. Therefore, sarin and VX consist of equal amounts of stereoisomers, denoted as (+) and (-)-sarin, and (+)- and (-)-VX, respectively. In the case of soman, an additional chiral center resides in the 1,2,2-methylpropyl (pinacolyl) moiety, leading to the presence of four stereoisomers. Synthetic soman, i.e., a mixture of the four stereoisomers, is denoted as C(±)P(±)-soman, whereas the individual four stereoisomers are denoted as as C(+)P(+), C(+)P(-), C(-)P(+), and C(-)P(-), in which C stands for chirality in the pinacolyl moiety and P for chirality around phosphorus.

Separation of the various stereoisomers of the nerve agents for analytical purposes became feasible with the advent of optically active coating materials for columns as used in chiral capillary gas chromatography (GC) and in high performance liquid chromatography (HPLC). For example, the four stereoisomers of soman are fully separated with GC on capillary columns coated with a derivative of L--valine bound to a siloxane backbone (Chirasil-L--Val), whereas the two stereoisomers of sarin are completely separated by means of GC on a capillary column coated with β-cyclodextrin (Cyclodex B). Sofar, (+)- and (-)-VX could not be separated by means of capillary gas chromatography, but HPLC on a so-called Chirocel OD-H column yields complete separation of the two stereoisomers of this agent.

Using the above-mentioned analytical procedures to monitor progress, the four stereoisomers of soman, as well as (-)-sarin, could be isolated on a mg-scale for toxicological purposes by using judicious combinations of synthetic and enzymatic separation techniques. In case of C(±)P(±)-soman, synthetic resolution of the stereoisomers of (±)-pinacolyl alcohol and subsequent synthesis of soman from these stereoisomers gave C(+)P(±)-soman and C(-)P(±)-soman, i.e., two diasterestereoisomeric mixtures of two soman stereoisomers. These pairs were separated enzymatically by incubation of C(+)P(±)- and C(-)P(±)-soman with α-chymotrypsin, which binds the P(-)-stereoisomers of soman. In this way C(+)P(+)- and C(-)P(+)-soman, respectively, could be isolated. Incubation with rabbit plasma hydrolyzes the P(+)-stereoisomers and provides therefore C(+)P(-)- and C(-)P(-)-soman. Similarly, incubation of (±)-sarin with α-chymotrypsin gave optically pure (-)-sarin. The two stereoisomers of VX are easily obtained synthetically from optically resolved precursors.

Table 1

Stereoselectivity in Anticholinesterase Activity and Acute Lethality of Nerve Agent Stereoisomers

Nerve agent stereoisomer	Rate constant for inhibition of AChE[a] M^{-1} min^{-1} (25 C)	LD_{50} (mouse) μg/kg
C(+)P(-)-soman	$2.8x10^8$	99[b]
C(-)P(-)-soman	$1.8x10^8$	38[b]
C(+)P(+)-soman	$< 5 x10^3$	> 5000[b]
C(-)P(+)-soman	$< 5x10^3$	> 2000[b]
C(±)P(±)-soman		156[b]
(-)-sarin	$1.4x10^7$	41[c]
(+)-sarin	$< 3x10^3$ [d]	
(±)-sarin		83[c]
(-)-VX	$4x10^8$	12.6[c]
(+)-VX	$2x10^6$	165[c]
(±)-VX		20.1[c]

[a] Rate constants for the stereoisomers of soman were measured with AChE from electric eel at pH 7.5, whereas those for sarin and VX stereoisomers were obtained with AChE from bovine erythrocytes at pH 7.7. [b] Subcutaneous administration. [c] Iv administration. [d]Estimated from an experiment with optically enriched sarin (64% enantiomeric excess).

With sufficient amounts of the various stereoisomers of the major nerve agents available it became feasible to investigate the acute lethality of

these stereoisomers. *A priori*, it should be expected that the degree of lethality will correlate with the inhibitory potency towards AChE. Therefore, bimolecular rate constants of inhibition of AChE with these stereoisomers were measured, as well as their LD_{50}-values in mice. A summary of the results is given in Table 1. Apparently, the P(-)-stereoisomers of soman and sarin inhibit AChE with rate constants which are 4-5 orders of magnitude higher than those of the corresponding P(+)-stereoisomers. Concomitantly, it appeared that the P(-)-stereoisomers of soman are at least two orders of magnitude more acutely lethal than the P(+)-counterparts. For practical purposes the difference in acute lethality is such that the P(+)-stereoisomers should be regarded as a nontoxic impurity in synthetic soman, taking into consideration that the lower limit for the acute lethality of the P(+)-stereoisomers is difficult to determine in view of possible *in vivo* racemization. The same extreme differences will probably hold for (+)- and (-)-sarin, although this cannot be made explicit since methods to isolate optically pure (+)-sarin are not yet available.

By contrast with soman and sarin, the rate of inhibition of AChE by (+)-VX is only two orders of magnitude less than that of the (-)-stereoisomer. In this case the LD_{50} of the (+)-stereoisomer could also be determined, revealing that (-)-VX is only 8-fold more acutely lethal than the (+)-stereoisomer.

The detailed three-dimensional structure of the active site of human AChE based on X-ray analysis and molecular modeling allowed to draw a detailed model of the Michaelis complex for the inhibition of the enzyme by the soman stereoisomers, the stability of which should be regarded as a reflection of the reactivity of the stereoisomer. It appears that the extremely low reactivity of the P(+)-stereoisomers is due to steric constraints which prevent accomodation of the bulky t-butyl group in the pinacolyl moiety and practically exclude it from the acyl pocket of the active site "gorge".

Analysis of Nerve Agents in Biological Samples

Toxicokinetic investigations of nerve agents are only relevant if these agents can be analyzed at minimum levels in blood or tissue samples which are still toxicologically relevant. Such relevance in case of anticholinesterases should be related to their capacity to inhibit the enzyme AChE. Since nerve agents inhibit this enzyme with rate constants up to 10^8–10^9 $M^{-1}min^{-1}$ it can be derived that blood levels down to a few $pg.ml^{-1}$ blood (appr. 10^{-10} M) can still cause significant inhibition over a period of time of several hours. Obviously, the need for such extremely low minimum detectable concentrations requires analytical procedures which provide utmost detection limits and selectivity. Moreover, as elucidated in the previous paragraph, differential analysis of the various stereoisomers of a nerve agent is also required.

The relatively volatile nature of nerve agents, the extremely low detection limits of modern detectors for gas chromatography and the recent advances in chiral separation in gas chromatography have led to the extensive use of this technique for toxicokinetic investigations of nerve agents. Primarily, the procedure was developed for analysis of the four stereoisomers of soman, based on separation of these stereoisomers on a capillary Chirasil-L-Val column. As shown in Figure 1, this column separates the C(+)P(+)- and C(-)P(+)-stereoisomers of soman perdeuterated in the pinacolyl moiety from the four stereoisomers of soman. Hence, the deuterated stereoisomers are highly useful internal standards for quantitation of soman stereoisomers, without recurring to the use of expensive mass spectrometric detection systems. In stead, highly sensitive alkali flame (NPD) and pulsed flame photometric (PFPD) detectors can be used with absolute detection limits for nerve agents of 1-5 pg. In order to further increase the selectivity of the analytical procedure, a two-dimensional system was introduced in which a cut containing the analytes is trapped from a precolumn, e.g., a CPSil 8CB column, into a cold trap from which this cut is re-injected onto the chiral column by means of flash heating.

The pronounced volatility of nerve agents, especially of sarin and soman, prevents their concentration into a sufficiently small sample volume of ca. 1–5 μL for injection into the two-dimensional gas chromatograph. Therefore, an on-line large volume injection system was introduced based on application of of the analytes in an organic solvent, up to a volume of 500 μL on Tenax absorption material, from which the solvent is blown off selectively. Next, the analytes are thermally desorbed from the Tenax absorbent into a cold trap for subsequent injection into the two-dimensional system by means of flash heating.

For analysis of the stereoisomers of sarin, the

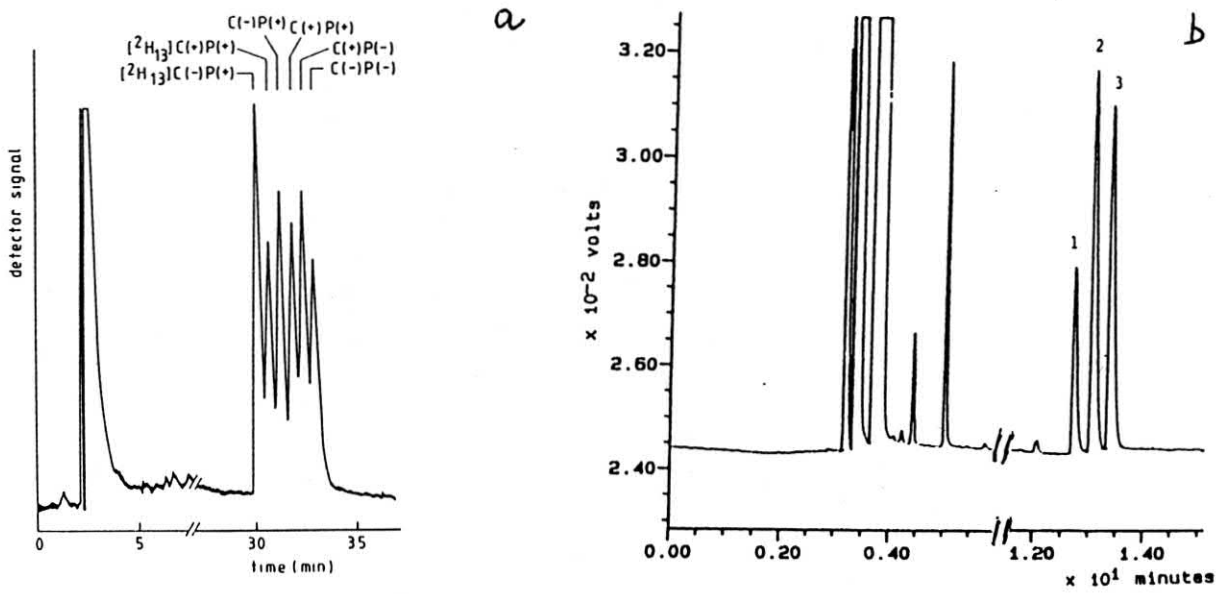

Fig. 1: Gas chromatographic sepation of the four stereoisomers of soman and two deuterated stereoisomers on an Chirasil-L-Val column (a) and of the two stereoisomers of sarin and two deuterated stereoisomers on a CycloDexB column (b).

optically active L-Chirasil-Val column is replaced by an optically active CyclodexB column (Fig. 1). This column separates the two stereoisomers of sarin from the two stereoisomers of sarin which are perdeuterated in the isopropyl moiety. Therefore, these deuterated stereoisomers are convenient internal standards for analysis of sarin stereoisomers in biological samples.

In vivo, the stereoisomers of soman and sarin are subject to rapid processes of elimination, including spontaneous and enzymatically catalyzed hydrolysis and phosphylation of protein binding sites. Theses processes should be "frozen" at the moment that the sample is taken for a period of time that is sufficient for further work-up. Stabilization procedures of nerve agent stereoisomers in biological samples were developed with stringent validation for all separate stereoisomers since these have widely differing rates of degradation. It appeared that spontaneous and enzymatic hydrolysis of nerve agent stereoisomers can be sufficiently suppressed by immediate acidification of the sample to pH 4 with an acetate buffer. This was validated by adding known amounts of soman to rat blood samples that had been pre-incubated with soman in order to "saturate" irreversible binding sites and from which excess of soman had been removed. However, it then appeared that fluoride ions in the blood, present either from natural sources or from hydrolysis of soman, reactivated soman from phosphylated binding sites such as carboxylesterases (CaE's) which led to substantially higher levels of soman in the samples than added for the purpose of validation. This complication was effectively suppressed by addition of aluminum sulfate which binds fluoride ions, mostly in the complex [AlF_2^+]. Finally, "leakage" of soman stereoisomers to unoccupied phosphylation sites, which can be an overriding phenomenon when investigating the toxicokinetics at low level exposures, is blocked effectively by adding a large excess of O-neopentyl methylphosphonofluoridate (neopentyl sarin). This agent saturates the unoccupied binding sites without interfering with the gas chromatographic analysis.

The combined use of acidification to pH 4, addition of aluminum sulfate and of neopentyl sarin proved to be sufficient to stabilize the stereoisomers of sarin and soman. In subsequent work-up, the analytes and internal standard are extracted from the stabilized blood or tissue sample by means of solid phase extraction and elution with ethyl acetate, for gas chromatographic analysis. The same work-up and analytical procedure can be used for homogenized brain and diaphragm tissue samples.

The procedure for bioanalysis of (±)-VX is more straightforward than for soman and sarin. Alkali isadded to the sample in order to deprotonate the amino moiety of VX. Next the agent is extracted with n-hexane.Finally, the total of the two stereoisomers of VX is analyzed by means of gas chromatography with NP detection. For chiral analysis, the enantiomers are first separated with HPLC on

the earlier mentioned Chirocel column, followed by gas chromatography of the fractions that contain the separate (+)- and (-)-VX stereoisomers. A structural analog of VX appears to be a convenient internal standard for such analyses.

Intravenous toxicokinetics

Initial investigations on the toxicokinetics of nerve agents were performed after intravenous (iv) administration of doses corresponding with multiple LD_{50}-values. This route of administration provides basic toxicokinetic data, which can subsequently be compared with results for more realistic routes of administration, e.g., the subcutaneous (sc), percutaneous (pc), and respiratory routes. With gradually improving methods of bioanalysis, the administered doses could be lowered. Nevertheless, data obtained at multiple LD_{50}-values are highly relevant since these pertain to exposure scenarios where immediate medical treatment of casualties should be applied.

Animal species selected for initial investigations were rats, guinea pigs, and marmosets, with the latter species serving as a primate model for man. In order to perform toxicokinetic measurements at high doses, the anaesthetized animals were provided with a tracheal cannula for artificial respiration and with a carotid cannula. Shortly before administration of nerve agent in the dorsal penis vein, the animals were atropinized intraperitoneously (ip) and blood samples were taken from the carotid cannula at various points of time after intoxication for analysis of nerve agent stereoisomers. Blood levels of the individual nerve agent stereoisomers in rats and guinea pigs were measured at each time point randomly in at least six animals, while complete toxicokinetic curves were measured in each individual marmoset.

The LD_{50}-values of C(±)P(±)-soman are highly species-dependent, since the amount of carboxylesterases (CaE'ss) in the blood is species-dependent. These enzymes act as scavengers of nerve agent by means of irreversible binding and are present in large amounts in the blood of rats, in significantly smaller amounts in guinea pigs and are almost absent in the blood of marmosets. In accordance herewith, the LD_{50}-values decrease in the order rat > guinea pig > marmoset. As a consequence of these abnormally high amounts of binding sites in the blood of rats we found that this species is an unsatisfactory model for toxicokinetic investigations that should be relevant to primates, in particular to man. The guinea pig appeared to be a reasonable model for man, at least from a toxicokineic point of view.

The toxicokinetics of the relatively nontoxic P(+)-stereoisomers of soman and sarin will not be dealt with. These stereoisomers disappear from the blood within seconds or at most a few minutes, mostly due to rapid enzymatic hydolysis.

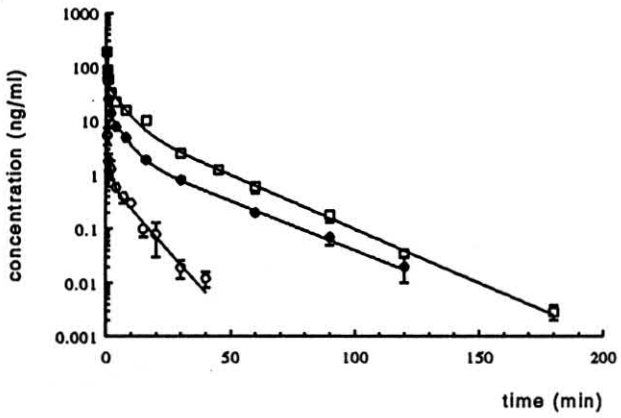

Fig. 2: Semilogarithmic plot of the concentrations in blood (± s.e.m; n = 6) of C(-)P(-)-soman versus time after iv administration of 0.8 (22 µg/kg), 3, and 6 LD_{50} of C(±)P(±)-soman to anaesthetized, atropinized, and mechanically ventilated guinea pigs (o 0.8 LD_{50}; • 2 LD_{50}; 6 LD_{50}).

Figure 2 gives the concentrations in blood of the highly toxic C(-)P(-)-stereoisomers of C(±)P(±)--soman at doses varying from 0.8-6 LD_{50} in guinea pigs. In contrast with the C(±)P(+)-stereoisomers, the highly toxic C(+)P(-)- and C(-)P(-)-stereoisomers of soman can be measured for periods of almost one hour up to several hours, depending on the dose, in spite of very steep initial decline of all blood levels due to rapid distribution and covalent binding. The toxicokinetic curves are best described with three-exponential equations, except for those at the lowest dose (0.8 LD_{50}), for which the data can be fitted to a two-exponential equation. Areas under the curve (AUC) and terminal half lives are calculated from these equations according to standard equations.

For comparison, Figure 3 gives the toxicokinetic curves for (-)-sarin and C(-)P(-)-soman in guinea pigs after an intravenous dose of 0.8 LD_{50} of soman

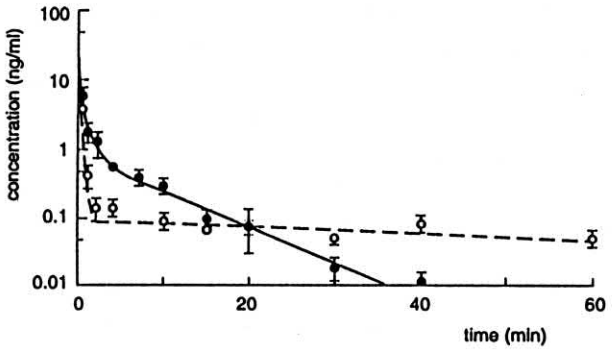

Fig. 3: Semilogarithmic plot of the mean concentrations in blood (± s.e.m.; n = 6) of (-)-sarin (o) versus time after iv bolus administration of 0.8 LD_{50} (19.2 µg/kg) of (±)-sarin to anaesthetized, atropinized, and mechanically ventilated guinea pigs. For comparison the concentration-time course of C(-)P(-)-soman at an equitoxic dose (iv) of C(±)P(±)-soman (0.8 LD_{50}; 22 µg/kg) is also shown.

and sarin. It appears that the distribution phase of sarin is an order of magnitude more rapid than for soman whereas, surprisingly, the elimination phase is an order of magnitude slower for (-)-sarin.

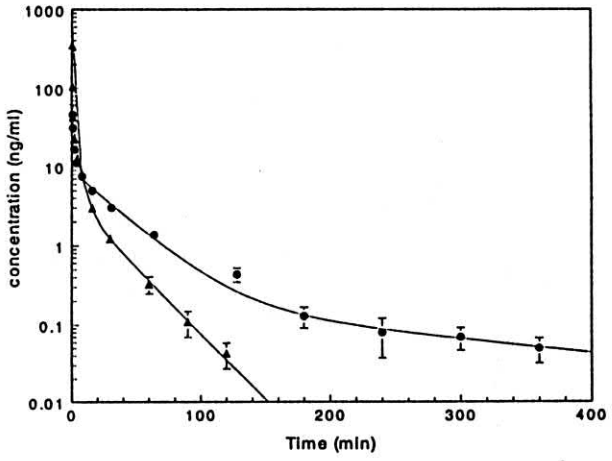

Fig. 4: Mean concentration-time course of (-)-VX (•) and C(±)P(-)-soman (∇) in blood of anesthetized, atropinized and artificially ventilated guinea pigs after iv administration of doses corresponding with 2 LD_{50} of (±)-VX (56 µg/kg) and the same absolute dose of C(±)P(±)-soman (55 µg/kg). It is assumed that the concentrationn of (-)-VX is 50 % of the total concentration of (±)-VX.

In contrast with G-agents, few toxicokinetic investigations with V-agents have been performed. For various reasons it is worthwile to compare the toxicokinetics of these two types of nerve agents. Several pathways for degradation of G-agents appear to be less effective for V-agents. For example, phosphoryl phosphatases hydrolyze the P(+)-stereoisomers of G-agents rapidly, but hardly degrade V-agents. Covalent scavenging by CaE'ss is a major pathway for the degradation of the P(-)-stereoisomers of G-agents, while these enzymes are much less effective in binding V-agents. Unless alternative *in vivo* pathways are available, these relative reactivities suggest a greater *in vivo* persistence of V-agents than of G-agents. Our first results corroborate this suggestion (van der Schans *et al*, 2000). Figure 4 gives the toxicokinetic curves for the summated C(±)P(-)-stereoisomers of soman and of (-)-VX in guinea pigs at the same absolute dose of 55 µg/kg of C(±)P(±)-soman and of (±)-VX, which corresponds for the latter agent with 2 LD_{50}. Apparently, VX is even far more persistent than (-)-sarin. In clear contrast with the results for soman and sarin, stereospecificity in the elimination of the two stereoisomers of VX is hardly observed.

The derivation of the time period during which acutely toxic levels of (summated) C(±)P(-)-soman stereoisomers are present is based, somewhat arbritarily, on a scenario of intoxication in which an animal resumes spontaneous respiration presumably due to ca 5 % reactivation by oxime (or protection by carbamate) of completely inhibited AChE in diaphragm. Since the concentration of AChE in diaphragm of guinea pigs is ca 2-2.6 nM, this reactivated fraction corresponds with ca 150–200 pM AChE.

Based on a bimolecular rate constant for inhibition of AChE by C(±)P(-)-soman of ca. 10^8 $M^{-1}min^{-1}$, it is calculated that this reactivated fraction of AChE can be re-inhibited by 150 pM of C(±)P(-)-soman with a half life of ca 1 h. An order of magnitude lower concentration of C(±)P(-)-soman can only cause toxicologically insignificant re-inhibition.

Therefore it is assumed that 150 pM C(±)P(-)- -soman represents approximately the lowest concentration having toxicological relevance. In a more generalized way it may be reasoned that an area under the curve (AUC) of 30 $pg.ml^{-1}$ x 60 min = 1.8 $ng.min.ml^{-1}$ in the last part of the blood level curve is needed for toxicological relevance. The period of time in between intoxication and the point on the time axis at which this area starts can be regarded as the period of time in which toxicologically relevant levels of C(±)P(-)-soman are present.

Table 2

Time Periods of Acutely Toxic levels After Intravenous Administration

Nerve Agent	Species	Dose (x LD_{50})	Acutely Toxic Level until (min)
C(±)P(-)-soman	Rat	6	317
		3	95
		1	37
	Guinea Pig	6	126
		2	104
	Marmoset	6	74
		2	49
(-)-VX	Guinea Pig	2	380
		1	210
	Marmoset	1	400

Table 2 gives a summary of the time periods of acutely toxic levels of nerve agents in various species and intravenous doses. Obviously, the time period is rather long at high dosage (6 LD_{50}) in rats, which may explain the earlier mentioned re-intoxication phenomena as observed by Wolthuis *et al.* However, the "toxic periods" are much shorter at lower dose in rats and at all dosages of soman in guinea pigs and marmosets. In contrast, the acutely toxic levels are rather long lasting for VX in guinea pigs at relatively low dosages corresponding with at most 2 LD_{50}. Presumably due to a less efficient metabolism than in guinea pigs, the toxicologically significant period of time is extremely long at even 1 LD_{50} in marmosets. This may spell difficulties in the treatment of percutaneous intoxications with VX.

Inhalation toxicokinetics at acutely toxic levels

In case of intoxications with nerve agents under realistic conditions, the primary route of entrance into the body of volatile nerve agents such as sarin, tabun, soman, and GF is the respiratory route. The latter route is almost as effective as parenteral administration, with ca 70 % of an inhaled dose of (±)-sarin being retained in guinea pigs, dogs, monkeys, and humans. It was anticipated that the shapes of the inhalation toxicokinetic curves of nerve agent stereoisomers differ considerably from those for other routes of exposure, which may have impor-

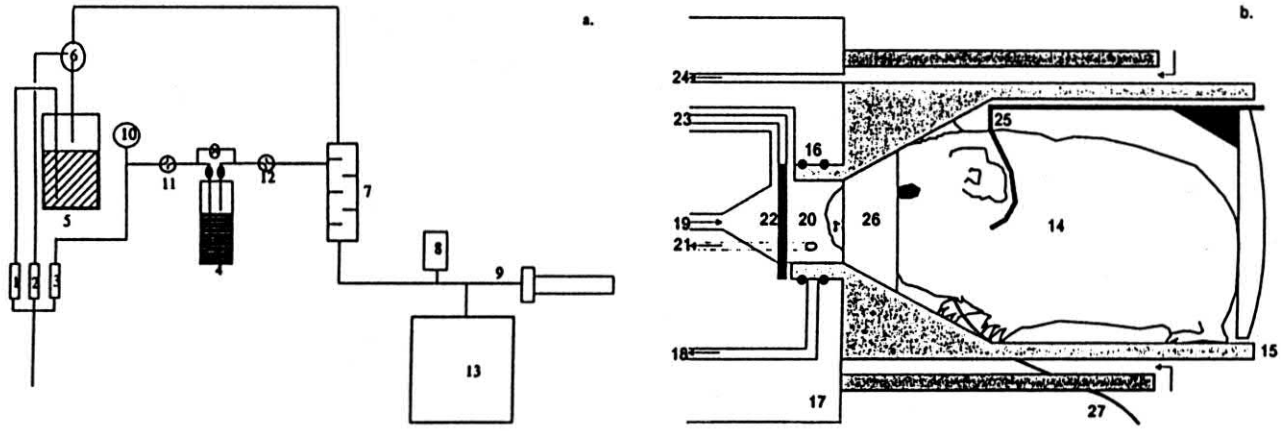

Fig. 5: (a)Apparatus constructed for generation of nerve agent vapor. (1), (2), and (3) mass flow controllers; (4) vial containing the nerve agent; (5) thermostatted water bath; (6) and (7) mixing chambers; (8) temperature/relative humidity meter; (9) towards the exposure modules; (10) overpressure security; (11) and (12) splash heads; and (13) gas chromatograph with gas sampling valve. (b) Guinea pig (14) positioned in the modified Battelle tube (15). (16) O-rings for gastight connection of the tube to the body of the exposure apparatus (17); (18) tubing with a critical orifice, which is connected to an underpressure check for gastight connection of the tube; (19) tubing through which the nerve agent is transported to the exposure chamber; (20) front chamber of the modified Battelle tube, from which the animal breathes; (21) tubing with a critical orifice, which is the outlet of the front chamber; (22) wire mesh resistance; (23) differential pressure measuring device; (24) tubing with a critical orifice, which sucks air from the "underpressure chamber" surrounding the tube; (25) fork for positioning the animal; (26) rubber mask; and (27) carotid artery cannula. Arrows indicate flow directions.

tant consequences for the efficacy of pretreatment and therapy of intoxications with these agents. Therefore, Langenberg *et al* investigated the inhalation toxicokinetics of C(±)P(±)-soman, using an apparatus which they constructed for the continuous generation of nerve agent vapor in air, nose-only exposure of guinea pigs, and monitoring of respiratory minute volume and respiratory frequency during exposure. Figure 5 gives a schematic representation of this apparatus, as well as a short explanation on the functioning of the various elements.

The inhalation and subsequent absorption of nerve agent vapor, largely in the upper part of the respiratory tract may involve a time period of a few seconds or minutes, up to several hours in case of low level exposure. Initial investigations of the inhalation toxicokinetics involved exposure periods of 4–8 min which was regarded as a compromise between the often shorter exposure time to volatile agents in case of chemical warfare and the desire to measure in a managable time frame the increasing blood levels due to inhalation and absorption. Since it was considered as too involved to use mechanically ventilated animals in inhalation toxicokinetics, the investigations were restricted to sublethal doses in the range of 0.4–0.8 LCT_{50} of C(±)P(±)-soman and (±)-sarin, inhaled by anaesthetized and atropinized guinea pigs.

The effect of inhaled dose on the toxicokinetics is shown in Figure 6 where the time course of the concentration of C(-)P(-)-soman is given for an 8 min exposure to 0.4 and 0.8 LCT_{50}.

The kinetics of C(+)P(-)- and C(-)P(-)-soman were described mathematically as a discontinuous proces, with an equation for the exposure period and an equation for the postexposure period. Our data suggest that the systemic penetration of C(-)P(-)--soman during nose-only exposure is very rapid, since this stereoisomer can be measured in blood at 30 s after starting the exposure. Moreover, the concentration of this stereoisomer does not increase further after terminating the exposure. In contrast herewith, there is a lag time of several minutes before the C(+)P(-)-stereoisomer can be detected in blood. Furthermore, as observed earlier for iv administration, the concentrations in blood and the AUC of C(+)P(-)-soman are consistently lower than those of the C(-)P(-)-stereoisomer, despite the 22% excess of the C(+)P(-)-stereoisomer over the C(-)P(-)--stereoisomer in C(±)P(±)-soman. Based on the 36--fold higher reaction rate of the C(+)P(-)stereoisomer with guinea pig plasma CaE'ss, this phenomenon can at least partly been explained by preferential binding of the C(+)P(-)-stereoisomer, e.g., to CaE'ss at the absorption site(s) in the respiratory tract and in blood.

Our data (not given) show that the apparent elimination half life of the C(-)P(-)-stereoisomer after respiratory exposure to 0.8 LCt_{50} in 8 min is somewhat longer than for the equitoxic iv dose. This suggests that, despite rapid absorption, some depot formation occurs at the absorption site, from which absorption continues after termination of the exposure.

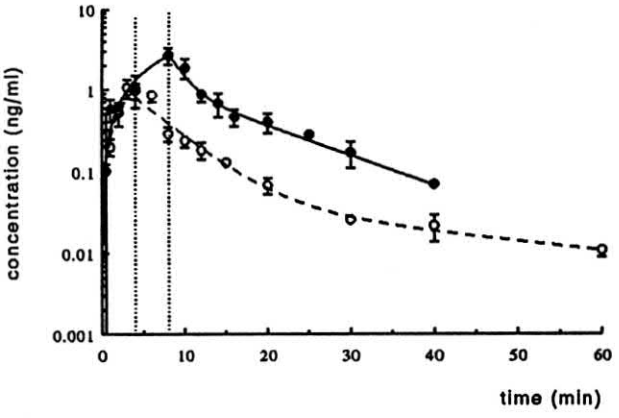

Fig. 6: Semilogarithmic plot of the mean concentrations in blood (SEM, n = 6) of C(-)P(-)-soman vs time during and after nose-only exposure of anesthetized, atropinized, and restrained guinea pigs to C(±)P(±)-soman vapor in air yielding 0.4 (o) and 0.8 (•) LCt50 in 8 min. The dotted line marks the end of the exposure period.

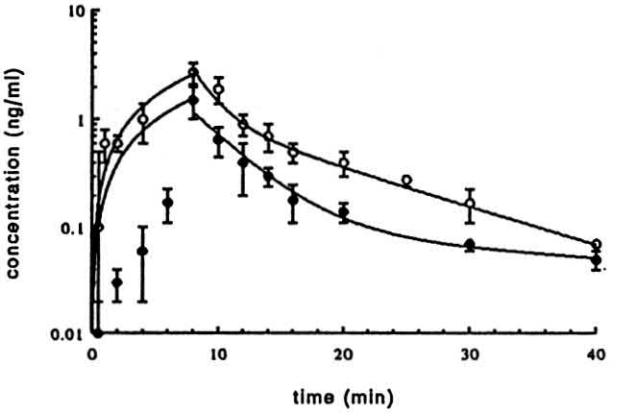

Fig. 7: Mean concentrations in blood (±s.e.m.; n = 6) of (-)--sarin (•) and of C(-)P(-)-soman (o) vs time after nose-only exposure of anesthetized and atropinized guinea pigs to 0.8 LCt_{50} of (±)-sarin and C(±)P(±)-soman, respectively, in the course of 8 min.

Experiments on the inhalation toxicokinetics of (±)-sarin have been restricted to 8-min exposures of guinea pigs to 0.8 and 0.4 LCt50 of this agent (Spruit et al, 2000). See Figure 7 for comparative results with C(±)P(±)-soman. As in the case of soman, there is a rapid absorption of the agent during inhalation, Obviously, the maximum concenration of (-)sarin after respiratory exposure is also comparable to that of C(-)P(-)-soman for an equitoxic exposure. The terminal half life of (-)-sarin appears to be nearly an order of magnitude longer than that of C(-)P(-)-soman, as in the case of iv bolus administration.

Inhalation toxicokinetics upon low level exposure

Investigations on the toxicokinetics of nerve agents have centered on lethal and supralethal doses of nerve agent.However, the controversy on the possible relationship between the so-called Gulf War Syndrome and exposure to traces of nerve agent shortly after the Gulf War has emphasized that knowledge on the acute and delayed effects of trace exposure to nerve agents is almost nonexistent. For example, miosis, rhinorrhea, dyspnea and tightness of the chest was observed in rescue workers and medical personnel in hospitals due to secondary exposure to small amounts of agent subsequent to the terrorist attack with sarin in Matsumoto and in the metro of Tokyo. Sarin vapor could be detected in houses up to 12 h after the attack with sarin in Matsumoto. Some victims in this city reported their first symptoms as late as 20 h after the incident, presumably due to the cumulative effect of persistent low level exposure.

In order to initiate a quantitative basis for the toxicology of low dose exposure to nerve agents, we investigated the toxicokinetics of the four stereoisomers of C(±)P(±)-soman upon nose-only exposure of anaesthetized, atropinized and restrained guinea pigs to 20 ppb (160 $\mu g.m^{-3}$) of C(±)P(±)-soman over a 5 h exposure period, providing blood levels of the toxic C(±)P(-)-soman stereoisomers at Ct-values accumulating from 0–48 $mg.min.m^{-3}$. Concomitantly, the progressive inhibition of AChE in erythrocytes was measured.

The exposures were performed using the apparatus as described in Figure 5 with minor adaptations. The concentrations of the C(+)P(-)- and C(-)P(-)-soman stereoisomers, measured during and after

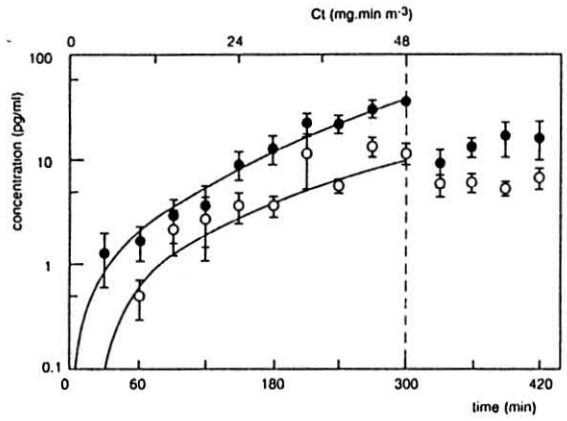

Fig. 8: Semilogarithmic plot of the mean concentrations (± SEM, n = 6) in blood of C(-)P(-)-soman (●) and C(+)P(-)-soman (O) versus time during nose-only exposure of anesthetized, atropinized and restrained guinea pigs to 160 ± 16 $\mu g.m^{-3}$ of C(±)P(±)-soman for 300 min and up to 120 min after exposure. Accumulated Ct-values are also shown. The solid lines represent optimal fits of bi-exponential functions to the data. The dotted line marks the end of the exposure period.

exposure, are given in Figure 8. A bi-exponential equation sufficed to describe the gradually increasing concentrations of the C(+)P(-)- and C(-)P(-)-soman stereoisomers, when adopting a lag time of 30 min for the C(+)P(-)-stereoisomer. It was not attempted to describe the time-concentration course in the 120 min post-exposure period. The blood levels decrease clearly in the first 90 min post-exposure but remain remarkably constant over the next 90 min period. One intriguing explanation, which needs validation, is the formation of a "depot" of intact soman, for example in the epithelial tissue of the respiratory tract, from which the C(+)P(-)- and C(-)P(-)-stereoisomers diffuse into the bloodstream.

In case of short term respiratory exposure, the C(-)P(-)-soman stereoisomer penetrated almost immediately in the bloodstream, whereas the appearance of the C(+)P(-)-stereoisomer lagged a few minutes behind. In the present case of low level respiratory exposure, it takes ca 30 min before even the C(-)P(-)-stereoisomer has penetrated, while it takes another 30 min before the C(+)P(-)-stereoisomer appears in measurable concentrations in the bloodstream. Thus, as should be expected, the preferential scavenging of C(+)P(-)-soman becomes more outspoken upon lowering the doses of C(±)P(±)-soman. In this extreme case of low dose exposure, the AUC of the C(+)P(-)-stereoisomer is more than 3-fold lower than that of the C(-)P(-)-stereoisomer, in spite of the 22% excess of the

former stereoisomer in C(±)P(±)-soman. It should also be noted that enzymes such as CaE'ss which scavenge C(+)P(-)-soman preferentially, are abundantly available in the epithelial tissue of the upper respiratory tract where, by analogy with sarin, most of the soman is presumably absorbed.

It is remarkable that intact C(-)P(-)-soman becomes detectable in blood after a 30 min exposure to 160 $\mu g.m^{-3}$ (20 ppb), i.e., at a total dose of 4.8 $\mu g.min.m^{-3}$ which is only two orders of magnitude higher than the Ct-value allowed by the U.S. Army during an 8 h occupational exposure to sarin. Inhibition of AChE (as measured "classically" by a decrease in activity) is still marginal under these conditions, but becomes clearly detectable at 60 min, i.e., at Ct = 9.6 $\mu g.min.m^{-3}$. The present investigations are at the limit of possibilities for measuring intact agent in blood upon low level exposure. A further reduction of the dose can only be investigated on a basis of nerve agent accumulated by internal scavengers like BuChE and CaE'ss. Recently, methodology for such an approach has been developed in which sarin is released from sarin-inhibited BuChE by means of fluoride ion-induced reactivation and subsequent isolation and analysis of released sarin. In this way, $\geq$ 0.01% inhibition of BuChE in blood of primates can be quantified. Preliminary investigations indicate that such a degree of inhibition occurs at or only slightly above the occupational exposure limit.

Percutaneous toxicokinetics

Recent systematic investigations on the toxicokinetics and metabolism of VX (Van der Schans *et al*, 2000) include a study on the toxicokinetics of (±)-VX after application of a dose corresponding with 1 LD (125 µg/kg, pc) on the skin of hairless guinea pigs, which corresponds with the most relevant route of penetration of VX in case of chemical warfare. As shown in Figure 9, the concentration of (±)-VX in blood gradually increases up to values of 140 pg/ml at 6 h after application. A lethal level of AChE inhibition in blood (> 90%) is reached not earlier than 4 h after application. While the actual concentration in blood starts to decrease after 6 h, the inhibition of AChE is still increasing. These results indicate an extremely long period of toxicological relevant concentrations, with concomitant problems in treatment of such intoxications.

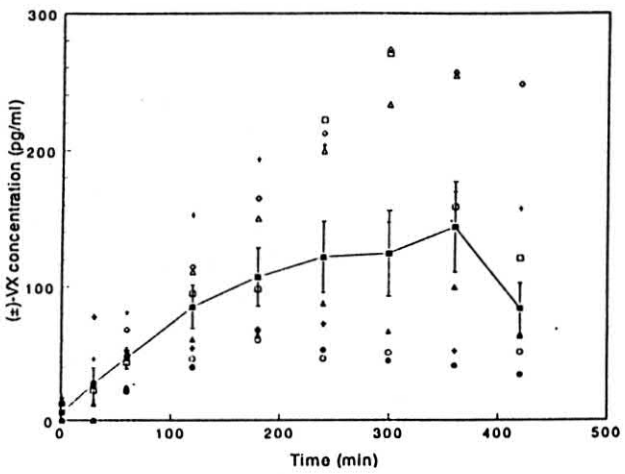

Fig. 9: Mean concentration-time course of (±)-VX (pg/ml, ±s.e.m., n = 7–8) in blood of anesthetized, atropinized, and artificially ventilated hairless guinea pigs after pc application of a dose of 125 µg/kg which corresponds with 1 LD_{50}. Small data points pertain to individual animal experiments.

Future directions

With the methodology that has been developed for the analysis of nerve agents like soman, sarin and VX, minimal detectable concentrations in the range of 1–50 $pg.ml^{-1}$ blood have been obtained. These concentrations are at the lower limit of possibilities at the present or foreseeable state of the art in analytical chemistry. For most investigations on nerve agents, these procedures suffice to investigate the toxicokinetics at a wide range of doses and for all practical routes of exposure. For example, many practical applications of toxicokinetic measurements can be expected in investigations dealing with the efficacy of new antidotes ,e.g., in development of enzymatic scavengers for nerve agents.

The exposure studies of soman at low level have shown that respiratory exposures for several hrs to ca 20 ppb of nerve agent are approximately the utmost that can be reached with regard to toxicokinetics based on *in vivo* measurement of intact nerve agent. If the toxicokinetics at even lower exposure levels should be investigated, e.g. at levels that are in the range of the occupational exposure limit, one has to rely on measurement of nerve agent accumulated by internal scavengers such as BuChE and CaE's. Methodologies for this approach have been developed for sarin and are being developed for other nerve agents, mostly based on release of the protein-bound nerve agent with fluoride ions and

subsequent analysis of the generated phosphofluoridate. Alternatively, mass spectrometric analysis of hydrolyzed fragments of the phosphylated proteins should be considered as a promising approach in the near future.

It should be expected that further quantitative measurements on elimination routes of nerve agents, in combination with the wealth of available toxicokinetic data, will enable the further development of physiologically based modeling of toxicokinetics. Further model developments are needed, in particular for the respiratory and percutaneous exposure routes. Ultimately, the modeling will enable reliable interspecies extrapolation of toxicokinetic results, including extrapolation to man which is the ultimate goal.

References

For references to original papers see:

1. H.P. Benschop and L.P.A. de Jong: Toxicokinetics of Nerve Agents. In: "*Chemical Warfare Agents: Toxicity at Low Levels* (eds. S.M. Somani and J.A. Romano), CRC Press (Boca Raton), 2001, chapter 2.
2. *See also:* H.E.T Spruit, J.P. Langenberg, H.C. Trap, H.J. van der Wiel, R.B. Helmich, H.P.M. van Helden, and H.P. Benschop: Intravenous and inhalation toxicokinetics of sarin stereoisomers in atropinized guinea pigs, Toxicol and Appl. Pharmacol., 169, 249-254 (2000).
3. M.J. van der Schans, B.J. Lander, G.W.H. Moes, H.J. van der Wiel, J.P. Langenberg, and H.P. Benschop: Toxicokinetics of (±)-VX and results of an exploratory study on the *in vitro* metabolism of (±)-VX. Proceedings of the meeting of NATO TG-004 Task Group on Prophylaxis and Therapy of Chemical Agents, 2-5 October 2000, Netherlands Defence College, Rijswijk, The Netherlands (available on CD-ROM).

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