

## PROTECTION AGAINST SOMAN-INDUCED LETHALITY OF THE ANTIDOTE COMBINATION ATROPINE-PRALIDOXIME-PRO-DIAZEPAM PACKAGED AS A FREEZE-DRIED FORM

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### ABSTRACT

The purpose of this study performed in mice was to evaluate, during the time-course of intoxication with the organophosphonate nerve agent soman, the efficacy, in terms of survival, of the combination atropine-pralidoxime-pro-diazepam packaged either as a freeze-dried form or a liquid form compared with atropine-pralidoxime-diazepam. Each antidote combination was given immediately after poisoning. At various time-points during the first 24 h following the intoxication, LD<sub>50</sub> of soman was assessed in animals treated with the different antidote combinations. Then, the protective ratio of each combination was calculated comparatively to control-intoxicated animals. The results show that a) lyophilization does not alter the protective efficacy of the combination atropine-pralidoxime-pro-diazepam, b) replacing diazepam with pro-diazepam gives a protective efficacy increased by about 30% to the three-drug autoinjector therapy (from 15 min after poisoning), c) this increased efficacy persists until 6 h after treatment but decreases thereafter, and d) at 24 h after poisoning, the protective efficacy of the combination atropine-pralidoxime-pro-diazepam, although diminished, is still comparable to that of atropine-pralidoxime-diazepam. Altogether, these results demonstrate that the freeze-dried form of the combination atropine-pralidoxime-pro-diazepam is suitable for emergency self-treatment of organophosphorus poisoning and possesses advantages, in term of early protective efficacy, compared to the current therapy atropine-pralidoxime-diazepam.

### INTRODUCTION

Soman, an organophosphonate compound, is a potent irreversible inhibitor of acetylcholinesterase in both the central and peripheral nervous systems, which can cause severe incapacitation and death. Soman-induced clinical symptoms include salivation, diarrhea, lacrimation, tremors, convulsions, and seizures. Recent pathophysiological studies have revealed that exposure to soman may result in central nervous system and myocardial lesions (McDonough et al., 1995).

The currently recommended therapy against organophosphonate intoxication is based on pretreatment with pyridostigmine, a reversible inhibitor of acetylcholinesterase that undergoes a spontaneous reactivation, followed by treatment with a three-drug regimen consisting of a) atropine, an anticholinergic drug; b) pralidoxime, a reactivator of organophosphorus-inhibited

acetylcholinesterase; and c) diazepam, a benzodiazepine anticonvulsant. This three-drug regimen is packaged in a liquid form in separate autoinjectors which must be administered intramuscularly. An autoinjector includes 2 mg atropine, 7.5 mg diazepam, and 350 mg pralidoxime and must be used by the soldier when first signs of intoxication appear (tremors, salivation, chewing etc.). A second autoinjector must be injected 10–15 minutes later if the signs of poisoning are still present.

It is known that the use of a benzodiazepine such as diazepam reduces the severity of soman-induced convulsions and prevents or reduces subsequent neuropathology (Hayward et al., 1990; Lallement et al., 2000; Lipp, 1972, 1973; Lipp and Dola, 1980). Since diazepam is not water soluble, an organic solvent has to be incorporated into the formulation of the triple-injection solution. This lack of water solubility limits the use of diazepam for intramuscular (i.m.) injection when pharmacologically effective blood levels are required rapidly. Moreover, as argued by Maidment and Upshall (1990), the injection solvent used for diazepam likely slows the absorption of this drug from the injection site. Conversely, the water soluble pro-drug, pro-diazepam (avizafone [CAS: 65617-86-9] or lysyl, peptido-aminobenzophenone diazepam pro-drug) has been developed to be one component in an aqueous drug mixture with atropine sulfate and pralidoxime for the therapy of nerve agent poisoning. Pro-diazepam, *in vivo*, undergoes a rapid hydrolysis by an aminopeptidase to give lysine and diazepam (Maidment and Upshall, 1990; Upshall et al., 1990). The half-life of conversion of avizafone to diazepam is about 4 min in humans (Upshall et al., 1990).

Several studies have shown the protective effects of pro-diazepam against organophosphorus poisoning in rodents (Clement and Broxup, 1993 and other references in Maidment and Upshall, 1990). More recently, we compared in primates the protective efficacy of the liquid formulations of the mixtures atropine-pralidoxime-pro-diazepam and atropine-pralidoxime-diazepam against an acute intoxication with 8 LD<sub>50</sub> of soman (Lallement et al., 2000). We thus demonstrated that to obtain similar protections of the central nervous system, pro-diazepam must be injected at a dose 1.5-fold higher than that of diazepam (doses expressed in  $\mu\text{mol/kg}$ ). However, we also observed that relative bioavailability and clearance (absorption, elimination) of plasmatic diazepam differ depending on whether pro-diazepam or diazepam itself is injected to the animal. As argued (Lallement et al., 2000), these pharmacokinetics differences may influence the time-efficacy of the emergency treatment (latency for diazepam to control seizures, duration of the protective efficacy of the autoinjector self-treatment). Since these results were obtained in primates, it is likely that such pharmacokinetics differences will also exist in humans even if larger quantities of drugs will be used.

Conversely, in order to increase the duration of preservation of the combination atropine-pralidoxime-pro-diazepam, a freeze-dried form of this antidote association has been recently manufactured by the “Pharmacie Centrale des Armées, Orléans, France” (see details in Zabe et al., 2003).

The present study was thus undertaken with a double purpose: a) to compare the protective efficacy against soman of the combination atropine-pralidoxime-pro-diazepam packed either as a liquid form or as a freeze-dried form, and b) to precisely evaluate the efficacy, in terms of survival, of the combination atropine-pralidoxime-pro-diazepam during the time-course of a nerve agent intoxication comparatively to atropine-pralidoxime-diazepam.

## MATERIALS AND METHODS

To achieve the double purpose of this study (see end of the Introduction), we evaluated and compared, at different times during the first 24 h after an acute intoxication, the LD<sub>50</sub> of soman in control mice and in intoxicated mice, then immediately treated with one of the three antidote combinations of interest (atropine-pralidoxime-pro-diazepam packaged as a liquid form, atropine-pralidoxime-pro-diazepam packaged as a freeze-dried form, and the current therapy atropine-pralidoxime-diazepam packaged as a liquid form). Moreover, to verify that these combinations gave, if any, strictly comparable protections of intoxicated animals, we also evaluated and compared, at 24 h time-point, the LD<sub>20</sub> and the LD<sub>80</sub> of soman in control mice and in intoxicated animals treated with one of the three antidote combinations of interest.

### Animals

The “Principles of Laboratory Animal Care” (National Institutes of Health publication number 85-23, revised 1985) were followed in all experiments and the experimental design of the study was approved by the Ethical Committee of our Institute.

Male Swiss mice weighing 30–35 g served as subjects. They were housed under a 12 h/12 h dark/light cycle, with full spectrum light provided between 0700 and 1900 hours, and were given food and water *ad libitum*.

### Chemicals

The freeze-dried form of atropine-pralidoxime-pro-diazepam was obtained from the Pharmacie Centrale des Armées (Orléans, France). To prepare liquid mixtures, atropine sulphate and pralidoxime were purchased from Sigma (France) and Société d’Etudes et de Recherches Biologiques (France), respectively. Diazepam was obtained from Roche laboratories (France) and pro-diazepam from Roche (UK). Soman (purity >98% by gas chromatography) was obtained from Centre d’Etudes du Bouchet (France).

### Experimental Design

Four experimental groups (n=42 per group) were organized. In each group, seven doses of soman were tested (six mice per dose) with a constant ratio of 1.14 between the dose levels. Detailed doses of soman given in each group are given in table 1. The ratio of 1.14 between the doses used allows determination of the LD<sub>50</sub> of soman with a satisfactory precision (see results).

Group 1 (control): Animals injected only with soman at doses varying from 74 to 163 µg/kg (subcutaneous [s.c.] injection).

Group 2: Animals subcutaneously injected with soman at doses varying from 110 to 241 µg/kg and treated by i.m. injection 1 min after poisoning with atropine sulphate (0.053 mg/kg in saline), pralidoxime (9.3 mg/kg in saline), and diazepam (0.2 mg/kg in the specific vehicle of Roche Laboratories). Atropine and pralidoxime were injected in the right limb while diazepam was given in the left one. The doses of atropine, pralidoxime, and diazepam adopted are equivalent to the injection of two autoinjectors in humans (according to French military doctrine).

Groups 3 and 4: Animals subcutaneously injected with soman at doses identical to those for group 2 and treated by i.m. injection 1 min after poisoning with atropine sulphate (0.053 mg/kg), pralidoxime (9.3 mg/kg), and pro-diazepam (0.53 mg/kg). In group 3, the liquid combination of these three drugs was prepared from the chemicals issued from the sources (as described above). In group 4, the liquid combination of atropine, pralidoxime, and pro-diazepam was prepared in saline from the freeze-dried form supplied by the Pharmacie Centrale des Armées (Zabe et al., 2003). As mentioned, in both groups, pro-diazepam was injected at 0.53 mg/kg (i.e., a dose of 1.05  $\mu\text{mol/kg}$ ). This corresponds, as stated in the introduction, to 1.5-fold the dose of diazepam (expressed in  $\mu\text{mol/kg}$ ) used in group 2, which was 0.2 mg/kg (i.e., 0.7  $\mu\text{mol/kg}$ ).

In each group, mortality rates due to the various doses of soman were noted at the following time-points after intoxication: 15, 30, 45 min; 1 h; 1 h 15 min; 1 h 30 min; 1 h 45 min; 2 h; 2 h 30 min; 3 h; 3 h 30 min; 4, 5, 6, and 24 h (in the legend of figure 1, these times are expressed in min). At each time-point of observation and in each group, the  $\text{LD}_{50}$  of soman and its confidence limits at 95% were then calculated using probit analysis (Finney, 1971).

Thereafter, for a given time-point, the following was observed:

- The  $\text{LD}_{50}$ s of soman observed in the four groups were statistically compared in pairs using  $\chi^2$  test from logarithmic values obtained by the probit analysis (significance was set at  $p < 0.05$ ) (Schwartz, 1994).
- The protective potency afforded by each of the three antidote associations was calculated using the following protective ratio:  $\text{LD}_{50}$  in treated animals/ $\text{LD}_{50}$  in control animals (group 1).

Moreover, in each of the four groups, at the 24 h time-point, beside the  $\text{LD}_{50}$ , we also calculated by probit analysis the  $\text{LD}_{20}$  and  $\text{LD}_{80}$  of soman, and their confidence limits. Then,  $\text{LD}_{20}$  and  $\text{LD}_{80}$  were statistically compared between the four groups as described above ( $\chi^2$  test).

## RESULTS AND DISCUSSION

Toxicity of soman in the four experimental groups during the first 24 h after poisoning is presented in table 2A. During this period,  $\text{LD}_{50}$  of the nerve agent was always significantly increased in the three groups receiving antidote combinations compared to control animals of group 1, proving that antidote combinations are effective. Moreover, table 2B shows that, at the 24 h time-point, there was no significant difference of lethal potency of soman (assessed through the determinations of its  $\text{LD}_{20}$ ,  $\text{LD}_{50}$ , and  $\text{LD}_{80}$ ) between the three groups receiving antidote combination. Altogether, this first observation confirms (Lallement et al., 2000) that incorporation of pro-diazepam at a dose 1.5-fold higher than that of diazepam (dose expressed in  $\mu\text{mol/kg}$ ) confers, 24 h after poisoning, strictly similar protective potencies on the atropine-pralidoxime-pro-diazepam and atropine-pralidoxime-diazepam combinations.

Despite similar protective efficacies for the three treated groups at 24 h time-point, a more accurate analysis of the protective potency of the three antidote combinations, during the first 24 h after poisoning and especially during the first 6 h, reveals obvious differences between group 2 and groups 3 and 4.

In this view, table 2A shows that, whatever the time after poisoning (from 15 min to 6 h), lethal toxicity of soman (assessed through its LD<sub>50</sub>) remains statistically unchanged between the groups 3 and 4; an observation also valid at the 24 h time-point (table 2B). Analysis of the time-course of the protective ratio of each antidote combination during the first 24 h after intoxication corroborates these data (figure 1). Indeed, during this period, protective ratios in groups 3 and 4 are always very similar. All of this indicates that the lyophilization process does not alter the protective efficacy of the combination atropine-pralidoxime-pro-diazepam.

On the other hand, as indicated in table 2A, during the first 6 h after poisoning, the LD<sub>50</sub> of soman was always significantly increased in groups 3 and 4 compared to group 2. This demonstrates that, during the first 6 h after poisoning, the protective efficacies achieved by the combination atropine-pralidoxime-pro-diazepam (issued from commercial sources or from the freeze-dried form) are significantly better than that given by atropine-pralidoxime-diazepam. This point is confirmed in figure 1 since during the first 6 h after poisoning, protective ratios in groups 3 and 4 were always, on average, 25 to 30% higher than those observed in group 2 (ratios varying from about 2 to 2.2 in groups 3 and 4 vs. 1.7 to 1.75 in group 2). Remarkably, compared to atropine-pralidoxime-diazepam, the obvious protective superiority of the combination atropine-pralidoxime-pro-diazepam was observable as from 15 min after treatment. This could be due to the fact that plasmatic diazepam level is known to be achieved faster after injection of pro-diazepam than after diazepam itself (Maidment and Upshall, 1990; Capacio et al., 2001; Lallement et al., 2000).

As argued by Maidment and Upshall (1990), the short C<sub>max</sub> value of plasmatic diazepam observed after injection of pro-diazepam may be due to its water solubility and its subsequent rapid absorption. Moreover, the injection solvent used for diazepam might also slow the absorption of this drug from the injection site (Maidment and Upshall, 1990). Altogether, the present early protective superiority (as from 15 min after treatment) of the combination atropine-pralidoxime-pro-diazepam reinforces the previously described advantage (Lallement et al., 2000) for the use of pro-diazepam instead of diazepam in the emergency treatment of organophosphorus poisoning since seizures and subsequent neuropathology will be counteracted more rapidly.

As discussed above, one day after intoxication, lethal potency of soman is globally similar in the three groups receiving antidote combination. Thus, compared to atropine-pralidoxime-diazepam, the superior protective efficacy of the combination atropine-pralidoxime-pro-diazepam noted during the first 6 h after poisoning is no longer observable at 24 h time-point. As shown in tables 2A and 2B, this is likely related to the obvious decrease of the LD<sub>50</sub> of soman in groups 3 and 4 between the 6 and 24 h time-points compared to group 2. Data in figure 1 corroborate this finding. Indeed, the better protective ratios observed during the first 6 h after poisoning in groups 3 and 4 obviously decline until 24 h time-point at values approaching that of group 2, this latter remaining stable throughout the experiment. The increase in protective efficacy afforded by atropine-pralidoxime-pro-diazepam between 6 and 24 h after poisoning could be due to the fact that plasmatic diazepam level is known to decline more rapidly after injection of pro-diazepam than after administration of diazepam (Lallement et al., 2000). However, from an applied point of view, the fact that, compared to atropine-pralidoxime-diazepam, the improved protective efficacy afforded by atropine-pralidoxime-pro-diazepam declines after the 6 h time-point does not constitute a re-inhibitory defect. Indeed, it is very likely that 6 h will be sufficient to institute a delayed medical support with classical intravenous injections or perfusions of atropine, pralidoxime, and benzodiazepine. Moreover, even diminished, the protective effect of atropine-pralidoxime-pro-diazepam is still

equivalent, 24 h after poisoning, to that of atropine-pralidoxime-diazepam. And finally, the improved protective efficacy afforded by the combination atropine-pralidoxime-pro-diazepam, at least until 6 h after intoxication with soman, will certainly be accompanied by an effective protection against the development of seizures and subsequent neuropathology; this latter usually appears in the first 1–6 h after poisoning (Lemercier et al., 1983).

In conclusion, this study shows several successful results obtained with the replacement of the combination atropine-pralidoxime-diazepam by atropine-pralidoxime-pro-diazepam for emergency self-treatment of organophosphorus poisoning. First, replacing diazepam by a 1.5-fold higher dose of pro-diazepam (dose in  $\mu\text{mol/kg}$ ), from 15 min after poisoning, increases the protective efficacy by about 30% to the three-drug autoinjector therapy. Second, the increased efficacy of the combination atropine-pralidoxime-pro-diazepam is observable until 6 h after poisoning (i.e., during the period in which development of seizure activity and subsequent neuropathology develops from exposure to the nerve agent). Third, even though diminished at 6 h, the protective efficacy of the combination atropine-pralidoxime-pro-diazepam is still comparable to that of atropine-pralidoxime-diazepam 24 h after intoxication. And lastly, the lyophilization process does not alter the protective efficacy of the combination atropine-pralidoxime-pro-diazepam.

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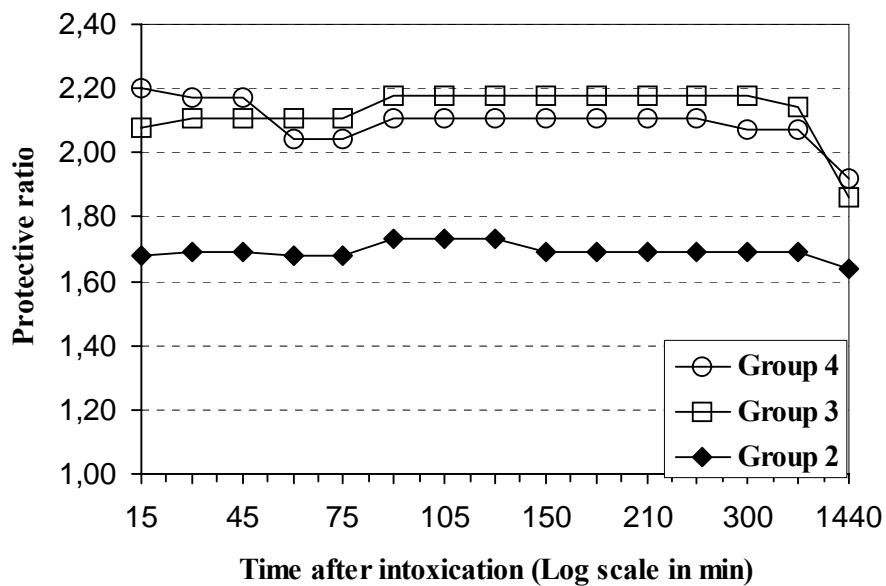
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**Figure 1. Protective ratio of each antidote combination during the time-course of the intoxication**



**Notes:**

For a given time-point, protective ratio is calculated as follows:  $LD_{50}$  in treated animals/ $LD_{50}$  in control animals of group 1. Group 2 is treated with atropine-pralidoxime-diazepam. Groups 3 and 4 are treated with atropine-pralidoxime-pro-diazepam issued from commercial sources or from the freeze-dried form, respectively.

**Table 1. Doses and groups used in experiments**

	<b>Group 1<sup>*</sup></b>	<b>Group 2<sup>†</sup></b>	<b>Group 3<sup>‡</sup></b>	<b>Group 4<sup>§</sup></b>
<b>Soman dose µg/kg</b>	74	110	110	110
	84	125	125	125
	96	142	142	142
	110	163	163	163
	125	186	186	186
	142	212	212	212
	163	241	241	241

<sup>\*</sup> Controls.

<sup>†</sup> Treated by i.m. injection 1 min after poisoning with atropine sulphate (0.053 mg/kg in saline), pralidoxime (9.3 mg/kg in saline), and diazepam (0.2 mg/kg in the specific vehicle of Roche Laboratories). The doses of atropine, pralidoxime, and diazepam adopted are equivalent to the injection of two autoinjectors in humans.

<sup>‡</sup> Treated by i.m. injection 1 min after poisoning with atropine sulphate (0.053 mg/kg), pralidoxime (9.3 mg/kg), and pro-diazepam (0.53 mg/kg) prepared from the commercial sources.

<sup>§</sup> Treated by i.m. injection 1 min after poisoning with atropine sulphate (0.053 mg/kg), pralidoxime (9.3 mg/kg), and pro-diazepam (0.53 mg/kg) prepared in saline from the freeze-dried form supplied by the Pharmacie Centrale des Armées (Zabe et al., 2003).

**Table 2A. Toxicity of soman in the different experimental groups during the time-course of an intoxication**

Time after poisoning	LD <sub>50</sub> of soman (µg/kg)			
	Group 1 <sup>*</sup>	Group 2 <sup>†</sup>	Group 3 <sup>‡</sup>	Group 4 <sup>§</sup>
<b>15 min</b>	104.2 (94.1–115.4)	175.5 <sup>  </sup> (167.2–184.2)	216.3 <sup>  , #</sup> (203.3–230.1)	229.4 <sup>  , #, ns</sup> (204.0–257.9)
<b>from 30 to 45 min</b>	95.6 (87.3–104.7)	162.0 <sup>  </sup> (153.1–171.4)	202.1 <sup>  , #</sup> (192.9–211.7)	207.0 <sup>  , #, ns</sup> (176.3–243.0)
<b>from 1 h to 1 h 15 min</b>	95.6 (87.3–104.7)	160.6 <sup>  </sup> (152.3–170.3)	202.1 <sup>  , #</sup> (192.9–211.7)	195.4 <sup>  , #, ns</sup> (180.3–211.8)
<b>from 1 h 30 min to 2 h</b>	92.7 (83.8–102.6)	160.6 <sup>  </sup> (152.3–170.3)	202.1 <sup>  , #</sup> (192.9–211.7)	195.4 <sup>  , #, ns</sup> (180.3–211.8)
<b>from 2 h 30 min to 4 h</b>	92.7 (83.8–102.6)	156.9 <sup>  </sup> (151.4–162.6)	202.1 <sup>  , #</sup> (192.9–211.7)	195.4 <sup>  , #, ns</sup> (180.3–211.8)
<b>5 h</b>	92.7 (83.8–102.6)	156.9 <sup>  </sup> (151.4–162.6)	202.1 <sup>  , #</sup> (192.9–211.7)	192.3 <sup>  , #, ns</sup> (171.6–215.5)
<b>6 h</b>	92.7 (83.8–102.6)	156.9 <sup>  </sup> (151.4–162.6)	198.8 <sup>  , #</sup> (185.0–213.8)	192.3 <sup>  , #, ns</sup> (171.6–215.5)

**Table 2B. Variations in measures of LD at the 24 hour point for each group**

<b>24 h after poisoning</b>	<b>Group 1<sup>*</sup></b>	<b>Group 2<sup>†</sup></b>	<b>Group 3<sup>‡</sup></b>	<b>Group 4<sup>§</sup></b>
<b>LD<sub>20</sub> (µg/kg)</b>	81.6 (71.1–93.7)	138.8 <sup>  </sup> (131.7–146.4)	120.5 <sup>  , ns</sup> (96.6–150.2)	135.0 <sup>  , ns, ns</sup> (121.1–150.5)
<b>LD<sub>50</sub> (µg/kg)</b>	92.7 (83.8–102.6)	151.6 <sup>  </sup> (145.5–157.9)	172.1 <sup>  , ns</sup> (155.6–190.4)	177.8 <sup>  , ns, ns</sup> (173.8–181.8)
<b>LD<sub>80</sub> (µg/kg)</b>	105.3 (92.3–120.2)	173.8 <sup>  </sup> (166.8–180.9)	215.8 <sup>  , ns</sup> (206.2–221.7)	216.2 <sup>  , ns, ns</sup> (202.5–225.7)

Notes: Toxicity is assessed during the first 24 h after poisoning through the determination of the LD<sub>50</sub> of soman in the different experimental groups. Moreover, at 24 h time-point, the LD<sub>20</sub> and the LD<sub>80</sub> of the nerve agent are also determined.

Values given are LDs and confidence limits at 95% (in parentheses) determined by probit analysis.

Statistical comparisons in pairs of the LDs by  $\epsilon$  test from logarithmic values obtained by probit analysis.

\* control

† treated with atropine-pralidoxime-diazepam (see details of doses in table 1).

‡ treated with atropine-pralidoxime-pro-diazepam prepared from commercial sources (see details in table 1).

§ treated with atropine-pralidoxime-pro-diazepam issued from the freeze-dried form (see details in table 1).

|| significant difference vs. control at  $p < 0.001$

# significant difference vs. group 2 at  $p < 0.01$

<sup>ns</sup> no statistical difference vs. group 2

<sup>ns</sup> no statistical difference vs. group 3