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Detection of sarin in plasma of rats after inhalation intoxication

LUCIE BARTOSOVA, MARCELA BIELAVSKA, & JIRI BAJGAR

[1]*Faculty of Military Health Sciences, Trebesska 1575, Hradec Kralove, Czech Republic*

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Abstract

Presently used methods for detection and diagnosis of the severity of intoxication with organophosphorus (OP) compounds are mostly those that quantify inhibition of blood cholinesterases. It was found that when plasma inhibited with OP compounds is incubated in the presence of a high concentration of fluoride ions, the organophosphate is released from the enzyme thus yielding a phosphofluoridate, which can be analyzed by gas chromatography and NP detection. In our study, the concentration of sarin released after fluoride ions were added to the plasma of sarin-poisoned rats was determined. Sarin amounts in plasma measured after refluoridation and plasma butyrylcholinesterase activity in ten rats, that were exposed to sarin vapors at concentration of 1.25 μg/L (E1 group) and 2.5 μg/L (E2 group) respectively, for 60 min. In the E2 group the concentration of sarin in plasma was nearly 2-fold higher than in the E1 group. These results correspond well with the concentrations of sarin vapors to which the animals were exposed. Both experimental groups of animals showed significant decreases in butyrylcholinesterase activity by more than 30%–36.4% (E1 group) and 47.0% (E2 group). The method of fluoride-induced reactivation provides a very good marker for monitoring sarin intoxication in laboratory animals determined previously mostly by ChE determination which does not allow any information on sarin amounts in plasma.

Keywords: *Organophosphorus compounds, acetylcholinesterase, inhibition, sarin, plasmalevels, rats*

Introduction

Organophosphorus (OP) compounds are used in industry as plasticators, hydraulic fluids, in veterinary and human medicine as drugs or as compounds for research of nervous functions and they can be also misused in armed conflicts and terrorist attacks as chemical warfare agents [1].

Presently used methods for detection and diagnosis of the severity of intoxication by OP compounds are mostly those that quantify inhibition of blood cholinesterases (ChEs). These methods do not allow identification of the OP compounds to which people were exposed and when inhibition of acetylcholinesterase (EC 3.1.1.7, AChE) activity in the red blood cells is less than 20% they are not reliable evidence for OP exposure. Even if intoxication is not treated, the activity of blood ChEs increases because of *de novo* synthesis of enzymes and spontaneous hydrolysis of inhibited enzymes, thus these methods are not suitable for retrospective analysis of exposures to OP compounds [2]. The intact compound and its metabolites can only be measured shortly after the exposure [3].

OP compounds mainly react with three enzyme groups in the mammalian body: cholinesterases [ChEs: AChE and butyrylcholinesterase (EC 3.1.1.8 BuChE)], carboxylesterases (aliesterases, EC 3.1.1.1, CaEs) and OP-hydrolyzing enzymes called A-esterases (arylesterhydrolases, EC 3.1.1.2). The ChEs and CaEs in blood are inhibited by OP compounds, but their role in the mechanisms of the toxic effect is not clear. These enzymes act as alternative binding sites for OP compounds and thus reduce the amount of OP compound available to react with AChE in the target sites [4]. The protective role

Correspondence: L. Bartosova, Department of Toxicology, Faculty of Military Health Sciences, Trebesska 1575, 500 01, Hradec Kralove Czech Republic. Tel. +420 973 251 534. Fax. +420 495 512 430. Email: bartosova@pmfhk.cz

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of BuChE may be confirmed by the fact, that people having a low level of BuChE have a more serious course of intoxication with OP pesticides [5]. The role of CaEs in the binding of OP compound has been thought to be critical, since the capacity of CaE binding sites in the mammalian body greatly exceeds that of ChEs [4]. CaEs, whose only known function is detoxify a wide variety of xenobiotic esters, have much higher affinity to nerve agents and have a more accessible active centre than AChE [6]. Especially in the blood of rodents (and rabbits), unlike in primates, CaEs are present at high concentrations [7]. The A-esterases such as chlorpyrifos oxonase and paraoxonase protect against anticholinesterase toxicity by catalytically inactivating oxons. The amounts and activities of these three enzyme groups vary greatly in different tissues and species [4]. Their affinities for OP compounds depend on the tissue, species and OP compound. The effects described are underlined especially in intoxications with highly toxic agents like sarin, soman etc. Exogenous administration of plasma-derived ChE in both rodent and non-human primate models, has been successfully used as a safe and efficacious prophylactic treatment to prevent poisoning by OP compound [8].

It was found that when plasma ChEs inhibited with OP compounds is incubated in the presence of a high concentration of fluoride ions, the organophosphate is released from the enzyme thus yielding a phosphofluoridate [9]. As a result of the high nucleophility of the fluoride ions toward the (bound) phosphoryl moiety the fluoride ion is capable of releasing the OP moiety from the enzyme. The covalent bond between the OP moiety and the enzyme will be replaced by a covalent bond between fluoride and the OP moiety, leading to the formation of a phosphofluoridate [10] (Figure 1), which can be analyzed by gas chromatography and NP detection or mass selective detection. Sample clean up through solid phase extraction and solvent transfer to ethylacetate is necessary to enable GC analysis [9].

In the case of human plasma that was treated with sarin, a high concentration of fluoride ions can reactivate inhibited AChE and BuChE. In our study, the concentration of sarin released after addition of fluoride ions and the BuChE activity in plasma of sarin-poisoned rats was determined.

Methods and materials

Chemicals and materials

Sarin (87.5%, *O*-isopropyl methylphosphonofluoridate) and diisopropylfluorophosphate (DFP) were obtained from Zemianské Kostolany, Slovak Republic. All other chemicals used were of p.a. purity. Isopropanol dried (IPA), acetic acid (189 mM), sodium acetate trihydrat (10.8 mM), sodium fluoride (12.5 M), ethylester acetate (EA) were from Lachema, a.s. (Neratovice, Czech Republic). Heparin (5 000 IU/mL) was obtained from Léčiva (Praha, Czech Republic). Water was purified by reverse osmosis and filtration using Aqua 50 apparatus (Goro, s.r.o., Czech Republic). SepPak C_{18} cartridges type "Classic" 360 mg/cartridge were from Waters Associates (Millipore Corporation, Milford, MA, U.S.A.).

Animals

Female albino Wistar rats weighing 170–190 g were purchased from Biotest Konárovice (Czech Republic). They were kept in an air-conditioned room with lights on from 7 a.m. to 7 p.m. and were allowed access to standard food and tap water *ad libitum*. The rats were divided into groups of five animals. Handling of the experimental animals was done under supervision of the Ethics Committee of the Purkyne Military Medical Academy, Czech Republic.

Animal experiments

Before inhalation exposure to sarin, blood was collected initially from the tail vein for animals in both experimental groups. The second blood collection was after inhalation exposure to sarin. Each group of rats was exposed to sarin vapors in an inhalation chamber [11,12] for 60 min. The sarin concentrations in the inhalation chamber were 1.25 μg/L for the E1 group and 2.5 μg/L for the E2 group.

Preparation of samples for calibration

Solutions of sarin and DFP for calibration were prepared at following concentrations: 5, 10, 50 μg/mL sarin in IPA and 10 μg/mL DFP in IPA. An aliquot (10 μL) of a standard solution was added to rat plasma (0.9 mL; animal non-exposed to sarin

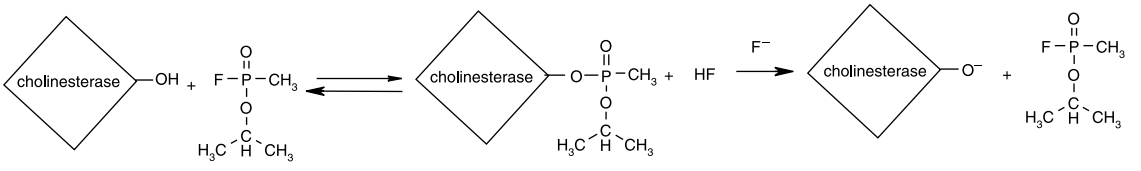

Figure 1. Scheme of fluoride-induced reactivation of sarin-inhibited cholinesterase

vapors) in a polytetrafluoroethylene test-tube and the mixture was incubated at 37°C for 10 min. Samples were then processed as described below under fluoride-induced regeneration.

Preparation of rat plasma samples

Rat plasma was acquired by centrifugation (2000×g, 10 min) of fresh heparinized blood of rats which was collected immediately after exposure to sarin vapors and then quickly frozen.

Fluoride-induced regeneration of sarin from binding sites in plasma. Rat plasma was mixed with an acetate buffer (3 mL, pH 3.5) consisting of 189 mM acetic acid and 10.8 mM sodium acetate trihydrate. A solution (80 μL) of 12.5 M potassium fluoride in water was added and the mixture was incubated at 25°C for 15 min. Then an aliquot (10 μL) of the standard solution of DFP (10 μg/mL) was added as an internal standard (ISTD) and the mixture was well shaken.

Solid phase extraction of sarin. Sarin was isolated from the incubated mixture by direct elution with 1.2 mL of EA from a SepPak C_{18} column, which was pre-rinsed with EA and water. The EA layer was separated from the aqueous phase by freezing the water with dry ice/acetone. The EA extract was stored in GC vials at −20°C until GC analysis.

BuChE activity in plasma. The activity of inhibited BuChE in plasma was determined in TRIS – HCl buffer (pH 7.6) at 22°C by the spectrophotometric method [13] using butyrylthiocholine iodide as a substrate and 5,5′-dithio– bis (2-nitrobenzoic) acid as the chromogen. The activities were expressed as μcat/mL blood (cat/L= mol of substrate hydrolysed/s/L).

GC analysis of sarin. Analyses were performed by using an HP 6890 gas chromatograph equipped with an CP-Sil 8CB column (length 10 m, i.d. 0.32 mm, film thickness 1.2 μm), an alkali flame ionization (NP) detector and an HP ChemStation, Rev. A.06.01.(403) (Hewlett Packard, Wilmington, DE, U.S.A.). The flow of the carrier gas helium was 3.7 mL/min and the flows of hydrogen, air and make-up gas (nitrogen) through the detector were 2.60 and 10 mL/min, respectively. The volume of splitless injection was 1 μL. For each chromatographic run, the column was kept at 60°C for 4 min and then subsequently heated to 200°C at 40°C/min. The injector and detector blocks were held at 250°C and 280°C, respectively.

Quantitative analysis of sarin was based on comparison of the peak area ratio of sarin and the internal standard DFP with those obtained for mixtures of standard solutions of the two organophosphates.

Statistics. Data are given as the mean ± SEM unless otherwise noted. Results were analyzed by Student's *t*-test. Correlation between the plasma butyrylcholinesterase inhibition and sarin amounts in plasma after fluoride-induced reactivation was tested by calculating Pearson's correlation coefficient, *r*. The level of significance was set at $p < 0.05$ in all cases.

Warning

In view of its extreme toxicity and volatility, sarin should only be handled in specialized and approved laboratories where trained medical personnel are continuously present.

Results

The gas chromatographic analysis of sarin was calibrated using mixtures of the standard solutions of the organophosphate and the internal standard DFP containing 5, 10 or 50 μg/mL of sarin and 10 μg/mL of DFP. An example of a chromatogram obtained for these analysis is given in Figure 2.

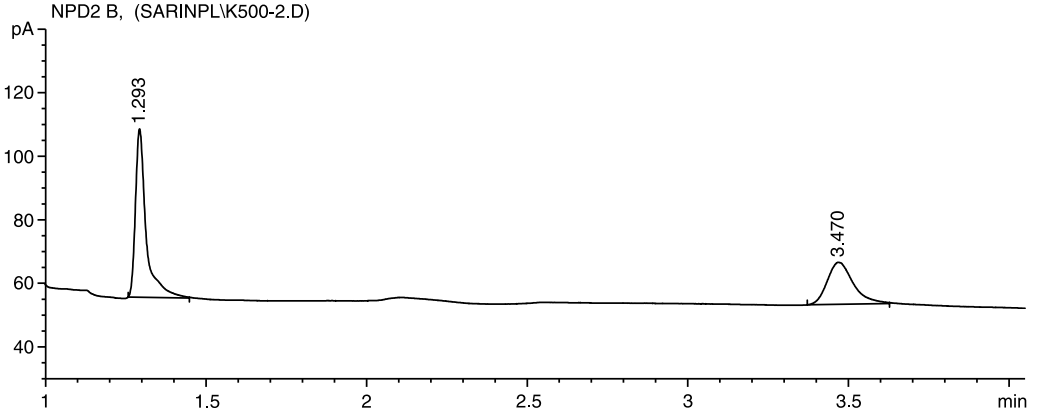

Figure 2. Gas chromatographic analysis of an ethyl acetate extract of a sample of rat plasma (0.9 mL) incubated with sarin (5 μg/mL) and subsequently treated with 12.5 M potassium fluoride; DFP (second eluting peak) was used as an internal standard.

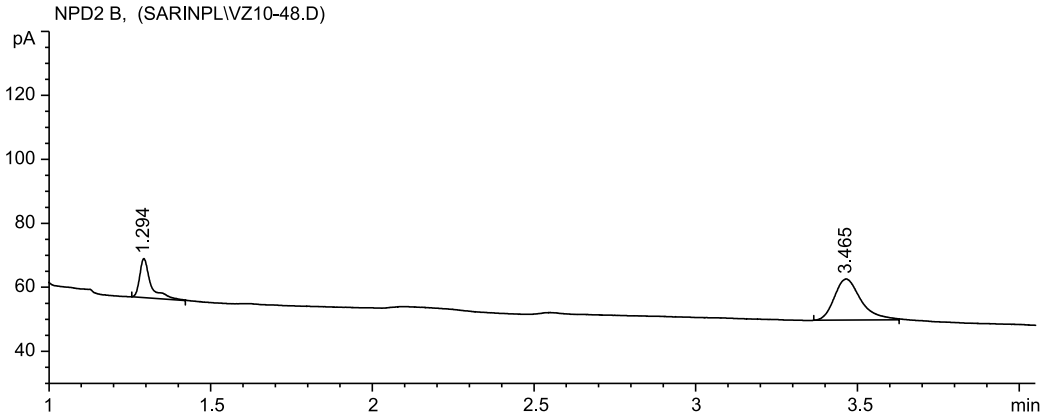

Figure 3. Gas chromatographic analysis of an ethyl acetate extract of a rat plasma from an animal intoxicated wih 1.25 μg/mL of sarin in an inhalation chamber for 1 h (animal no. 1); DFP (second eluting peak) was used as an internal standard.

A linear relationship between the peak area ratios for the two organophosphates and the concentrations of sarin was obtained. The calibration curve was $y = 0.0032x + 0.0375$ (y- peak area ratio, x- sarin concentration (5 μg/mL, 10 μg/mL, 50 μg/mL)); $R^2 = 0.9988$; recovery for sarin 76.0%; recovery for DFP 86.8%. R_t values were 1.293 min for sarin (first elution peak) and 3.470 min for DFP (second elution peak).

In our study we have measured the sarin concentration in plasma of ten rats, which were exposed to sarin vapors at concentrations of 1.25 μg/L and 2.5 μg/L respectively, for 60 min (Figure 3).

Concentrations of sarin determined in the plasma of experimental animals are presented in Table I. In the E2 group the concentration of sarin in plasma was 454 $ng \cdot ml^{-1}$ that means that it is nearly 2-fold higher than in the E1 group (262 ng/mL). These results correspond well with the concentrations of sarin vapors to which the animals were exposed (1.25 μg/L and 2.5 μg/L).

BuChE activities in plasma for both groups of experimental (E1-2) animals are presented in Figure 5. BuChE activities 30 min before inhalation exposure were in the range 28 – 34 μcat/mL. Following inhalation intoxication with sarin, significant decreases in plasma BuChE activities were observed in both experimental groups. Inhalation exposure to sarin resulted in a decrease to 63.6% of normal BuChE activity in the E1 group and 53.0% in the E2 group (Figure 4). Difference between BuChE activities of both experimental groups after exposure to sarin vapors was not significant.

The correlation between the plasma BuChE inhibition and sarin amounts in plasma of rats after intoxication with sarin was evaluated (Figure 5). We observed a positive correlation ($r = 0.741$; $p < 0.05$) between BuChE inhibition and sarin amounts in plasma.

The rats were observed for incidence of the typical signs of nerve agent intoxication (salivation, fasciculations, tremor, convulsions, respiratory distress) during exposure to sarin vapors as well as after ending the exposure (before the second blood withdrawal). No animal from both groups displayed any signs of nerve agent intoxication.

Table I. Concentration of sarin in rat plasma after fluoride-induced reactivation. Values are means ± SEM, n = 5.

Concentration of sarin vapors in inhalation chamber (μg/L)	Concentration of sarin in rat plasma (ng/mL)
1.25	262 ± 12
2.50	454 ± 77

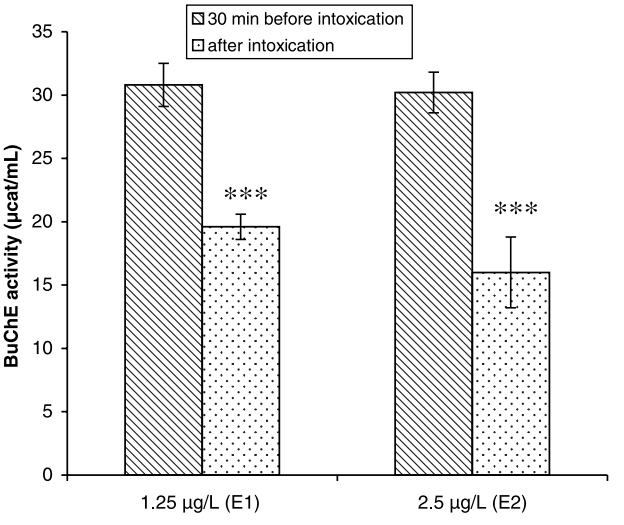

Figure 4. Butyrylcholinesterase activity in plasma of rats intoxicated with sarin. E1 – experimental animals exposed to sarin vapors at concentration 1.25 μg/L; E2- experimental animals exposed to sarin vapors at concentration 2.5 μg/L. Results represent mean ± SD, n = 5 animals/group. Statistical significance: *** $p < 0.005$ (each animal was it's own control).

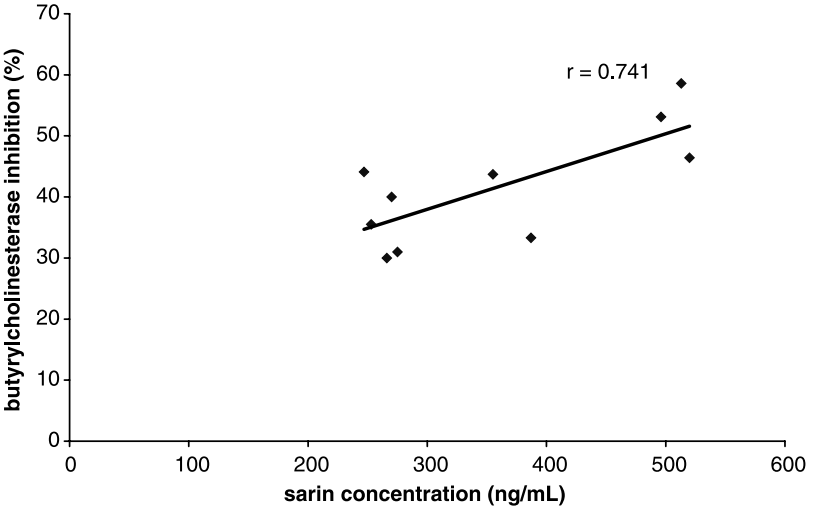

Figure 5. Correlation between plasma butyrylcholinesterase inhibition and sarin amounts in rat plasma after intoxication with sarin. These data were fitted using linear regression analysis. A significant ($r = 0.74$; $p < 0.05$) positive linear correlation occurred.

Discussion

Nerve agents can bind not only to BuChE but also to other binding sites in plasma, such as albumin [14] or CaE [6]. Man seems to have a quite low serum CaE activity, in contrast to the mouse, guinea-pig or rat. The CaE activity in the plasma of adult rat is more than 4-fold higher than that in human plasma [4,15]. Prior in vivo inhibition of plasma CaE with selective inhibitors such as 2-[o-cresyl]-4H-1,2,3-benzodioxaphosphorin-2-oxide (CBDP) has been shown to potentiate the acute toxicity of several agents [4,16]. The wide interspecies differences in the nerve agent LD_{50} values (e.g. soman) in control animals could be reduced to a common LD_{50} value in CBDP-treated animals whose CaE had been selectively inhibited [4,6]. This would be helpful with regard to remarkable differences of basal enzyme activities of man as compared with mouse, rat or guinea pig, the species widely used in research on OPC toxicity.

The normal value of BuChE activity in pooled blank European serum is 13.3 IU/mL [2], while the BuChE activity in rat plasma is nearly fourteen-fold higher. Our results of normal BuChE activity in rat plasma - 1800 IU/mL are very well comparable with those of Mioduszewski et al. – 1750 IU/mL [17]. Unfortunately the data about the amount of BuChE in rat plasma to calculate a maximum amount of sarin that could be generated from completely inhibited BuChE are not available. Upon our results we can only speculate, that it could be even double the amount that was measured in the E2 group (53% inhibition of BuChE; amount of sarin - 455 ng/mL).

There are sex differences in the activity of BuChE and CaE (but not AChE) both in rodents and in man [18,19]. Sterri and Fonnum speculated that the two-fold higher BuChE activity in the female rat is probably due to a doubling in the number of enzyme molecules, which should also double the number of binding sites for OP compounds [18].

The greater importance of CaE vs. BuChE, as a detoxifying resource of OP compounds, has been emphasized by several investigators [6,20]. Maxwell, et al., [6] suggested this may be due to the greater frequency of CaE binding sites. Because CaE activity was not measured in this study, it would be difficult to speculate about the possible role of CaE in accounting for much higher amounts of sarin released after fluoride-induced reactivation in our study with rats in comparison with already published data using marmosets and guinea-pigs [21].

There are many biochemical parameters changed during OP poisoning – more or less specific and/or sensitive [22] e.g. esterases, alkaline phosphatase, number of leucocytes etc. Also acid metabolites of OP can serve as a chemical marker for monitoring OP exposure in humans [23]. The method of fluoride-induced reactivation is very good for diagnostic purposes: currently diagnosis of OP poisoning is based on determination of ChE activity (either in the blood or erythrocytes or plasma). However, the measurement of ChE activity (in the case of decreased activity) indicates the exposure to ChE inhibitors only without any specification of inhibitor, and in the acute phase of organophosphate poisoning, low serum acetylcholinesterase (> 50% of minimum normal value) does not show a significant relationship to the severity of poisoning [24]. The method of fluoride-induced reactivation allows us to differentiate/specify which OP caused inhibition (in our case sarin) and therefore is more specific in comparison with simple ChE determination only. The concentration of sarin determined in our experiments (262 and 455 ng/mL $= 1.87 \cdot 10^{-6}$ and $3.25\ 10^{-6}$ mol/L) in connection

with plasma BuChE inhibition is very well comparable with previous results determining ChE inhibition in rats poisoned intravenously with sarin [25]. This supports an idea about not only qualitative but also quantitative assessment of OP causing inhibition as it was demonstrated for victims of the sarin terroristic attack in Tokyo [26].

Although the essential application of this method is in retrospective monitoring of people exposed to OP compounds, in studies with laboratory animals this method is very useful, because it enables a comparison between some markers of intoxication with OP compounds (e.g. cholinesterase inhibition) with real levels of OP compounds in the plasma of the animals.

In conclusion, this method is a very good marker for monitoring sarin intoxication in laboratory animals carried out till now mostly by ChE determination which does not allow any information on sarin plasma levels.

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