

THE REACTIVATION EFFECT OF PRALIDOXIME IN HUMAN BLOOD ON PARATHION AND PARAOXON-INDUCED CHOLINESTERASE INHIBITION

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ABSTRACT

In this investigation the reactivation of cholinesterases by pralidoxime in parathion and paraoxon intoxication in plasma and erythrocytes were studied. For this purpose, human plasma and erythrocytes were incubated with various concentrations of parathion (0.1-10 μ M) and paraoxon (0.03-0.3 μ M) at 37 °C for 10 min. Then, pralidoxime (10-300 μ M) was added to the samples and incubated for 10 min before cholinesterases assay.

The results showed that effects of parathion and paraoxon were dose dependent. These agents inhibited more than 85% of butyrylcholinesterase (BChE) and acetylcholinesterase (AChE) activity and the inhibitory effect of paraoxon was 10 times more than parathion. BChE activity was significantly higher than the control at 100 μ M of pralidoxime and it reduced inhibitory effects of parathion to less than 50% and of paraoxon to 42% of control. When pralidoxime (10 μ M) was added to erythrocytes, the inhibitory effects of two organophosphates were reduced to less than 15%. At higher concentrations of pralidoxime (>100 μ M), both BChE and AChE activities were inhibited.

Keywords: Plasma, Erythrocytes, Cholinesterases, Parathion, Paraoxon, Pralidoxime

INTRODUCTION

Organophosphates (OPs) have largely been used throughout the world as agricultural pesticides use and chemical warfare agent (nerve agents). OP poisoning may occur in a variety of situations and is a serious health problem due to the lack of control on their importation, sale and safe uses. The number of intoxications with OPs is estimated at some 3,000,000 per year, and the number of deaths and casualties 300,000 per year (1-5).

OPs inhibit the action of cholinesterases by phosphorylation of serine residues in the active site of the enzyme and the inhibited enzyme is unable to metabolize acetylcholine. As a result excessive nicotinic and muscarinic receptors of acetylcholine are stimulated excessively. The two principal human cholinesterases are acetylcholinesterase (AChE) found primarily in nervous tissue and erythrocytes and butyrylcholinesterase (BChE) found in liver and plasma. Both enzymes are of pharmacological and toxicological importance, BChE is used to detect the early, acute effects of OP poisoning while AChE is used to evaluate long-term or chronic exposure (6-9).

The main drugs used in the treatment of OP poisoning are anticholinergics such as atropine and pralidoxime. Pralidoxime is the oxime most often used world-wide and occurs in two common forms: pralidoxime chloride (2-PAM) and

mesylate (P2S)(10). It reactivates cholinesterase by removing the phosphoryl group bound to the esteratic site. The great majority of its effects are on the peripheral nervous system, since its lipid solubility is low and entry into the central nervous system (CNS) limited (11), but may also reverse the CNS effects of OP (12).

Pralidoxime has been shown to be effective in sarin-poisoned mice, rats, rabbits and dogs (5,13). Also, it has therapeutic efficacy against acute toxicity of dichlorvos (14). This drug is not effective in soman and tabun poisoning (5, 15-17). Pralidoxime becomes ineffective as an antidote when administered more than 24 to 48 hours post-exposure as a result of aging of the phosphate-ester bond. Pralidoxime also slows the process of aging of phosphorylated acetylcholinesterase to a nonreactivable form and detoxifies certain OPs by direct chemical actions (5, 18-19). In spite of numerous studies, the mechanism of action of pralidoxime in human OP poisoning is still unknown. Also, further studies are required to investigate the effects of high doses of pralidoxime in common known of OP poisoning. Parathion is a widely insecticides, which is readily absorbed. In human liver, parathion is readily oxidized to paraoxon, which is the active form of parathion (20). The purpose of the present investigation was to determine the activity of

pralidoxime in human blood in terms of reactivation of parathion and paraoxon-induced inhibition of plasma and erythrocyte cholinesterases.

MATERIALS AND METHODS

Chemical

All reagents used were of analytical grade. Pralidoxime, Butyrylthiocholine (BTC), acetylthiocholine iodide (ATC), 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), parathion (O, O'-diethyl O-p-nitrophenyl phosphothioate) and paraoxon (O, O'-diethyl O-(p-nitrophenyl phosphate) were purchased from Sigma Chemical Company. Other chemicals were obtained from Merck.

Pralidoxime was dissolved in distilled water at a stock concentration of 0.1 M and was diluted to desired concentrations before use. Stock solutions (10 mM) of parathion and paraoxon were dissolved in absolute ethanol and then working solutions of organophosphates (1mM) were prepared in ethanol/water (40% v/v). The final ethanol concentration in assay medium was less than 0.5% and at such a low concentration it did not inhibit the enzyme activity.

Sample preparation

Peripheral venous blood was taken from healthy, young male human volunteers aged 18 to 30 years. Blood was sampled in EDTA and immediately centrifuged at 2500 rpm for 15 min. Plasma were removed and stored in 1 ml aliquots at -20°C . Prior to analysis, plasma was diluted 1:10 and then 100 μl was used for enzyme assay.

Packed erythrocytes were obtained after washing freshly drawn erythrocytes three times with five volumes of phosphate buffered saline (PBS), centrifuged as above and after removal of the white buffy layer were divided into equal portions and frozen at -20°C until use. On the day use erythrocytes were suspended in lysis buffer (0.5 g/dl saponin in 50 mM phosphate pH 7.4, 1 mM EDTA) and diluted 1:100 with PBS and 100 μl was used for enzyme assay.

Organophosphate inhibition and oxime-induced reactivation

Plasma and erythrocytes were incubated with various concentrations of parathion (0.1-10 μM) and paraoxon (0.03-0.3 μM) at 37°C for 10 min. After incubation, pralidoxime (10 μM for erythrocytes and 100 μM for plasma) was added and samples were incubated at 37°C for 10 min prior to assay. A control was incubated accordingly, with corresponding volumes of water and ethanol. Also, different concentrations of pralidoxime (10-100 μM) in the absence and presence of plasma or erythrocytes cholinesterase

were incubated at 37°C for 10 min. Enzyme activity were assayed in all samples.

Assay of cholinesterases

AChE and BChE activities were measured spectrophotometrically at 37°C at 410 nm by a slight modification of Ellman method (21) using acetylthiocholine iodide (ATC, 10 mM) as the substrate for the red blood cell and butyrylthiocholine iodide (BTC, 10 mM) for the plasma. Measurement of the cholinesterases activity was optimized and carried out in 0.05 M phosphate buffer, pH=7.4 containing 0.42 mM DTNB. One unit of activity is defined as the amount of enzyme that catalyzes hydrolysis of 1 μmol of substrate at 37°C .

Statistical analysis

All calculations were performed using Instat statistical software. Results were analyzed by analysis of variance (ANOVA), paired t-test, and unpaired t test. *p* values less than 0.05 were considered statistically significant. Results were expressed as means \pm SD, with *n* denoting the number of experiments performed.

RESULTS

The effect of various concentrations of pralidoxime (10-300 μM) in the absence and presence of erythrocytes or plasma cholinesterase activity at 410 nm are shown in Fig. 1. As it is shown, raising pralidoxime concentration in the absence of plasma or erythrocytes cholinesterase activity, gave a straight upward line but when pralidoxime was added to plasma (up to 100 μM) and erythrocytes (up to 10 μM), the absorbance is increased to a value exceeding those of control. However, at higher concentrations of drug, the absorbance is significantly decreased in comparison with control.

The effects of different concentrations of parathion and paraoxon on human plasma BChE inhibition and the effect of pralidoxime are shown in Figs. 2 and 3. In the absence of pralidoxime, increase in the concentration of two OPs decreased BChE activity and at higher concentrations more than 85% the BChE activity was inhibited (compared to control, $P < 0.001$). The effect of paraoxon was more than parathion and at high concentrations a saturation effect was observed. Pralidoxime (100 μM) increased BChE activity in comparison with control and reduced inhibitory effects of parathion to less than 50% and of paraoxon to 42% of control (Table 1).

Figs. 4 and 5 represents the results of parathion and paraoxon erythrocytes AChE activity. As it is seen, the effects of parathion and paraoxon on erythrocytes are similar to plasma and in the presence of pralidoxime (10 μM), the inhibitory effects of two Ops were reduced to less than 15% (Table 1).

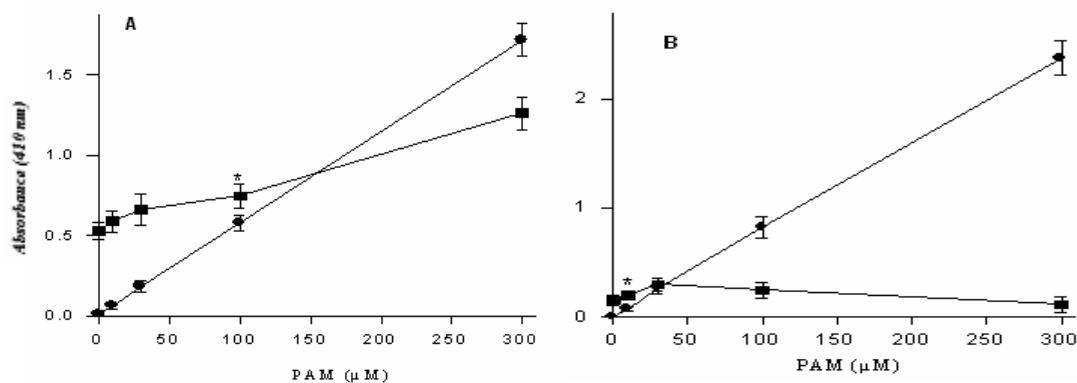


Figure 1. Changes in the absorbances of various concentrations of pralidoxime (PAM) in the absence (●-●) and presence (■-■) of plasma (A) and erythrocytes (B) cholinesterases activity at 410 nm. * $P < 0.05$ vs control (zero PAM) and the same concentrations of PAM in absence of plasma or erythrocytes cholinesterase activity (n=5).

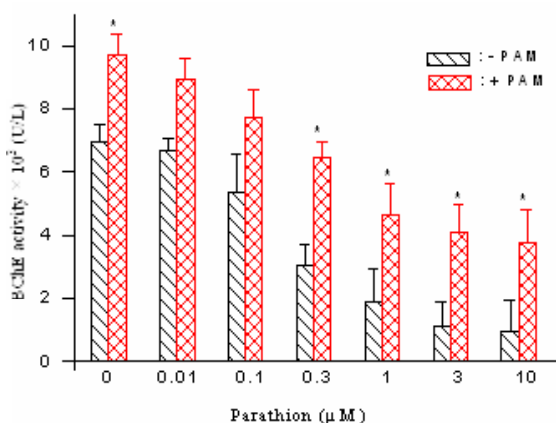


Figure 2. The effect of parathion on human plasma BChE activity in the absence and presence of pralidoxime (PAM). * $P < 0.05$ vs control and the same concentrations of parathion without PAM (n=10).

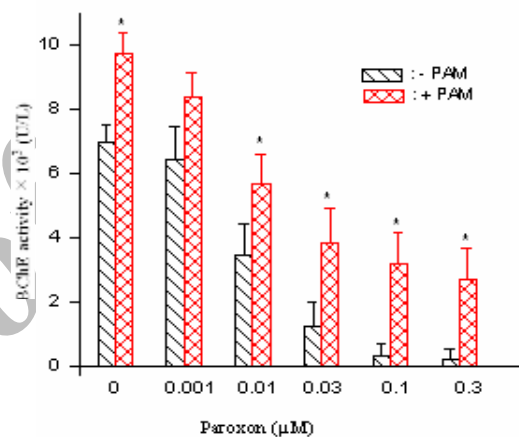


Figure 3. The effect of paraoxon on human plasma BChE activity in the absence and presence of pralidoxime (PAM). * $P < 0.05$ vs control and the same concentrations of parathion without PAM (n=10).

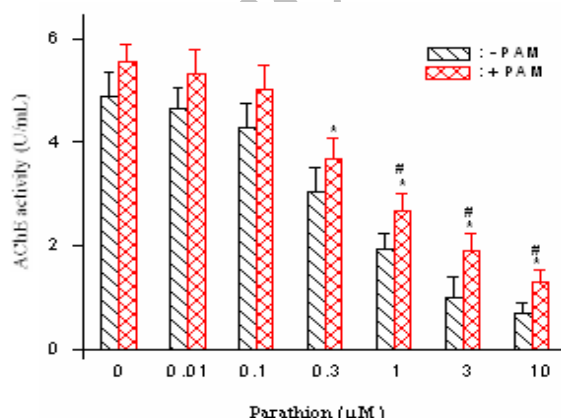


Figure 4. The effect of parathion on human erythrocytes AChE activity in the absence and presence of pralidoxime (PAM). * $P < 0.05$ vs control; # $P < 0.05$ vs the same concentrations of parathion without PAM (n=10).

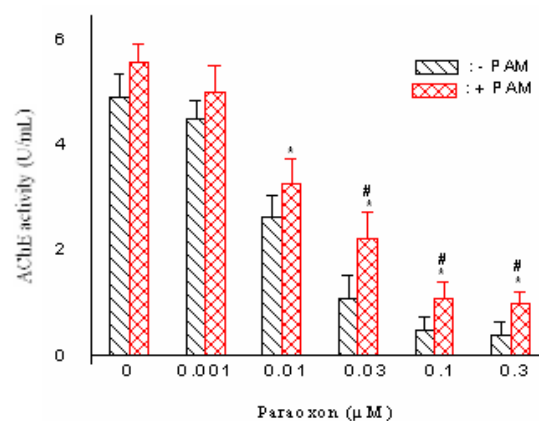


Figure 5. The effect of paraoxon on human erythrocytes AChE activity in the absence and presence of pralidoxime (PAM). * $P < 0.05$ vs control; # $P < 0.05$ vs the same concentrations of paraoxon without PAM (n=10).

Table 1. Percentage inhibition of human plasma and erythrocytes cholinesterases activity by paraoxon and parathion in the absence and presence of pralidoxime (10 μ M for erythrocytes; 100 μ M for plasma).

Inhibitor	%Inhibition				
		Befor treatment		After treatment	
		Plasma	Errythrocytes	Plasma	Errythrocytes
Parathion (μ M)	0.01	3.6	5.01	-	-
	0.1	22.66	12.39	-	-
	1.0	73.01	60.11	33.08	45.13
	10.0	86.18	85.97	45.72	73.68
Paraoxon (μ M)	0.001	7.52	8.36	-	-
	0.01	50.09	46.62	18.26	33.57
	0.1	95.43	90.20	53.86	78.03
	0.3	96.76	92.08	61.11	79.89

DISCUSSION

The OPs are a group of toxins that exert their effects by inhibition of AChE and BChE. Pralidoxime is indicated as an adjunct in treatment of moderate and severe poisoning caused by OP pesticides and some chemicals used as nerve agents during chemical warfare (9,12). The effects of oximes in human OP poisoning have not been well studied.

Spectroscopic examination of various concentration of pralidoxime on plasma and erythrocytes cholinesterase activity revealed that the effect of pralidoxime is dose-dependent. At concentration of 10 μ M (for erythrocytes) and 100 μ M (for plasma) of pralidoxime, the absorbance was significantly increased in comparison with the control. Therefore, these concentrations were chosen for the study of its reactivating effects. The absorbance decreased significantly at higher concentrations of pralidoxime (>30 μ M for erythrocytes and >100 μ M for plasma). This finding is in agreement with the results of the previous reports that at high doses, pralidoxime may inhibit acetylcholinesterase and cause neuromuscular blockade (9, 22).

Pralidoxime hydrolyzes acetyl- and butyrylthiocholine, the substrates which are used in the assay of AChE and BChE, respectively. The thiocholine that is produced forms a yellow complex when Ellman's reagent is used in the assay (even in the absence of plasma and thus of any enzyme). Cholinesterase activities determined photometrically after pralidoxime therapy can be erroneously high (23-24). Also, the Ellman method does not allow the exact determination of AChE in erythrocytes due to interference with hemoglobin absorption and only highly diluted blood samples can be used (1:100), which reduces the assay sensitivity. Therefore, the effect of high concentrations of pralidoxime on AChE activity should be investigated by other methods (24-26).

The results show that the effect of parathion and paraoxon were dose dependent. Both parathion and its active metabolite, paraoxon were able to inhibit more than 85% of BChE and AChE activity, the inhibitory effect of paraoxon was 10 times higher than parathion. The metabolism of parathion to paraoxon significantly enhances anticholinesterase properties (7, 20). The maximal inhibition of brain acetylcholinesterase activity was not immediate with parathion, reflecting the time required for bioactivation of the phosphorothionates as well as the effectiveness of the aliesterases to inactivate much of the hepatically generated oxons. In contrast, brain acetylcholinesterase activities were more quickly inhibited following administration of paraoxon, which do not require bioactivation (27-28). The proportion of plasma parathion to paraoxon varied from 0.3-30, pointing also to varying paraoxon elimination by paraoxonase-1 activity (29). It has been reported that the potency difference may be attributed to the differences in the interaction of the leaving group with the active site, whereby the association of the leaving group moiety with the anionic site may produce an environment that affects the rate of phosphorylation of the active site by inducing conformational changes in the tertiary structure of the enzyme (30). This in vivo potency difference is primarily a reflection of the higher capacity for metabolic detoxification of parathion compared to paraoxon (31).

Pralidoxime increased BChE activity and reduced the inhibitory effects of parathion and paraoxon on BChE and AChE, which is agreement with previous reports (32-34). In the present study, pralidoxime was added 10 minutes after that cholinesterases were exposed to OPs. However, the onset of action of pralidoxime occurs within 10 to 40 minutes (18, 22, 35) and the minimum therapeutic concentration of pralidoxime in plasma is 23.17 μ M (4 μ g/ml) (4, 33, 36). In the in vitro

model, a partial recovery of acetylcholinesterase activity was observed with concentrations of 0.066mM of pralidoxime, probably useful enough to prevent death in most cases *in vivo*. However, much more effectiveness was observed with pralidoxime concentrations up to 0.70mM (37). Among different OP insecticides, it has been reported that *in vivo* cholinesterase recovery is probably related to differences in absorption, biotransformation, and, most importantly, to spontaneous enzyme reactivation (38).

Our results are in contrast with previous reports that pralidoxime has no effect on BChE level and only normalizes AChE levels (39) and also with other studies in animals about the enhancement of toxicity of OPs by pralidoxime (22, 40-41). It has also been reported that pralidoxime has no therapeutic value (42-46). These controversies may be due to the difference in experimental conditions and techniques of analysis. However reactivation of inhibited AChE by oximes will likely be absent or limited, in the case of: (I) poor affinity for the particular OP-AChE complex; (II) insufficient dose or duration of treatment; (III) persistence of the OP within the patient and therefore rapid reinhibition of newly reactivated enzyme; and (IV) aging of the inhibited AChE. Therefore such a generalized statement cannot be justified from the published results and we believe that the evidence supporting or against the use of oximes is not yet established (47-50).

Our results are in agreement with a prospective study, which has shown that AChE reactivation was only observed in the pralidoxime group, although it was not statistically significant. There were no deaths in the pralidoxime group and high doses of pralidoxime revealed no serious side effects (2, 51). Also, pralidoxime significantly decreased mortality and increased RBC-AChE activities at 30 min until 48 h in rats acutely exposed to large doses of paraoxon (52). Proponents of oximes responded that these physicians were using too low a dose and that a loading dose of at least 30 mg/kg followed by an infusion of >8 mg/kg/h was required for clinical benefit (11,53). Therefore, it seems that the therapeutic effect of pralidoxime depends on the plasma concentration of the OPs agent, with the benefits being minimal at high blood levels of OP. In conclusion, the present findings indicate that pralidoxime reactivates *in vitro* parathion and paraoxon-inhibited BChE and AChE. However, further studies are required to investigate the effects of high doses of pralidoxime on erythrocytes by other methods. Also, pralidoxime increased BChE activity and it may be useful in the pretreatment of poisonings of OP compounds.

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