# THE EFFECTS OF OXIMES IN THE ASSAY OF ACETYLCHOLINESTERASE ACTIVITY IN LYSED ERYTHROCYTES IN VITRO

M. Abdollahi., Y. Behmanesh and M. Akhgari

Department of Toxicology and Pharmacology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

*Abstract — Organophosphorus compounds are known to inhibit the esteratic site of acetylcholinesterase by phosphorylation. The phosphorylated esteratic site of acetylcholinesterase undergoes hydrolytic regeneration at a slow or negligible rate. Nucleophilic agents such as hydroxylamine, hydroxamic acids, and oximes reacctivate the enzyme more erapidly than does spontaneous hydrolysis. The red cell cholinesterose activity was assayed using dithio bis-2-nitrobenzoic acid (DTNB) commonly known as Ellman's reagent. The principle of this assay method is the rate of hydrolysis of acetylthiocholine (substrate) by a red cell suspension. Thiocholine that is produced, forms a yellow complex, when Ellman's reagent (DTNB) is used in the assay. This was tested in vitro in lysed erythrocyte samples of 35 healthy persons who had no known exposure to cholinesterase inhibitors, after the observation of immediate increase in absorption of light at 440 nm. All of data were statistically analyzed using one-way ANOVA and student t-test. A value of $p<0.01$ was considered. Results of this study show an increased absorbance in 440 nm, for pretreated samples with pralidoxime. This was observed by doses of (0.1, 0.5, 1,2 mmol, $p<0.01$). It was also a good dose dependent increase in absorbance at 440 nm for pralidoxime, ($r=0.940$, $p<0.01$). Also there is a significant increase in absorbance at 440 nm for samples pretreated by obidoxime at doses of (0.1, 0.5, 1,2 mmol). There is also a good correlation between absorbance at 440 nm and variou doses of obidoxime ($r=0.946$ , $p<0.01$). It is concluded that oximes can hydrolyzes the substrate, which then would be a source of error in determination of acetylcholinesterase activity and must be taken into account.*

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*Key words: Acetylcholinesterase; pralidoxime; obidoxime; enzyme determination; Ellman's reagent; erythrocyte*

## INTRODUCTION

Cholinesterases [acetylcholinesterase (AChE) EC 3.1.1.7 and butyrylcholinesterase (BuChE) EC 3.1.1.8] are inhibited by organophosphorus (OP) through phosphylation (i.e phosphorylation or phosphonylation) of their esteratic active site serine [1]. Phosphylated cholinesterases can be reactivated by nucleophilic agents, of which oximes are of pharmacological interest as antidotes against OP posioning (2).

The reactivation of AChE with oximes occurs at a million times the rate of that with hydroxylamine as a nucleophil agent. Pralidoxime and obidoxime have been approved to be effective antidotes for OP poisoning, especailly in conjunction with atropine (3). In moderate or severe anti-ChE intoxications the recommended dose of pralidoxime for adults is 1 to 2g infused intravenously (4). Our previous research showed that oximes have neither benefit in management of acute OP poisoning nor in reversing ChE activity (4). The increased incidence of human exposure to pesticides, occupationally or environmentally, has resulted in many requests for clinical laboratories to monitor such patients routinely. Many of these patients usually receive oxime as antidote in the first hours of admission. In many hospitals, exposure to OP pesticides is monitored by measuring AChE activity alone. It was reported previously that pralidoxime could interact with the assay of acetylcholinesterase activity using DTNB in vivo. In this respect, we designed this study to investigate the effects of two commonly used oximes in our country in the assay of AChE activity. Study was conducted as the same situation as in vivo, and done an in vitro examination, to predict that oximes, could affect on assay of AChE activity resulting a false number.

## MATERIALS AND METHODS

### Materials

Chemicals: Acetylthiocholine iodide (ATChI), Quinidine sulphate and 5,5'-dithio bis 2- nitrobenzoic acid (DTNB) were purchased from Sigma chemical co., (U.K.), Hyamine 1622 (Benzethonium chloride) was supplied by BDH chemicals Ltd., (U.K.) and Buffer salts, from Fisons scientific apparatus Ltd. Water was distilled and de-ionized.

The 5-thionitrobenzoate used to evaluate the micellar spectral shift was prepared by the reaction between DTNB and thiocholine generated by erythrocyte ChE from ATChI.

Pralidoxime and obidoxime were supplied from Laboratories SERB (France) and Merk (Germany) respectively.

### Reagents

DTNB reagent was prepared in 1 L of 33 mmol/L (5.74 g/L) K2HPO4 and 100 mL of 0.10 mol/L

KH2PO4 (1.36 g/100 mL). Enough amount of the latter was added to bring the pH of the former to 7.6. 0.27 mmol (107 mg) of DTNB and 20 mol (16 mg) of quinidine sulphate were prepared in 1 L of the buffer and stored in a dark bottle, at 40°C.

ATChI substrate (0.2 mol/L): 530 mg of ATChI was dissolved in 10.0 mL of water, and freezed 1.0 mL qliquots for subsequent use. Immediately before use, the substrate was thowed diluted with 1.0 mL of water, and mixed throughly.

Stopping reagent: This was either 43 mmol/L (20 g/L) Hyamine 1622 in water, which was stored at 40°C in dark bottle.

Samples: Blood specimenes used to assess the precision of the methods were obtained from 35 apparently healthy individuals who had no evidence of exposure to OP or anti-ChE agents.

## Procedures

Recommended assay procedure: This method is a modification of that of Ellman which described by George and Abernity (5,6). In this method the stopping reagent is Hyamine 1622. 20 mol of quinidine sulphate is added to the buffer per liter to selectively inhibit contaminating plasma ChE. The reaction mixture concentrations of DTNB, ATChI, and quinidine sulphate are 0.28 mmol/L, 3.2 mmol/L, and 20 mol/L, respectively. Hemolysates were prepared by adding 100 mL of packed erythrocytes (taken from centrifugated samples) heparinized blood to 6.0 mL of water. The effects of oximes on ATChI, were tested by adding 100 mL of pralidoxime or abidoxime with different concentrations (0.1, 0.5 , 2 mmol) to 500 mL of hymolysed erythrocytes in four glass test tubes. This solutions were named pretreated blood and incubated at 30°C for 30 minutes. A control study was done using the same blood sample without being pretrated by oximes. In next step, it was equilibrated 3.0 mL of DTNB ragent and 100 mL of ATChI, in six glass test tubes in water bath at 30°C for 10 seconds. At exactly 10 seconds intervals, 100 mL of previous pretreated samples, was added to this reagent tubes and incubated at 30°C for 10 minuts.

One tube was considered for blank wich has not oxime or blood. After 10 minutes, it was added 1.0 ml of Hyamine 1622 stopping reagent, and tubes were placed at room temprature. At the end, the absorbances of all test solutions vs the respective blank, at 440 nm, were measured.

All of the data were statistically analyzed using one-way ANOVA and student t-test. A value of $p<0.01$ was considered significant.

Apparatus: UV-visiable (shimadzu 160-A) spectrophotometer with cps-controller and scanning, was used through this study.

# RESULTS

Figure 1, illustrates the increase of absorbance in 440 nm, for pretreated samples with pralidoxime. There is a significant increase in absorbance by doses of (0.1 , 0.5 , 1 , 2 mmol, $p<0.01$). It was also a good dose dependent increase in absorbance at 440 nm for pralidoxime, ($r=0.940$, $p<0.01$), (Fig.2).

Also there is a significant increase in absorbance at 440 nm for samples pretreated by obidoxime at various doses (0.1 , 0.5 , 1 , 2 mmol), (Fig.3) and there is a good correlation between absorbance at 440 nm and various doses of obidoxime ($r = 0.946$, $p<0.01$), (fig.4).

# DISCUSSION

Oximes cleave the phosphate ester bond formed between the OP and AChE, resulting in reactivation of the enzyme (4). There are some important differences in potency, efficacy, peneteration to blood brain barier, administration and adverse effects between these two oximes. Therefore they were administered with respect to severity or time of poisoning in poisoned patients (2,4). In this respect we examined them for analysis. Our results indicated that both pralidoxime and obidoxime can induce a false increase in the absorbance at 440 nm which lead to an error in reporting of AChE activity. This false increase in AChE activity, might be a source of mistaken diagnosis, treatment or prognosis of human OP poisoning. Oximes are known ChE reactivators used in poisoning. They were not known to cause any effect on the natural substrate of the AChE, namely the acetylcholine (6). The transformation of DTNB and oxime from colorness to a yellow color when the substrate acetylthiocholine was added caused by the reaction of DTNB with thiocholine. Because there was no enzyme (AChE) in the solution to hydrolyze the substrate, the only was thiocholine could have been produced is by the action of oxime. It is suggested that there is a nucleophilic reaction between the oximes and the substrate which lead to production of thiocholine, then reacting with DTNB to give a rise in the absorbance. It is also concluded that type of oxime has not significant role in this interaction.

These data are partly supported by report of Nadarajah (1992), who indicated that pralidoxime can hyrolyze ATChI, the substrate used in the assay of red cell ChE. It was concluded that the effect of pralidoxime on ATChI could give a false alarm in the assay of, inhibited acetylcholinesterase activity (7). They found the same results as mentioned in our study but only by pralidoxime and in vivo assay.

Further studies to find the exact mechanism of interaction between oximes and the substrate in the assay of acetylcholinesterase activity are proposed.

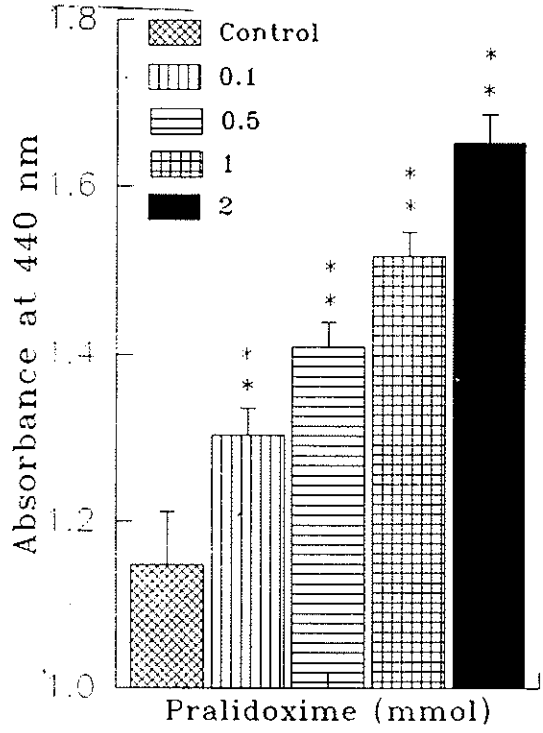


Fig. 1. Effects of different doses of pralidoxime on human RBC cholinesterase activity. ** Differences between control and treated samples are significant at p<0.01. n=35.

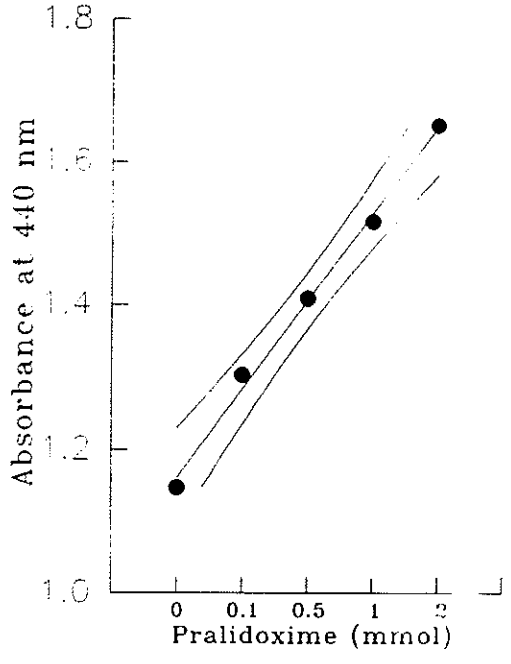


Fig. 2. Effects of different doses of pralidoxime on human RBC cholinesterase activity. r=0.940, P<0.01, n=35.

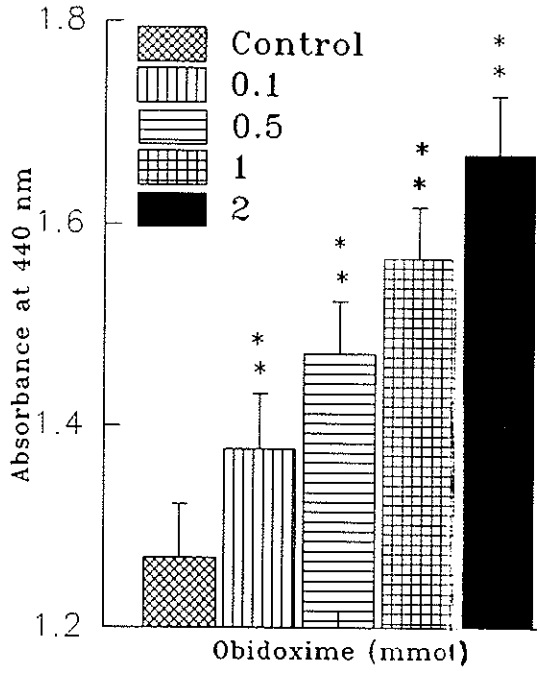


Fig. 3. Effects of different doses of obidoxime on human RBC cholinesterase activity. ** Differences between control and treated samples are significant at P<0.01. n=35.

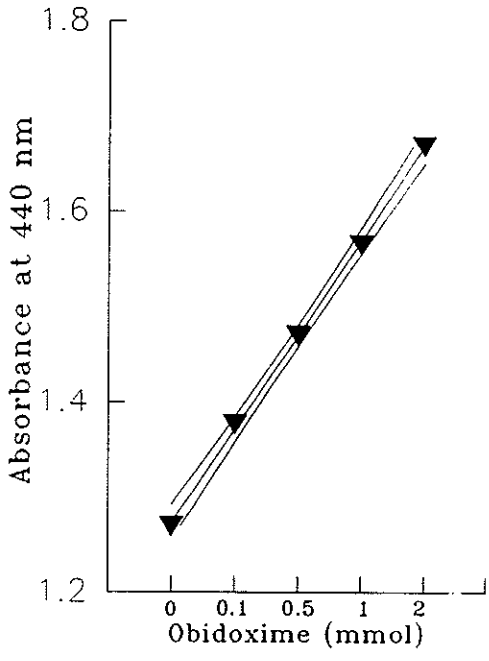


Fig. 4. Effects of different doses of obidoxime on human RBC cholinesterase activity. r=0.946, P<0.01, n=35.

# REFERENCES

1. Quinn, D.M. Acetylcholinesterase: Enzyme structure, reaction dynamics, and virtual transition states. Chem. Rev. 87: 955-979; 1986.

2. Ballantyne, B., Marrs, T.C. and Aldrige, W.N. Clinical and experimental toxicology of OP and Carbamates, Bath press Ltd., London, 565-566; 1992.

3. Raveh, I., Gurnwald, J., Marcus, D., Papier, Y., Cohen, E. and Ashani, Y. Human butyrylcholinesterase as a general prophylactic antidote for nerve agent toxicity. In vitro and in vivo quantitative characterization. Biochem. Pharmacol. 45 (12): 2456-2474; 1993.

4. Abdollahi, M., Jafari, A., Jalali, N., Balali-Mood, M., Kebriaeezadeh, A., Nikfar, Sh. A new approach to the efficacy of oximes in the management of acute OP posioning, Irn. J. Med. Sci., 20 (3&4): 105-109; 1995.

5.George, P.M., Abernethy, M.H. Improved Ellman procedure for erythrocytes cholinesterase. Clin. Chem. 29 (2): 365-368; 1983.

6. Ellman, G.I., Courtney, D.K., Anders, V., Featherston, R.H. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmac. 7: 88-95; 1961.

7. Nadarajah, B. The effect of pralidoxime in the assay of acetylcholinesterase using DTNB. J. Anal. Toxicol. 16: 192-193; 1992.