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DUAL SUBSTRATE MODEL FOR NOVEL APPROACH TOWARDS A KINETIC STUDY OF ACETYLCHOLINESTERASE INHIBITION BY DIAZINON*

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Limited reports as compared to other insecticides appear in the literature for acetylcholinesterase (AChE) inhibition by diazinon. In the current study, new kinetic parameters of AChE inhibition by diazinon have been investigated. The assay was done with bovine retinal AChE using two different substrate (ASCh) concentrations in the absence and presence of diazinon (0.08–1.28 mM). The optical density was monitored up to 25 min (reaction time) for the assay. New kinetic parameters (k'_{oms}, K'_{sms}, k_{oms}, K_{sms}, K'_{asms} and K_{asms}) were calculated from these experimental data.

Keywords: Acetylcholinesterase; Diazinon; Inhibition; Kinetics

Abbreviations: AChE, acetylcholinesterase; ASCh, acetylthiocholine; K'_{sms}, affinity constant; k'_{oms}, rate of reaction in the absence of inhibitor; K_{sms}, inhibition constant; k_{oms}, rate of reaction in the presence of inhibitor; K'_{asms}, enzyme–substrate dissociation constant; K_{asms}, enzyme–substrate–inhibitor dissociation constant

INTRODUCTION

Acetylcholinesterase (EC 3.1.1.7; AChE) is a hydrolytic enzyme which controls the transmission of nerve impulses by hydrolyzing acetylcholine

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(a neurotransmitter) in the nervous system. AChE in central and peripheral cholinergic synapses is the primary target of organophosphorus insecticides and therefore, it is the target enzyme for testing toxicity induced by organophosphate and carbamate insecticides.[1] It is well known that diazinon [*O*,*O*-diethyl *O*-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate] belongs to the group of organophosphate (OP) insecticides.[2–4] Pesticides, especially insecticides, are a major topic for research because of their toxic effects on non-target organisms such as fish, birds and mammals.[5] Determination of AChE activity is a useful tool in establishing the degree of toxicity caused by these agents.

The toxico-kinetics, tissue distribution, and anticholinesterase activity of diazinon have been investigated recently in the rat. Although both red blood cell (RBC) AChE and plasma cholinesterase (ChE) activities can be inhibited rapidly, the RBC AChE was more sensitive to diazinon than the plasma cholinesterase.[6] The influence of cimetidine on diazinon toxicity and toxicokinetics has been also investigated in rats. The acute toxicity of diazinon, as well as the inhibition of brain AChE and carboxylesterase have been studied by pre-treating rats with cimetidine prior to diazinon application ($50\,mg\,kg^{-1}$, i.p.). A comparison of toxico-kinetic parameters between control and cimetidine-treated animals showed that a major cause of the potentiation of diazinon may be related to the increased uptake of diazinon in the systemic circulation as well as by the brain.[7]

The toxicity effects of dimethoate, azinphos-methyl, diazinon, pirimiphos methyl, OP insecticides, and benomyl (a benzimidazole fungicide) singly and in a mixture have been studied in a human neuro-blastoma cell line, SH-SY5Y. The cells were incubated for 30 min and 4 h with pesticides concentrations ranging from 0.4 to 100 μg/mL. The suppression of AChE activity was maximum when a mixture of pesticides was used and was equal to the most active compound in the mixture. However, the mixture was more toxic to protein synthesis than the single components. Therefore, it is difficult to predict the toxicity of pesticide mixtures on the basis of these results from the data obtained using single components.[8]

The *in vitro* study of the hepatic metabolism of diazinon and the sensitivity of the brain AChE to diazinon inhibitory action have demonstrated a different toxicity spectrum in rainbow trout, guppy, zebra fish and carp. The zebra fish has the least sensitive AChE with a limited activation rate, thus making it a resistant entity. The data obtained indicated that diazinon toxicity differences among the fish family can largely be due to dissimilarities in liver metabolism and the sensitivity of the target enzyme.[9]

A variability in AChE activity and changes in the optomotor response have been utilized to study the effects of different concentrations of diazinon in bluegill Sunfish, *Lepomis macrochirus*. There was a significant decline in the AChE activity above 45 μg/L concentration. In case of optomotor behavior, the "following" responses in the fish at concentration of 30 μg/L were declined. This observation indicates that the behavioral bioassay has been more sensitive than other types of techniques.[10]

In a recent study, toxic effects of azamethiphos in two OP-resistant and one susceptible strain of houseflies, *Musca domestica L*, were investigated. The *Yachiyo* strain, which showed 1500-fold resistance to diazinon and had multiple mechanisms for OP-resistance, showed only 6-fold resistance to azamethiphos by topical application and 4-fold resistance by oral administration.[11] On the other hand no cross-resistance to diazinon was detected during a study of the high level of pyrethroid resistance in horn flies, *Haematobia irritans* (L.), selected with cyhalothrin.[12]

Nine fairways of a golf course located in Bellingham, Washington were treated with diazinon AG500 at an application rate of 2.2 kg active ingredient (AI) per hectare. The chemical application with a "bloomless" sprayer resulted in a variable distribution of diazinon residues on the turf (associated with a deep thatch layer) that ranged from 1.0 to 6.2 kg AI/hectare. The diazinon-treated turf was irrigated with 1.3 cm of water immediately following application. The post-irrigation diazinon residue levels ranged from 100 to 333 ppm. Eighty-five American wigeon (*Anas americana*) died after grazing on a treated fairway on the day of application following irrigation. Wigeon that died on a specified area showed 44–87% decline in AChE activity as compared to controls. Upper GI tract contents of 15 of the 85 dead wigeon contained 0.96–18.1 ppm diazinon.[13]

The activity of AChE and the density of muscarinic cholinergic binding receptors (mCBR) were measured in whole brains from normal Japanese quail and from quail after lethal intoxication with diazinon. During post-mortem decomposition, the ratio of AChE : mCBR activities remained constant at approximately 1.3 : 1 in normal brains while it was less than or equal to 0.5 : 1 with diazinon intoxication. Similar parallel measurement of AChE and mCBR could assist in the post-mortem diagnosis of death due to acute poisoning with anticholinesterase pesticides when control specimens are not available.[14]

Worek and co-workers studied various microscopic reaction parameters that contributed to the dynamic equilibrium of human erythrocyte AChE and the mouse diaphragm AChE inhibition, ageing and reactivation *in vitro*. Their data had been helpful to define more precisely the indications and

limitations of oxime therapy in OP poisoning. They reported that diethylphosphoryl-AChE resulted from intoxications with parathion, chlorpyrifos, chlorfenvinphos, diazinon and other OPs, which had been characterized by slow spontaneous reactivation and low propensity for ageing.[15]

Diazinon is widely used for the control of agricultural and household pests, the toxic effects of which are mainly due to inhibition of cholinesterases. In Saudi Arabia also, diazinon is commonly sprayed in homes, offices and stores, etc. for the control of insects and, therefore, it was thought of interest to investigate the effect of diazinon on the kinetic parameters of the AChE. The cow's eye has been employed as a model for testing the mutagenicity of excimer laser radiation and many other studies in opthalmology.[16] Whole eye has been also recommended for post-mortem cholinesterase analysis in *A. anguilla* due to its ease of collection, high cholinesterase activity and sensitivity to inhibition by pesticides.[17] Retinal degeneration has been reported along with chronic ocular degeneration and lesions in humans exposed to organophosphate insecticides.[18] Moreover, some of the organophosphate elicited ocular toxicity and serious changes in ciliary body as well as in the retina.[19] The presence of AChE activity in the retina was reported 54 years ago[20] and it has been extensively documented that the vertebrate retina, which is embryologically derived from the CNS, is rich in ACh (neurotransmitter) and cholinesterases.[21-24] The functional role of ACh in visual transmission in many mammalian retinas has been well documented,[25,26] and there is a wealth of data demonstrating the effect of cholinergic drugs and anticholinesterases on retinal function.[27] The effects reported by various groups of investigators appear to vary widely. Inhibition of AChE has been reported either to enhance or reduce the process of dark adaptation.[28] The decreases which have been noted in visual activity following exposure to anticholinesterases and a muscarinic cholinergic agonist may cause an increase in the flicker fusion frequency.[29] These effects have largely been attributed to the changes in accommodative ability.[30] Therefore, it was of interest to study the effects of diazinon on bovine retinal AChE activity.

MATERIALS AND METHODS

Materials

Acetylthiocholine iodide (ASCh, substrate) and 5,5′-dithiobis-(2-nitro)benzoic acid (DTNB) were purchased from Sigma Chemical Co. (USA). Triton

X-100 was purchased from Merk and bovine serum albumin was obtained from Fluka Chemika-Biochemika (Switzerland).

Enzyme Preparation and Assays

Retinas of young cows were obtained from a local abattoir. The whole retina from each eye was rapidly removed, rinsed in pre-chilled 0.9% saline solution, blotted and weighed. In each batch of enzyme preparation, twenty whole retinas were homogenized in pre-chilled 50 mM sodium phosphate buffer (pH 7.4). The extraction of retinal membrane-bound AChE has been described in a previous report.[31] AChE activity was determined by the spectrophotometric method of Ellman *et al.*[32]

Estimation of Protein

The protein content of the enzyme preparation was determined according to the method of Lowry *et al.* using bovine serum albumin as a standard.[33] The interference by Triton X-100 has been corrected as described previously.[34]

Graphics

The graphs were plotted by using GraFit program.[35] The value of the correlation coefficient was obtained by the linear regression analysis using the same program.

RESULTS AND DISCUSSION

Bovine retinal AChE activity was determined using two different substrate (ASCh) concentrations (0.025 and 0.25 mM) in the presence and absence of diazinon (0.08–1.28 mM/L) (Figure 1). The optical density was monitored up to 25 min (reaction time) in each assay. These results provide basic information for the calculations of all the kinetic parameters reported in the present study. A secondary plot of Figure 1 is presented in Figure 2, where the slope values of each plot in Figure 1 were plotted against the concentrations of diazinon for both assays as indicated above (Figure 2 inset). As shown in the inset plot, the plot for higher concentrations of ASCh was not linear, therefore, it was re-plotted against log concentration of diazinon and straight lines were obtained with correlation coefficients of 0.9346 and 0.9982 for the plot of ASCh concentration of 0.025 and 0.25 mM, respectively.

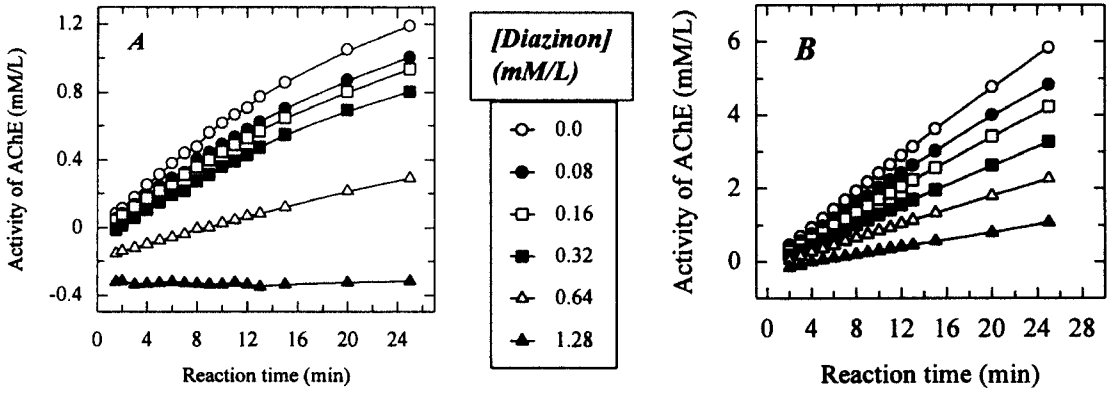

FIGURE 1 A plot of retinal AChE activity in the presence or absence of diazinon (0.08–1.28 mM) against reaction time. Plot A shows AChE activity at a low ASCh concentration (0.025 mM) and plot B represents assay at a high ASCh concentration (0.25 mM).

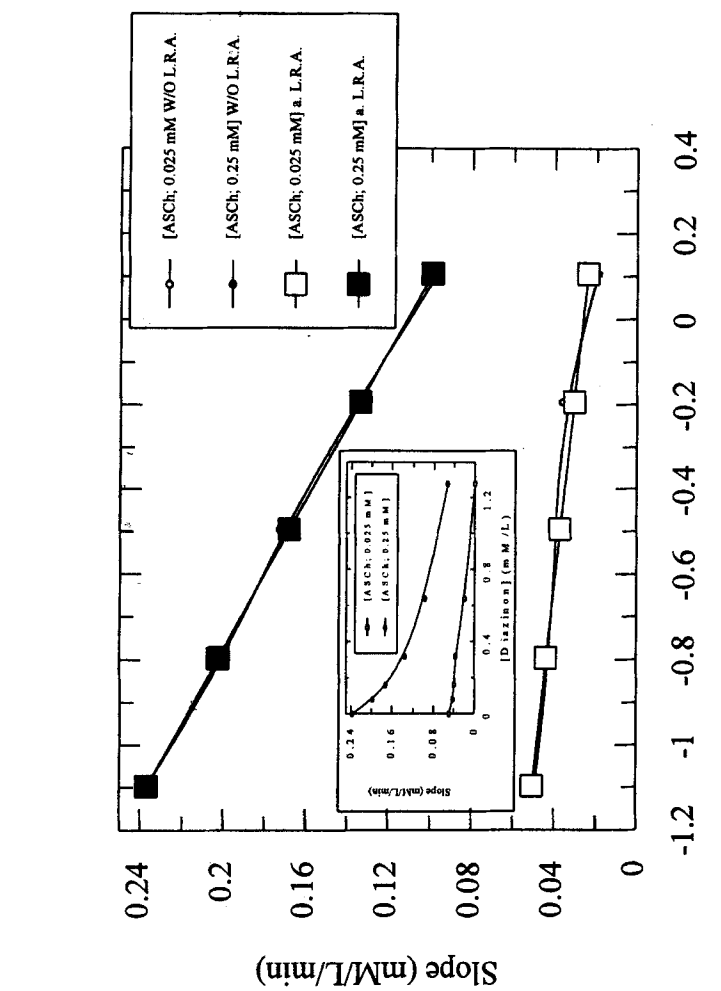

FIGURE 2 Secondary plot of Figure 1: Slope of each plot in Figure 1 versus log concentration of diazinon at two concentrations of ASCh as mentioned in legend box (W/O and LRA mean without and after linear regression analysis, respectively). Inset: Slope of each plot in Figure 1 versus concentration of diazinon.

Further analysis was carried out on the basis of the slope (k_s) and ordinate on the Y-axis (K_o) values obtained in the linear regression analysis of the two plots in Figure 2. Firstly, the reciprocal of k_s and K_o values was plotted versus the reciprocal of the ASCh concentrations (Figure 3(A)), which provided the $1/k'_{smax}$ value as intercept on the Y-axis, and the $-1/K'_{ss}$ value an abscissa (X-axis intercept) of the $1/k_s$ plot. The ratio of $1/k'_{smax}$ and $1/K'_{ss}$ was equal to K'_{sms} (affinity constant). The value of K'_{sms} was found to be 1.0 (mM · min) while the second plot of this figure (K_o) also provided the $1/K'_{omax}$, as an ordinate on the Y-axis, and the $-1/K'_{os}$, an abscissa (X-axis intercept) of the $1/K_o$ plot. The ratio of $1/K'_{omax}$ and $1/K'_{os}$ was equal to k'_{oms} (rate of reaction in the absence of inhibitor). The value of k'_{oms} was calculated to be 0.821 min^{-1}.

In case of part B of Figure 3, k_s and K_o were plotted against ASCh concentration. The first plot of k_s produced k_{smax}, an intercept, and $-K_{ss}$, intersection value on the abscissa. The ratio of k_{smax} and K_{ss} was equal to k_{sms} (inhibition constant). The value of K_{sms} was calculated to be 0.40 $(mM \cdot min)^{-1}$. The second plot of this figure (i.e. K_o) also gave the values for K_{omax}, an ordinate, and $-K_{os}$, an abscissa. The ratio of K_{omax} and K_{os} was equal to k_{oms} (rate of reaction in the presence of inhibitor), which was estimated to be 0.378 min^{-1}.

The ratio of k_s (slope) and K_o (ordinate) was obtained after linear regression analysis of the two plots in Figure 2 and was designated as K_{as} (association constant) which was plotted against the reciprocals of the two ASCh concentrations (Figure 4(A)). From this simple plot, again two parameters were obtained. One represents K_{asmax} as the intercept on the Y-axis, while the second is K'_{ass} given by the intercept on X-axis. The ratio of both yielded K'_{asms} (enzyme–substrate dissociation constant) and its value was calculated to be 0.175 $(mM/L)^2$. On the other hand, the curve obtained by plotting the reciprocal of K_{as} versus two concentrations of ASCh (Figure 4(B)) also resulted in two points, one was represented by $1/K_{asmax}$, which was the intercept on the ordinate, while the second was $-1/K_{ass}$, given by the intercept on the X-axis. The ratio of both values provided K_{asms} (enzyme–substrate–inhibitor dissociation constant), which was estimated to be 0.597 $(L/mM)^2$.

The nature of the inhibition of AChE by diazinon is non-competitive because $1/k_s$ and $1/K_o$ intersect at one point at high concentration of ASCh (Figure 3). The chemical structure of diazinon (Figure 5) illustrates the interaction of the diazinon with AChE and the proposed model (Figure 5) for interaction of diazinon with AChE in the presence of acetylcholine (as a substrate) is suggested in the light of previous reports in the literature.[1,36,37]

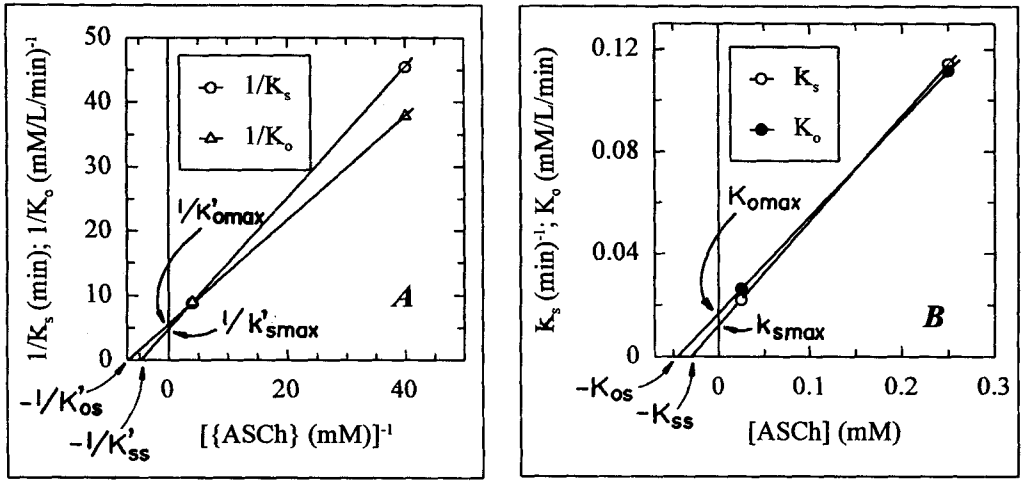

FIGURE 3 Secondary derivative plot of Figure 2. (A) Reciprocal of k_s (slope) and K_o (ordinate) versus reciprocal of ASCh concentrations. (B) The k_s and K_o versus two ASCh concentrations.

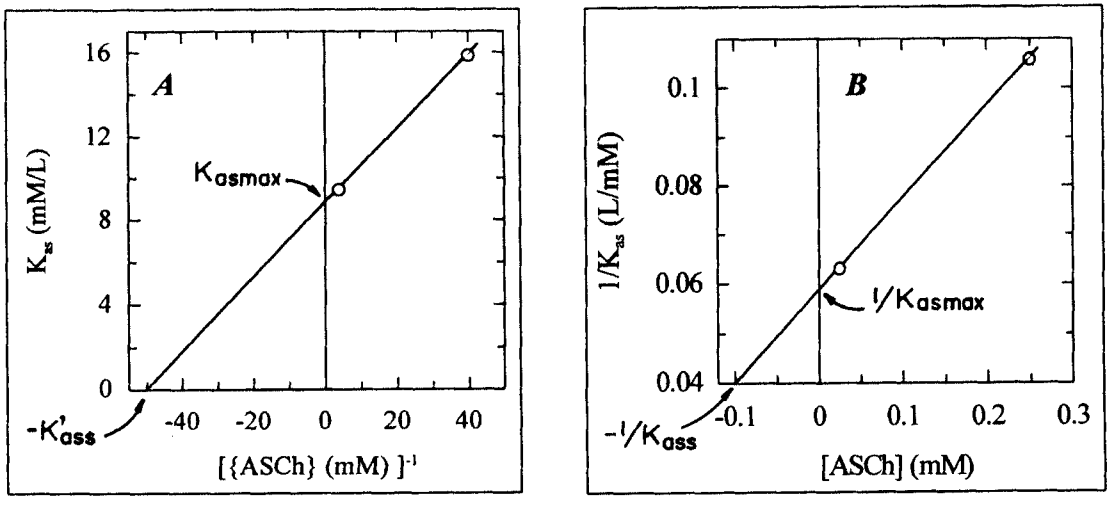

FIGURE 4 Secondary derivative plot of Figure 2. (A) K_{as} (association constant) versus reciprocal of two ASCh concentrations. (B) The reciprocal of K_{as} versus two ASCh concentrations.

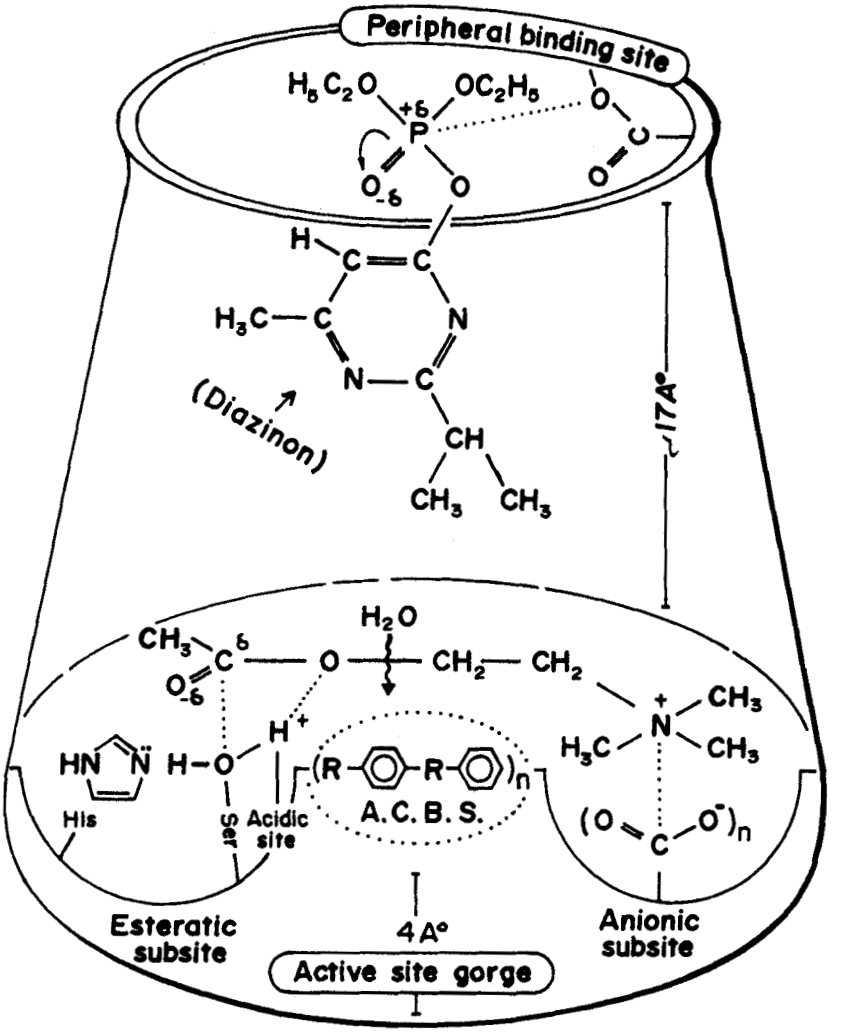

FIGURE 5 Proposed binding-site model for AChE with acetylcholine (substrate) and diazinon (inhibitor) used in the current study.

The values of the kinetic parameters, estimated in the current study, showed that diazinon is relatively a weak inhibitor of bovine retinal AChE as compared to other insecticides studied in our laboratory such as sevin and lannate, which have K_i value of 6.194 and 0.143 μM for camel retinal AChE, respectively.[38,39] Furthermore, a report that diazinon itself is a weak cholinesterase inhibitor yet its biologically active oxygen analog, diazoxon

(catalyzed by mixed-function monooxygenases, such as cytochrome *P*-450, *in vivo*) is a potent inhibitor of brain AChE.[9] Machin *et al.* have also suggested that the critical toxicity of diazinon is due to its extrahepatic activation into biologically active gradients.[7] Therefore, the effect of this insecticide cannot be ignored because it can affect exposed animals as well as human beings (wholly and in particular their eyes) due to spraying on crops and dwellings.

The current study is a unique in providing a rapid method for investigation of the novel kinetic parameters of a compound for inhibition of AChE by using only two substrate concentrations instead of multiple concentrations, so saving on chemicals, enzyme (which can be very expensive) and time. It is a promising general approach for the estimation of various kinetic parameters for the inhibition of different enzymes by a variety of chemicals, insecticides, herbicides and drugs. In the light of our previous studies on retinal AChE, we believe that the use of bovine retinal AChE is an easy and inexpensive model for the kinetic study of AChE inhibition by various drugs and chemicals, and it can be a useful approach to determine the effects of tested anti-AChEs on CNS enzymes, *ex vivo*.[40–47]

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