Original Paper

Towards Less Toxic Organophosphorus Pesticides: Predicting Equation of Acetylcholinesterase Activity

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ABSTRACT

This paper focuses on the applications of multivariate statistical techniques for designing cost-effective, low non-target nerve-damageable organophosphorus pesticides (OPs) used in Indian agriculture. The study was done with regard to the effect of seven OPs on acetylcholinesterase (AChE) activity in four different parts of rat brain: hypothalamus (H), striatum (S), cerebellum (CR), and cerebrum (C). Not all the parts of mammalian brain are equally sensitive to the same pesticide as was evident by direct plotting of inverse of AChE activity versus inverse of LD_{50} (lethal dose), and that of AChE activity versus P_{ow} values. The nature of dependence of AChE activity on the pesticide (P_{ow} i.e., octanol water partition coefficient values) and LD_{50} was determined by multiple regression analysis (MRA). This was supported by multiple correlation coefficient values, which indicate the measure of efficacy for different predicting equations. In most of the cases, the results appeared satisfactory. Thus, using MRA, model pesticides can be designed which are less toxic to non-target organisms such as mammals.

Key Words: Organophosphorus pesticide; Acetylcholinesterase; Multiple regression analysis

Introduction

Quantitative Structure Activity Relationship (QSAR) can be expressed in its most generalised form by the following equation: Biological activity = f(physicochemical and/or structural parameter). The overall objective is to find parameters from experiment or theory, which when substituted into one of the many forms of the equation along with the biological activity for a series of molecules, provide correlation which is statistically significant. When a good quality model is found it can be used to predict some other molecules which will then have greater activity in the defined biological system. For QSAR, one usually describes each analogue as a parent molecule to which substituents have been added. The change in potency when substituents are changed is correlated with the effect of same substituents on various types of physicochemical equilibrium constants, i.e., changes in logarithm of the octanol-water partition coefficient (P_{ow}). Therefore, for QSAR it is most meaningful to describe the biological properties of the molecule in terms of some equilibrium or rate constant. The statistical nature of QSAR enables one to test as many compounds as desired.[1]

Acetylcholine (ACh) is a neurotransmitter which helps in nerve impulse transduction. Acetylcholinesterase (AChE) is an enzyme which hydrolyzes ACh and makes passage for another nerve impulse. This enzyme is inhibited by organophosphorus compounds (OPs). Studies pertaining to the release of acetylcholine have already been conducted in different areas of rat brain, and major cholinergic pathways in central nervous system have been studied by immunohistochemistry.[2,3] Comparative studies have also been performed on AChE in salt-soluble and detergent-soluble parts of rat brain in male and female rats.[4] For interpreting the therapeutical effect of AChE inhibitor drugs on the brain, G4 and G1 molecular forms of acetylcholinesterase in rat cortex were isolated.[5] The effect of chlorpyrifos exposure on acetylcholinesterase activity, lipid peroxidation and activities of different

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ATPases have also been studied in rats.[6] It is therefore pertinent to find out whether there is any difference in acetylcholinesterase activity in different parts of the rat brain in the presence or absence of different organophosphorus pesticides.

Organophosphorus pesticides have comparable chemical structures as they all inhibit acetylcholinesterase activity in post-synaptic neurons. The quantitative correlation between pesticidal activity with physico-chemical parameters related to structure in the rational design of effective pesticides is often referred to as Quantitative Structure Activity Relationship (QSAR) study. The mathematical functions could be so chosen that certain physico-chemical properties influence the bioactivity and the structural modifications, which enhance such properties that would lead to generate potent compounds. A number of attempts have been made from different approaches.[7]

The objective of the present study was to find through Multiple Regression Analysis (MRA), a relationship of the dependence of AChE activity on parameters such as LD_{50} and P_{ow}.[8] From this, a model structure of an organophosphorus pesticide was predicted which would have minimal effect on any part of the mammalian brain. From the predicting equation, AChE of the pesticide having structure similar to the proposed model can be determined by knowing the LD_{50} and P_{ow} values. This will help reduce needless animal sacrifices in laboratories. It can also reduce the unnecessary production of large numbers of chemicals which adversely affect the environment, and thereby reduce the time of production of efficient and environment-friendly OPs. Lastly, it can also reduce the cost of production of a particular chemical by predicting its effects and side effects before production.

A contingent of seven commonly used OPs has been used in this study: methyl parathion, malathion, phorate, dimethoate, chlorpyriphos, monocrotophos and dichlorvos.

Materials and Methods

Animals and Experimental Design: Healthy male Albino Charles Foster rats (~100 g body weight) were treated with *ad libitum* food and water over 12 h day and night cycle for 30 days along with pesticide, observing ethical rules for animal care and handling. Hormonal variation being less in male rats as compared to females, they were chosen preferentially to test the effect of AChE. The rats were divided into 4 groups for 3 sublethal doses of pesticide. Group 1 was considered as the control and fed with palm oil. The rats of Groups 2, 3 and 4 were fed 1/20 LD_{50}, 1/10 LD_{50} and 1/5 LD_{50} doses of pesticide (dissolved in palm oil) respectively, orally for 30 days.

The effects of the first five pesticides mentioned under "Introduction" were used for initial calculations and predicting the equation. The effects of the last two pesticides were used to find the goodness of fit of the predicted equation.

Collection of Tissue: After 30 days of treatment, the rats were sacrificed. Four different parts of their brains, namely hypothalamus (H), striatum (S), cerebellum (CR) and cerebrum (C) were dissected out and collected at 0°C for homogenisation. Homogenisation was carried out in phosphate buffer (0.1 M Na_2HPO_4 and KH_2PO_4, pH 8.0). Brain homogenates of all the four different parts of rat brain were centrifuged at 8000 g.

Enzyme Assay: Supernatant specific spectrophotometric AChE assay[10] and protein estimation[11] were performed, and the effect of the pesticide on four parts of rat brain was observed.

Statistical Analysis: The statistical analysis was performed by utilising 2-way ANOVA for the four different regions of the rat brain. Statistical calculations were done based on the value of AChE activity of the seven organophosphorus pesticides, namely methyl parathion, malathion, phorate, dimethoate, chlorpyrifos, dichlorvos and monocrotophos. Student 't' tests were done and subsequent p values were calculated to determine the significance for each of the pesticides.[12] ANOVA, a powerful statistical technique for tests of significance was employed to test effect of three different lethal doses of pesticides (1/20 LD_{50}, 1/10 LD_{50}, 1/5 LD_{50}) of five pesticides (methyl parathion, malathion, phorate, dimethoate, chlorpyrifos) following standard method,[13] on AChE activity utilizing three observations (AChE activities) per cell (i.e., number of observations receiving a particular level of LD_{50} of a particular pesticide for a particular part of rat brain). AChE activity depends on two factors - Factor A: physical characteristics of pesticide (measured in terms of P_{ow} values) and Factor B: toxicity (measured in terms of LD_{50})

Multiple regression equation of AChE activity (x_1) on level of LD_{50} (x_2) and octanol-water partition coefficient (P_{ow}) values (x_3) were formulated by following the standard method,[14] and various regression coefficients, constants (b_2, b_3, a) were calculated. The standard error and confidence interval of various regression coefficients were also calculated.[15] All the calculations were done separately for four different parts of the brain.

Results

The results of the present investigation are laid out in **Tables 1** to **4**.

Discussion

All OPs affect the AChE activity in mammals and avians. This is the first observation on the effect of different OPs on AChE activity on different parts of rat brain at the doses of 1/20, 1/10, 1/5th of LD_{50} for 30 days. **Table 1** summarizes the effects of OPs on AChE activities (Δ OD/mg pr/hr) in different parts of brain, and the plotted curves prove that not all the parts of the brain are equally sensitive to OP toxicity.

Graphs were plotted showing changes in reciprocal of AChE activity with increase in reciprocal of LD_{50} at four different regions of rat brain at three different doses for five different pesticides. Similar type of exponential relationship was observed between reciprocal of AChE and reciprocal of LD_{50}, i.e., reciprocal of AChE decreases exponentially with reciprocal of 1/20, 1/10 and 1/5 LD_{50} values of different OPs. Although the effects of 1/20 LD_{50} amount of pesticide are almost similar to that of 1/10 LD_{50} in hypothalamus, striatum, and cerebrum, it is not true for cerebellum. The effects of 1/5 LD_{50} dose produce curves of different slopes in almost all parts of the brain. This shows that the experimental nature of dependence of AChE on LD_{50} is log linear.

P_{ow} is the parameter which shows how much a pesticide will penetrate the tissue. The AChE activity is thus related to octanol-water partition coefficient (P_{ow}) values. Since the dependence of AChE activity in four regions of the rat brain would be thus multiple in nature, it was decided to perform Multiple Regression Analysis using ANOVA model.

It is evident from ANOVA (**Table 2**) that variance ratio due to effect of pesticide (F_p), due to LD level (F_L) and due to interaction between P and L (F_{int}) is accepted both at 1% and 5% level of significance in case of Hypothalamus (H). It is also evident that variance ratio F_P and F_{int} are accepted both at 1% and 5% level of significance in case of Striatum (S), variance ratio due to effect of pesticide (F_p), due to LD level (F_L) and due to interaction between P and L (F_{int}) is accepted both at 1% and 5% level of significance in case of Cerebellum (CR), and F_P is accepted both at 1% and 5% level of significance, F_{int} is accepted at 5% level of significance but marginally accepted at 1% level of significance whereas F_L is not accepted at both 1% and 5% level of significance in case of Cerebrum (C). In hypothalamus and cerebellum, calculated values for F_P, F_L & F_{int} respectively have been found to be greater than the tabulated values of F_P, F_L & F_{int} for respective d.f (degrees of freedom) at 5% level of significance. Thus it can be concluded that –

1. There is significant effect of pesticide (measured in terms of P_{ow}) on AChE activity

2. There is significant effect of LD_{50} on AChE activity

3. There exists significant interaction effect between pesticide (measured in terms of P_{ow}) and LD level on AChE activity.

For striatum and cerebrum, in most of the cases the calculated F values are found to be greater than tabulated F values at 1% and 5% level of significance. From 2-way ANOVA, it is evident that factors affecting AChE activity, i.e., effect of pesticide (measured in terms of P_{ow}) and effect of LD_{50} are interdependent. To study their effect on AChE activity, a multiple regression equation involving level of LD_{50} (x_2) and P_{ow} values (x_3) was done.

Table 1 Acetylcholinesterase Activity in Different Parts of Pesticide-treated Rat Brain

Pesticide	**Area**	**Control**	**1/20 LD_{50} (mg/kg)**	**1/10 LD_{50} (mg/kg)**	**1/5 LD_{50} (mg/kg)**
Phorate	a) H	11.33±1.117	10.372±0.584*	7.077±0.767†	5.137±0.198♦
	b) S	14.912±0.731	13.956±0.933§	12.253±0.644†	10.956±1.526
	c) CR	2.559±0.346	2.350±0.250	1.802±0.154	2.248±0.098
	d) C	2.919±0.014	2.085±0.197♦	1.153±0.084♣	1.600±0.129♣
Methyl parathion	a) H	9.782±0.187	6.575±0.0.449♣	6.515±0.169♣	4.997±0.191♣
	b) S	14.912±0.794	5.711±0.453♣	2.835±0.066♣	2.215±0.104♣
	c) CR	5.535±0.06	3.772±0.191♣	3.506±0.209♣	3.301±0.017♣
	d) C	7.555±0.279	4.922±0.211♣	5.632±0.023♣	3.293±0.085♣
Chlorpyriphos	a) H	8.043±0.229	1.995±0.067♣	1.712±0.018♣	1.019±0.036♣
	b) S	16.546±1.671	2.403±0.142♣	2.964±0.153♣	0.947±0.121♣
	c) CR	2.420±0.142	1.681±0.081♦	0.332±0.013♣	0.355±0.017♣
	d) C	2.131±0.343	1.456±0.156	1.408±0.014	0.759±0.023♦
Dimethoate	a) H	7.714±0.532	1.083±0.039♣	1.337±0.061♣	1.691±0.014♣
	b) S	13.625±1.658	1.959±0.134♣	2.222±0.364♣	3.317±0.318♣
	c) CR	1.899±0.203	1.002±0.038♦	0.695±0.023♣	0.454±0.017♣
	d) C	1.891±0.163	0.789±0.033♣	0.921±0.031♣	1.086±0.058♣
Malathion	a) H	11.558±0.450	11.544±0.111	11.820±0.264	9.843±0.309†
	b) S	16.540±0.880	15.864±0.321*	16.990±0.300*	19.690±0.201
	c) CR	4.469±0.328	4.648±0.117	4.545±0.110	4.240±0.132
	d) C	4.333±0.204	2.334±0.085♣	2.330±0.080♣	♣
Dichlorvos	a) H	8.461± 0.953	4.601± 0.155♦	4.228± 0.515*	3.840± 0.216♦
	b) S	13.934±3.804	7.535±0.200	7.606±1.007	5.756±0.484
	c) CR	2.795±0.360	2.776± 0.274	3.053± 0.566	1.741± 0.106
	d) C	1.681±0.474	2.471±0.132	1.056±0.207	2.880±0.301
Monocrotophos	a) H	7.714± 0.532	2.255±0.105♣	2.186± 0.231♣	1.713± 0.098♣
	b) S	13.625±1.658	10.675±0.471	4.11±0.214♣	2.852±0.220♣
	c) CR	2.420± 0.142	2.149 ± 0.139	1.316± 0.031♣	0.668± 0.037♣
	d) C	2.120±0.175	1.691±0.151	1.718±0.143	0.372±0.001♣

† p<0.05 ; * p<0.02 ; ♦ p<0.01 ; ♣ p<0.001 H: Hypothalamus, S: Striatum, CR: Cerebellum, C: Cerebrum
Results are expressed as Mean ± SEM of 6 rats in each cage

Table 2 2-Way Anova with Three Observations per Cell

Area	Source of Variation	Degrees of Freedom (d.f.)	Sum of Squares (SS)	Mean Sum of Squares (MSS)	Variance Ratio (F)	F Tabulated 1%	F Tabulated 5%
H	Pesticide (P)	4	644.377	161.094	160.133 (F_P)	4.02	2.69
	LD level (L)	2	31.807	15.903	15.808 (F_L)	5.39	3.32
	Interaction between P and L	8	36.869	4.609	4.581(F_{int})	3.17	2.27
	Residual Error	30	30.171	1.006			
	Total	44					
S	Pesticide (P)	4	1602.091	400.523	278.916 (F_P)	4.02	2.69
	LD level (L)	2	2.166	1.083	0.754 (F_L)	5.39	3.32
	Interaction between P and L	8	75.484	9.435	6.570(F_{int})	3.17	2.27
	Residual Error	30	43.087	1.436			
	Total	44					
CR	Pesticide (P)	4	106.787	26.697	157.041 (F_P)	4.02	2.69
	LD level (L)	2	2.623	1.311	7.712 (F_L)	5.39	3.32
	Interaction between P and L	8	5.51	0.689	4.053(F_{int})	3.17	2.27
	Residual Error	30	5.099	0.170			
	Total	44					
C	Pesticide (P)	4	72.688	18.172	45.092(F_p)	4.02	2.69
	LD level (L)	2	0.799	0.389	0.965 (F_L)	5.39	3.32
	Interaction between P and L	8	9.318	1.165	2.891(F_{int})	3.17	2.27
	Residual Error	30	12.097	0.403			
	Total	44					

H: Hypothalamus, S: Striatum, CR: Cerebellum, C: Cerebrum

The nature of dependence of AChE activity on A (physical characteristics of pesticide measured in terms of P_{ow}) and B (toxicity measured in terms of LD_{50}) is given by the multiple regression equation –

$x_1 = \ln Y = a + b_2x_2 + b_3x_3$

where $x_1 = \ln Y$ = AChE activity

x_2 = level of LD_{50} (lethal doses of different pesticides in mg/kg body weight)

x_3 = octanol-water partition coefficient (P_{ow}) value of particular pesticide

a = constant

b_2= partial regression coefficient of Y (AChE activity) on x_2 (level of LD_{50})

b_3= partial regression coefficient of Y (AChE activity) on x_3 (P_{ow} value)

The above analysis leads to the determination of standard error (SE) and confidence interval (CI) of b_2 and b_3 at different doses in the four regions of rat brain. It is to be noted from **Table 3** that as the value of $r_{1.23}$ increases

Table 3 Predicting Equation for 3 Different Doses of LD_{50} on 4 Different Parts of Rat Brain

Area	Level of LD_{50}	Predicting Equation with SE (Standard error) & CI (Confidence interval)	$r_{1.23}$ (Multiple correlation coefficient)	s (s^2 is the variance of X_1 for any fixed values of X_2 and X_3)
H	1/20	$\ln Y = 1.464 + 0.010x_2 - 8.350 \times 10^{-6}$ $Sb_2=3.396 \times 10^{-4}$ $Sb_3=2.72 \times 10^{-7}$ $CI= \pm 0.00146$ $CI=\pm 1.17 \times 10^{-6}$	0.474	0.021
	1/10	$\ln Y = 1.390 + 0.006x_2 - 9.662 \times 10^{-6}x_3$ $Sb_2=0.00007$ $Sb_3=1.16 \times 10^{-7}$ $CI= \pm 0.003$ $CI=\pm 4.99 \times 10^{-7}$	0.620	0.009
	1/5	$\ln Y=1.270 + 0.003x_2 - 1.421 \times 10^{-5}x_3$ $Sb_2=0.00001$ $Sb_3=3.90 \times 10^{-8}$ $CI=\pm 0.00004$ $CI=\pm 1.68 \times 10^{-7}$	0.810	0.003
S	1/20	$\ln Y = 1.786 + 0.010x_2 - 9.768 \times 10^{-6}x_3$ $Sb_2=2.911 \times 10^{-4}$ $Sb_3=2.33 \times 10^{-7}$ $CI=\pm 0.00125$ $CI=\pm 1.00 \times 10^{-6}$	0.583	0.018
	1/10	$\ln Y = 1.419 + 0.008x_2 - 3.640 \times 10^{-6}x_3$ $Sb_2=0.00006$ $Sb_3=1.03 \times 10^{-7}$ $CI=\pm 0.000258$ $CI=\pm 4.43 \times 10^{-7}$	0.630	0.008
	1/5	$\ln Y = 1.410 + 0.005x_2 - 1.644 \times 10^{-5}x_3$ $Sb_2=0.00002$ $Sb_3=5.20 \times 10^{-8}$ $CI=\pm 0.0000861$ $CI=2.24 \times 10^{-7}$	0.831	0.004
CR	1/20	$\ln Y = 0.732 + 0.009x_2 - 2.864 \times 10^{-6}x_3$ $Sb_2=1.94 \times 10^{-4}$ $Sb_3=1.55 \times 10^{-7}$ $CI=\pm 0.001$ $CI=\pm 6.67 \times 10^{-7}$	0.531	0.012
	1/10	$\ln Y = 0.560 + 0.005x_2 - 1.863 \times 10^{-5}x_3$ $Sb_2=0.00006$ $Sb_3=9.10 \times 10^{-8}$ $CI=\pm 0.000258$ $CI=\pm 3.92 \times 10^{-7}$	0.809	0.007
	1/5	$\ln Y = 0.431 + 0.003x_2 - 1.672 \times 10^{-5}x_3$ $Sb_2= 0.00002$ $Sb_3=6.50 \times 10^{-8}$ $CI=\pm 0.0000861$ $CI=\pm 2.80 \times 10^{-7}$	0.699	0.005
C	1/20	$\ln Y = 0.765 - 0.001x_2 - 4.132 \times 10^{-6}x_3$ $Sb_2= 2.80 \times 10^{-7}$ $Sb_3=1.94 \times 10^{-7}$ $CI=\pm 0.00104$ $CI=\pm 8.35 \times 10^{-7}$	0.240	0.015
	1/10	$\ln Y = 0.634 + 0.001x_2 - 3.859 \times 10^{-6}x_3$ $Sb_2=0.00006$ $Sb_3=1.03 \times 10^{-7}$ $CI=\pm 0.000258$ $CI=\pm 4.43 \times 10^{-7}$	0.230	0.008
	1/5	$\ln Y = 0.613 + 0.001x_2 - 1.019 \times 10^{-5}x_3$ $Sb_2=0.000008$ $Sb_3=2.60 \times 10^{-7}$ $CI=\pm 0.00003$ $CI=\pm 1.12 \times 10^{-7}$	0.731	0.002

SE, CI & s (H: Hypothalamus, S: Striatum, CR: Cerebellum, C: Cerebrum) from zero to unity, the multiple regression equation may be viewed as tending towards a perfect predicting formula. Smaller range of confidence interval indicates efficient estimation of b_2 and b_3.

From **Table 3** it is clear that though b_2 and b_3 are small, values of Y are sufficiently influenced by values of b_2 and b_3 because they are exponentially related. Therefore, their contributions are not insignificant. An attempt was made to find the lnY by putting the LD_{50} value and P_{ow} value (both of these values taken from literature) in the multiple regression equation. In this way, it was possible to compare the expected lnY values from regression equation and the experimentally observed lnY values from pesticide treated rat brain. Here, along with the first five pesticides the last two were considered to estimate as to whether they fit the predicting equation. **Table 4** shows the comparisons.

Table 4 Comparison of Pesticide Treatment

	Pesticide	**1/20 LD_{50}**		**1/10 LD_{50}**		**1/5 LD_{50}**	
		Expected	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**
H	Methyl parathion	4.336	6.575	4.027	6.515	3.564	4.997
	Malathion	9.143	11.544	9.865	11.820	8.715	9.843
	Phorate	4.055	10.372	3.725	7.077	3.187	5.137
	Dimethoate	4.909	1.083	4.678	1.337	4.149	1.691
	Chlorpyriphos	2.158	1.995	1.804	1.712	1.057	1.019
	Dichlorvos	4.477	4.601	4.187	4.228	3.710	3.840
	Monocrotophos	4.375	2.255	3.960	2.186	3.611	1.713
S	Methyl parathion	5.995	7.138	4.170	2.835	4.120	2.215
	Malathion	12.604	15.864	13.791	16.990	18.302	19.690
	Phorate	5.534	13.956	4.023	12.253	3.607	10.956
	Dimethoate	6.776	1.959	5.043	2.222	5.285	3.317
	Chlorpyriphos	2.617	2.403	3.303	2.964	1.047	0.947
	Dichlorvos	6.170	7.535	4.371	7.606	4.242	5.756
	Monocrotophos	6.031	10.675	4.208	4.110	4.191	2.852
CR	Methyl parathion	2.090	3.772	1.747	3.506	1.539	3.301
	Malathion	4.092	4.648	3.673	4.545	3.758	4.240
	Phorate	2.033	2.350	1.513	1.802	1.350	2.248
	Dimethoate	2.333	1.002	1.988	0.695	1.793	0.454
	Chlorpyriphos	1.702	1.681	0.342	0.332	0.382	0.355
	Dichlorvos	2.145	2.776	1.813	3.053	1.605	1.741
	Monocrotophos	2.098	2.149	1.770	1.316	1.560	0.668
C	Methyl parathion	2.143	4.922	1.883	5.632	1.842	3.293
	Malathion	1.987	2.334	2.186	2.330	2.477	2.600
	Phorate	2.075	2.085	1.828	1.153	1.704	1.600
	Dimethoate	2.121	0.789	1.933	0.921	1.943	1.086
	Chlorpyriphos	1.463	1.456	1.343	1.408	0.749	0.759
	Dichlorvos	2.140	2.471	1.898	1.056	1.872	2.880
	Monocrotophos	2.147	1.691	1.889	1.718	1.855	0.372

H: Hypothalamus, S: Striatum, CR: Cerebellum, C: Cerebrum

From the foregoing discussion it would be clear that this type of predicting equation ($\ln Y = a + b_2x_2 + b_3x_3$) can be used for any pesticide for predicting activity from given values of lethal dose (levels used in the experiment) and P_{ow} values for a particular portion of rat brain. Using this equation, a pesticide having minimum toxic effect on non-targets (farmers who come in direct or indirect contact with pesticides) can be predicted. This is an environment friendly approach, and can be extended to design drugs and many other valuable chemicals also.

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