

Kinetics For The Inhibition of Serum Acetylthiocholine Esterase Activity by Some prepared Phenobarbital Derivatives.

Zaizafoon N. (University of AL-Mustansiriyah, College of Science, Department of Chemistry).

Abstract:

This work addresses the kinetic analysis on the interaction of some prepared Phenobarbital derivatives (A,B,C,D) with human serum acetyl cholinesterase. It was found that these compound (A,B,D) does have inhibitory effects at different concentrations (10^{-4} , 10^{-6} , 10^{-8} , 10^{-10} M), and were observed to have elevated inhibition with increasing concentrations (10^{-10} to 10^{-4} M) for both compounds A and B. Inversely, inhibition was increased with decrease concentration from 10^{-4} to 10^{-10} M for D. The effects of each A,B and D was reversible in nature. All of the results for C compound were neglected. Michaelis- Menten constant and maximum velocity for hydrolysis of acetyl thiocholine iodide by AChE was determined in control and treated systems. Lineweaver-Burk plot and their secondary replots indicated that the nature of inhibition was (competitive at concentration 10^{-4} , non competitive at 10^{-10} for A), (non competitive at 10^{-4} , competitive at 10^{-10} for B), (non competitive at 10^{-4} , uncompetitive at 10^{-10} for D) respectively. The value of k_i was also estimated. The action mechanism of these types of compounds acting as inhibitors to the AChE is suggested.

Introduction:

Cholinesterases (ChE) are specialized carboxylic ester hydrolases that catalyze the hydrolysis of choline esters. Two types of ChE activity have been identified in mammalian blood and tissues. These are distinguished according to their substrate specificity and sensitivity to the selective inhibitors. The first is acetylcholinesterase (AChE, E.C.3.1.1.7), which is systematically called acetylcholine acetylhydrolase. Other names include true cholinesterase, specific cholinesterase, red blood cell cholinesterase, erythrocyte cholinesterase, and cholinesterase I. The second is butyrylcholinesterase (BChE, E.C.3.1.1.8), which is referred to systematically as acylcholine acylhydrolase. Other names include cholinesterase, pseudocholinesterase, non-specific cholinesterase, plasma cholinesterase, serum cholinesterase, propionylcholinesterase, benzoylcholinesterase, and cholinesterase II ⁽¹⁻⁴⁾.

Biological markers (biomarkers) were early defined as “cellular, biochemical or molecular alterations that are measurable in biological media such as human tissues, cells, or fluids⁽⁵⁾. More recently, the definition includes biological characteristics that can be objectively measured and evaluated as indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention⁽⁶⁾. One of the early biomarkers characterized in human environmental exposure is represented by the inhibition of the enzyme acetylcholinesterase (AChE)

as biomarker of effect on nervous system following complex exposure to organophosphorus compounds⁽⁷⁾. The present work aims to study the effect of phenobarbital derivatives on this biomarker in relation to the current and future use in environmental and occupational human health monitoring.

The catalytic triad (Ser-203, His-447, and Glu-334) (all numbers refer to the amino acid position of human acetylcholinesterase) have been shown to be located at the base of an active site gorge, with the peripheral anionic site in the rim of this gorge, including residues (Tyr-72, Tyr-124, Trp-286, Tyr-841 and Asp-74). Interestingly, these two sites have been found to be working in tandem, where the role of peripheral anionic site can decrease or increase activity at catalytic site. For example, at high concentrations both acetylcholine and acetylthiocholine have been shown to bind peripheral anionic site, leading to substrate inhibition as a result of reduced substrate hydrolysis through steric blockade and/or conformation change in Trp-86 and Tyr-133 residues within the active site gorge⁽⁸⁾.

Cholinesterase inhibitor (or "anticholinesterase") suppresses the action of the enzyme because the essential function chemicals that interfere with the action of AChE are potent neurotoxins, which caused excessive salivation and eye-watering in low doses, followed by muscle spasms and ultimately death (examples are : reversible inhibitor compounds that act as the reversible competitive or non-competitive inhibitors of cholinesterase the most likely to have therapeutic uses. These include (organophosphates, carbamates, phenanthrene derivatives and tacrine). Quasi-irreversible inhibitor compounds that act as quasi-irreversible inhibitors of cholinesterase are most likely to be used as chemical weapons or pesticides, and these include (sarin, soman and phenmedipham)⁽⁹⁾. Barbituric acid system is a structural

element of many drugs that have anticancer activity such as raltitrexed and thymidine and their activity as thymidylate enzyme inhibitors^(10,11). Heterocyclic of barbituric acid represent as a new class of antitumor drugs, it was found to inhibit the epidermal growth factor receptor (EGFR)^(12,13). Furthermore, barbituric acid exerts their antitumor activity through inhibition of DNA repair enzyme system, enzyme-mediated repair of strand lesion in DNA is an established mechanism for resistance toward antitumor DNA damaging drugs and radiotherapy⁽¹⁴⁻¹⁶⁾. Schiff's bases can be considered as useful tools to prepare many compounds from barbituric acid.

The present study involves the preparation of four compounds that derive from Phenobarbital which appeared to have biological importance through the antibacterial activity for three types of bacteria (*Escherichia coli*, *Proteus mirabilis*, *Staphylococcus aureus*). The compounds were prepared and characterized by spectrum methods (U.V, IR, and C.H.N) with the determination of $RF^{(17)}$. These derivatives were obtained from the preparation of ester by the reaction of 5-ethyl-5-phenylbarbituric acid with ethyl bromoacetate. The ester then reacts with hydrazine hydrate which utilizes its derivatives to prepare Schiff's bases.

Phenobarbital derivatives required a large tension according to biological activities that proved it, for example: these compounds were mostly utilized as anti-spasmodic,

with some of them as anti microbial and antifungal^(18,19). The importance of Schiff's bases due to the presence of substitutes N-benzylidine derivative requires this type of compound biological activity, such as: salicylidene aniline and its derivatives, and the last is very useful as antimicrobial, fungi static agents and exhibit antitubercular activity⁽²⁰⁾. There are many Schiff's base molecules that are useful in life such as (Rhodopin) which originated from the combination of vitamin A with macroprotein (opsin). In addition Schiff's bases have medical properties such as diuretic and cordiotonic, and these compounds were derivatized from furfural⁽²¹⁾.

Materials and Methods:

Materials:

Acetylcholinesterase activity was assayed by Ellman Method⁽²²⁾. The principle of the method is the measurement of the rate of production of thiocholine as acetylthiocholine is hydrolyzed. This was accomplished by the continuous reaction of the thiol with 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) to produce the yellow color of 5-thio-2-nitrobenzoic acid. The rate of color production is measured at 430nm.

All compounds studied were prepared as following: By refluxing mixture of (6.9gm)(5-ethyl-5-phenyl barbituric acid) with (10gm)(bromoethyle acetate) and 8 gm of sodium carbonate for (6hr.)^(23,24) the product was vaporized under vacuum pressure until appearance of white crystal (ester), then (4.5gm) from ester was treated with (2.5gm) of hydrazine hydrate and 25 ml of benzen and refluxing the mixture for (6hr.) a gain to produce pure hydrazine derivative, then (0.5gm) of product refluxing with (0.3gm) of aldehydes or ketons in (25ml absolute ethanol) for (4hr.) the mixture was cooled, dried and recrystallized with suitable solvent as shown in table (1).

Blood sampling:

Five ml of blood was drawn from the same subject by vein directly after the clotting, in centrifuge at 3000rpm for 10min., the serum sample was separated and used immediately as a source of enzyme.

Methods:

1) **Determination of AChE activity** : human serum AChE activity was determined using Ellman et al⁽²²⁾ method as follows:

(50μL) of DTNB solution (0.001M) is added to (2.25ml) of sodium phosphate buffer solution (PH=7.3, 0.2M), then (10μL) of serum is added, mixed well and (2ml) of the mixture is transferred to a measuring cell (1cm), then (34μL) of acetylthiocholine iodide (ASChI 0.06M) is added, the change in absorbency is measured before and after adding the substrate at (430nm) for (3min.). The enzyme activity is calculated as the concentration in μ mole of the substrate hydrolyzed to each (ml) of sample in (3min.) and expressed as (μmole/3min./ml).

2) **Determination of biological activity of phenobarbital derivatives**⁽²⁵⁾. A stock solution (0.01M) concentration of each compounds in table (1) is prepared

and then different concentrations ($10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-10}$)M of each compound are prepared by diluting with dimethyl sulfoxide (DMSO) as solvent. ChE activity is measured in human serum as follows:

(50 μ L) of DTNB solution (0.001M) is added to (2.25ml) of sodium phosphate buffer solution (PH= 7.3, 0.2M), 0.25ml of inhibitor is mixed with 2ml of the same buffer, then (10 μ L) of serum is added, mixed well and (2ml) of the mixture is transferred to a measuring cell (1cm), then (34 μ L) of (AChI 0.06M) is added, the change in absorbency is measured after adding the substrate at 430nm for 3min.. The inhibition percentage is calculated by comparing the activity with and without inhibitor and under the same conditions according to the equation:

$$\%Inhibition = 100 * \left(100 - \frac{\text{The activity in the presence of inhibitor}}{\text{The activity in the absence of inhibitor}}\right)$$

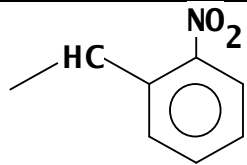
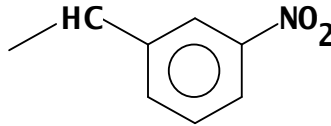
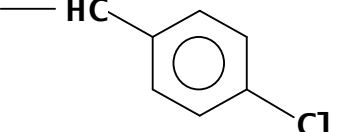
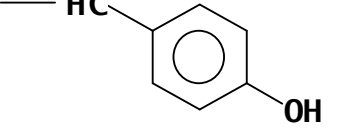
3) Determination the type of inhibition: A constant concentration of inhibitors ($10^{-4}, 10^{-10}$ μ L) (higher and lower) concentration of inhibitors. (10^{-2} M is excluded in the study due to precipitation during experiment) is used with different concentrations of substrate (0.02, 0.04, 0.06, 0.08M) to study the type of inhibition. These different concentrations are prepared from the stock solution (0.1M) AChI. The enzyme activity is determined with and without the inhibitors using the lineweaver – Burk equation by plotting $1/V$ vs. $1/[S]$ ⁽²⁶⁾. The following values were calculated 1) K_i , 2) Apparent V_{max} (V_{mapp}), 3) Apparent K_m (K_{mapp}), 4) type of inhibition.

Results and Discussion:

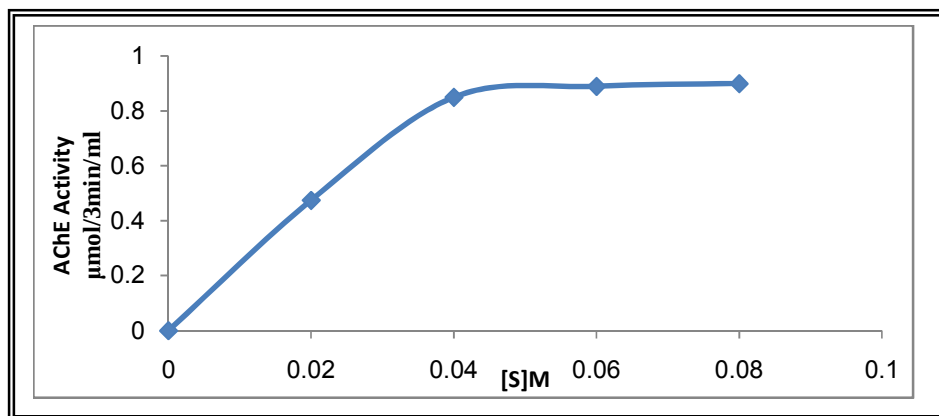
An AChE inhibitor is a chemical that inhibits the cholinesterase enzyme from breaking down acetylcholine, increasing both the level and duration of action of the neurotransmitter acetylcholine ⁽²⁷⁾.

AChE activity in present study was assayed in the absence and presence of Phenobarbital derivatives under different substrate concentrations. A series of compounds in table(1) is designed to investigate the effects of the Phenobarbital derivatives on AChE activity in vitro. First experiment tried to study the effect of solvent DMSO which did not show any inhibitory effect as found and as Jafferet *al* ⁽²⁸⁾ found too. Then examine the compounds A, B, C and D in the mixture at different concentrations ($10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-10}$ M).

Table (1): The derivatives of Phenobarbital used to inhibitedAChE.

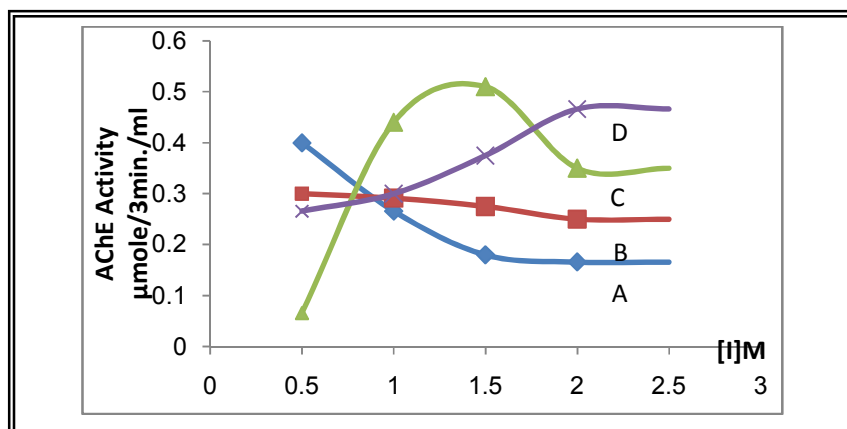
Comp. No.	Comp. name	R	Color	Yield%	m.p.c°	M. Formula	Purification solvents
A	5-ethyl-5-phenyl-1,3-di[N(2-nitrobenzilidin hydrazinyl)]barbituric acid		Yellow	50	(200-203)	C ₃₀ H ₂₇ N ₈ O ₉	Ethanol + Water
B	5-ethyl-5-phenyl-1,3-di[N(3-nitrobenzilidin hydrazinyl)]barbituric acid		Light yellow	40	(185-188)	C ₃₀ H ₂₇ N ₈ O ₉	Ethanol + Water
C	5-ethyl-5-phenyl-1,3-di[N(4-chlorobenzilidin hydrazinyl)]barbituric acid		Yellow crystal	45	(201-203)	C ₃₀ H ₂₅ Cl ₂ N ₆ O ₇	Acetone + Water
D	5-ethyl-5-phenyl-1,3-di[N(4-hydroxybenzilidin hydrazinyl)]barbituric acid		Dark yellow	55	(166-168)	C ₃₀ H ₂₈ N ₆ O ₇	Acetone + Water

Before each set of inhibition experiments, AChE activity was measured by using four different concentrations of acetyl thiocholine iodide (substrate) (0.02, 0.04, 0.06, 0.08) M as in figure (1).



Figure(1): The Michaelis-Menten plot of AChE with different concentrations of substrate without inhibitor.

The effect of four concentrations of each inhibitors (A,B,C and D) in AChE activity is illustrated in figure (2).



Figure(2): The relationship between concentrations of compound A,B,C and D on AChE activity .

We paid little attention to compound C, which shows unclear inhibition to enzyme activity figure(2) as a result of uncomplete dissolving of these compound in DMSO. From above figures we notice that the AChE activity was decreased significantly ($p \leq 0.05$) with increase in inhibitors (A & B) concentrations. In contrast, enzyme was inhibited significantly ($p \leq 0.05$) from concentration 10^{-4} to 10^{-10} in presence of compound D. Figure (3) showed the effect of maximum and minimum concentrations of inhibitors at different acetylcholine concentrations on AChE activity. .

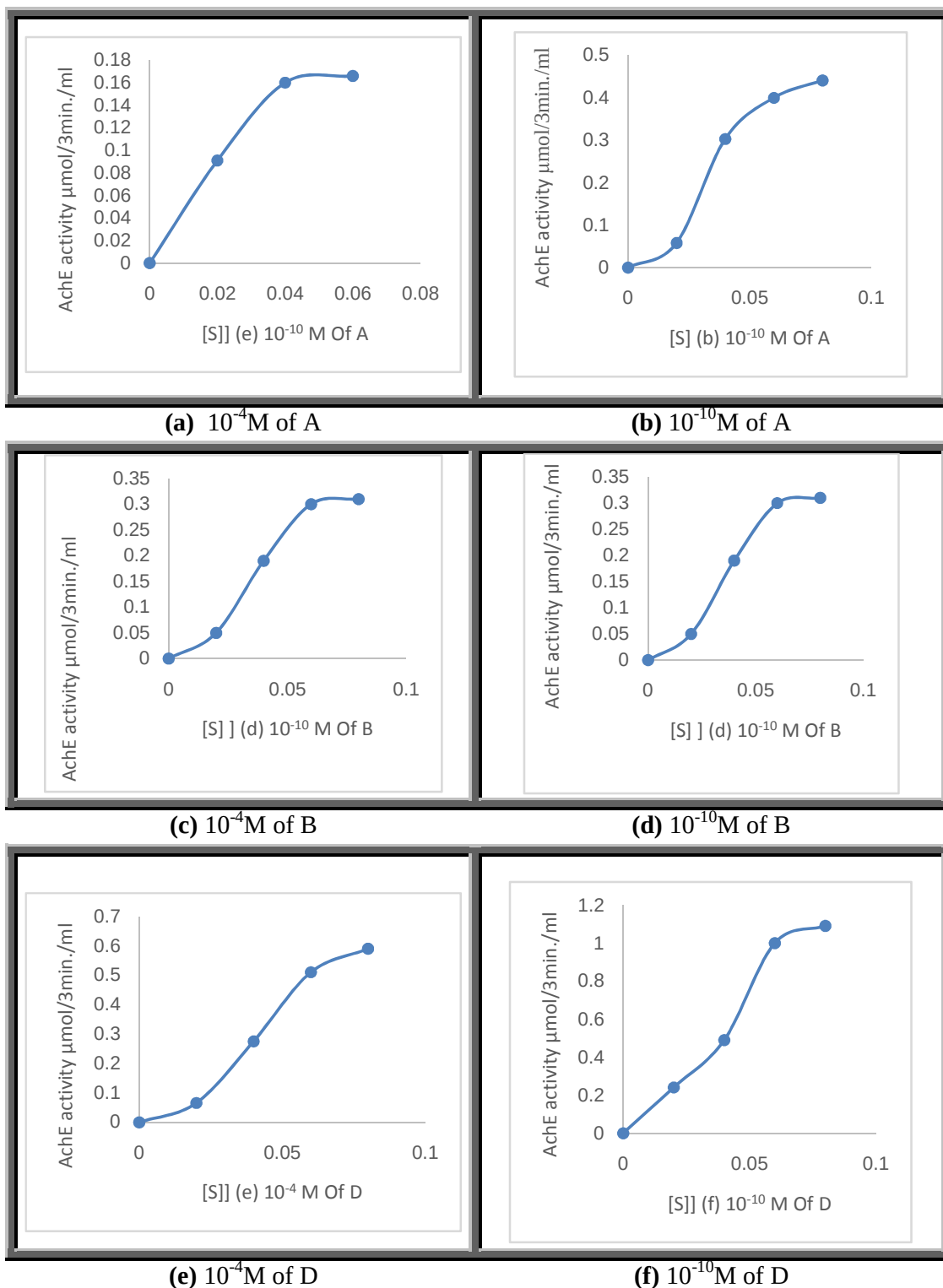


Figure (3): The Michaelis-Menten plots of AChE with different concentrations of substrate in presence of inhibitors.

The biochemical tests revealed that all compounds cause significant inhibitory effects on enzyme activity Table (2) compared with the measured normal values of the enzyme activity.

Table (2) showed that the highest significant inhibition percent was caused by compound A than others, these can be attributed to NO₂ group on ortho position of phenyl group which may lead to good orientation to active site gorge compared to rest compounds.

Table (2): The effect of different concentrations of compounds on the human serum AChE activity.

	Inhibition con. (M)	AChE activity $\mu\text{mol}/3\text{min.}/\text{ml}$ mean $\pm$ SD	%Inhibition
control	zero	2.4 $\pm$ 0.300	-
A	10 ⁻⁴	0.166 $\pm$ 0.002	80.08*
	10 ⁻⁶	0.180 $\pm$ 0.002	78.4*
	10 ⁻⁸	0.266 $\pm$ 0.0025	68.07*
	10 ⁻¹⁰	0.4 $\pm$ 0.033	51.99*
B	10 ⁻⁴	0.25 $\pm$ 0.022	69.99*
	10 ⁻⁶	0.275 $\pm$ 0.004	66.99*
	10 ⁻⁸	0.291 $\pm$ 0.002	65.07*
	10 ⁻¹⁰	0.3 $\pm$ 0.02	63.99*
D	10 ⁻⁴	0.5166 $\pm$ 0.003	37.99*
	10 ⁻⁶	0.375 $\pm$ 0.0035	54.99*
	10 ⁻⁸	0.3 $\pm$ 0.021	63.99*
	10 ⁻¹⁰	0.266 $\pm$ 0.0024	68.07*

Results with (p $\leq$ 0.05) considered significant.

The greatest significant inhibition was found at concentration (10⁻⁴ M) in A & B. Inversely, compound D showed greater significant inhibition at (10⁻¹⁰ M) as cleared in figure (4):

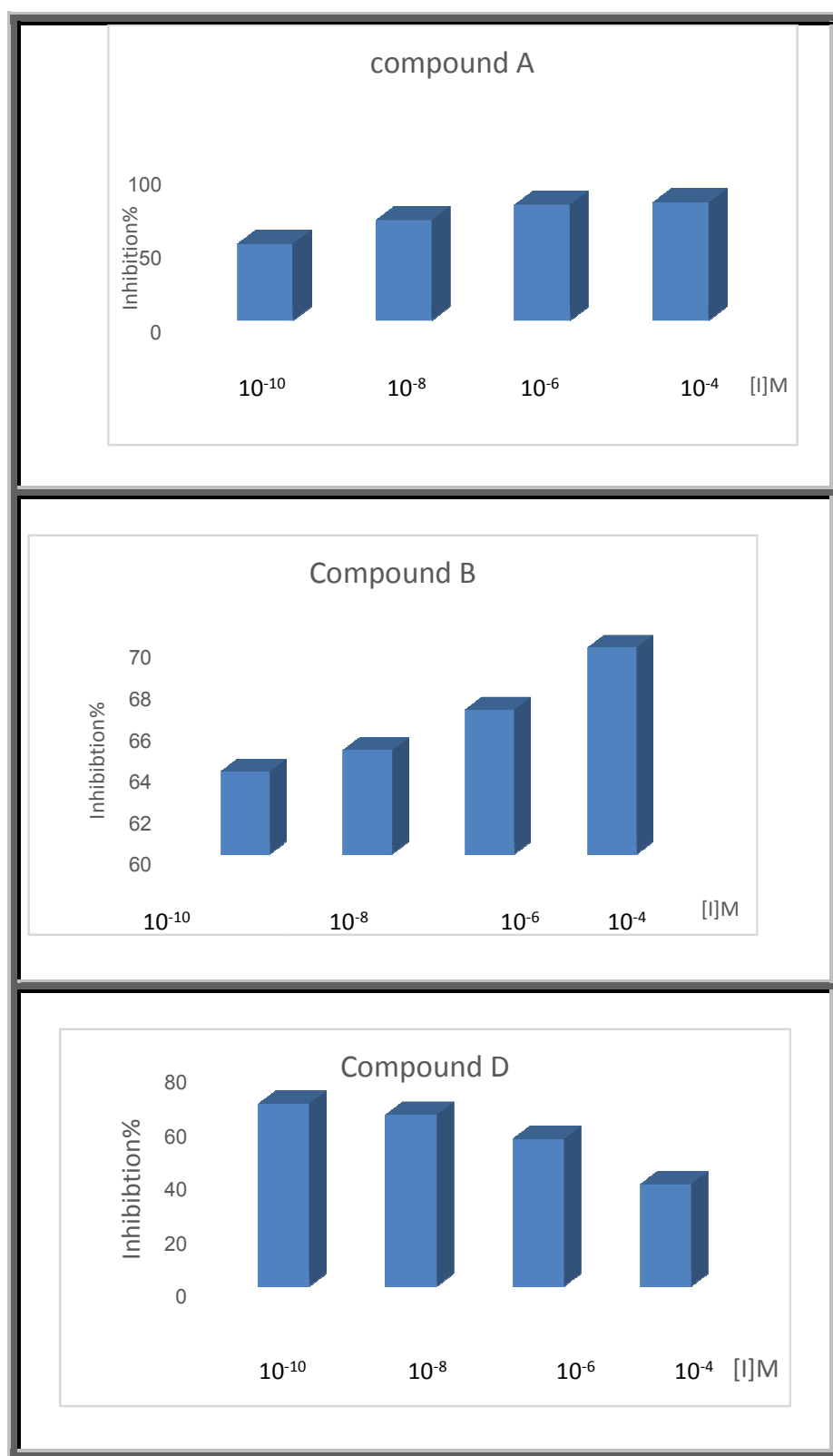


Figure (4):The Percentage of inhibition of AChE by different concentrations (10^{-4} , 10^{-6} , 10^{-8} , 10^{-10}) M of compounds (A,B and D)

It's observed that the substituted with 2-nitrobenzil (A) and 3-nitro benzyl(B) and 4-hydroxy benzyl (D) derivatives of Phenobarbital compound exhibits the main cause of inhibition , the different substitution of NO₂ group in A & B with OH group in D may explain inverse the inhibition from that in A and B according to the inhibitors concentrations .

Several findings also indicate the anticholinesterase effect of polycyclic aromatic hydrocarbons which are common environmental contaminants in surface waters, sediments, soils, and urban air. These compounds are formed during the incomplete combustion of fossil fuels, wood, and municipal waste incineration, from internal combustion engines⁽²⁹⁾. Kang and Fang⁽³⁰⁾ demonstrated that several polycyclic aromatic hydrocarbons inhibit AChE directly in vitro. The magnitude of the inhibition differs among the compounds tested and may be related to the number of aromatic rings in the molecule. Interestingly, polycyclic aromatic hydrocarbons are able to inhibit AChE activity in an additive manner together with organophosphate, being noncompetitive inhibitors of AChE⁽³⁰⁾.

In same line there are other studies that refer to the inhibitory effect of some classes of compounds on enzyme activity such as carbamates⁽³¹⁾, tacrine⁽³²⁾, prophen⁽³³⁾.

Study Type of Inhibition:

The second part of this study include determine the type of inhibition and kinetic parameters (K_m , V_{max} , and K_i) at different concentrations of substrate and under the same conditions by using Lineweaver-Burk equation and one plotted as shown in figures (5), table (3):.

Table (3): the kinetic properties of AChE with and without A,B and D compounds.

Sample	Inhibitor Concentration (M)	K_m (M) mean±SD	V_{max} (μ mol/ml/ min) mean±SD	K_i (M) mean±SD	Inhibition type
Control	Zero	0.0555±0.01	1.66±0.2	-	-
A	10 ⁻⁴	0.5±0.076	1.66±0.277	1.123×10 ⁻⁵ ±7.2×10 ⁻⁷	Competitive
	10 ⁻¹⁰	0.055±0.010	0.555±0.08	5×10 ⁻¹¹ ±5.9×10 ⁻¹⁵	Non comp.
B	10 ⁻⁴	0.055±0.011	0.526±0.073	4.615×10 ⁻⁹ ±9.1×10 ⁻⁷	Non comp.
	10 ⁻¹⁰	0.5±0.071	1.66±0.2	1.235×10 ⁻¹² ±4.3×10 ⁻¹⁴	Competitive
D	10 ⁻⁴	0.055±0.010	0.869±0.077	1.09×10 ⁻⁴ ±2×10 ⁻⁸	Non comp.
	10 ⁻¹⁰	0.035±0.009	0.454±0.060	3.73×10 ⁻¹¹ ±2.4×10 ⁻¹³	Un comp.

It is very clear from this presentation that K_m was higher or the same in the presence of Phenobarbital derivatives. This indicates that the affinity of ASCh (substrate) decreases in presence of each inhibitors for both concentrations (10^{-4} , 10^{-10})M in some cases.

The affinity is obviously influenced by several factors, for example size, three-dimensional structure, presence of groups which easily bind non-covalently to groups in or close to the active site etc. A high K_m means that the inhibitor fits very well into the active-site left of the enzyme. A consequence of such a good fit could be an orientation in space such that the covalent bonding to the serine residue is facilitated.

The V_{max} value for control sample was higher than in inhibited samples, so it is clear that the amount of active enzyme V_{max} present in non-inhibited system. The biochemical tests revealed that K_i (the binding affinity of the inhibitor) at 10^{-4} is higher than 10^{-10} for all studied compounds.

This difference in K_i values enables to conclude that not all of the assumptions underlying classic Michaelis-Menten equations are being obeyed and that the data are consistent with the kinetics of a tight-binding inhibitor.

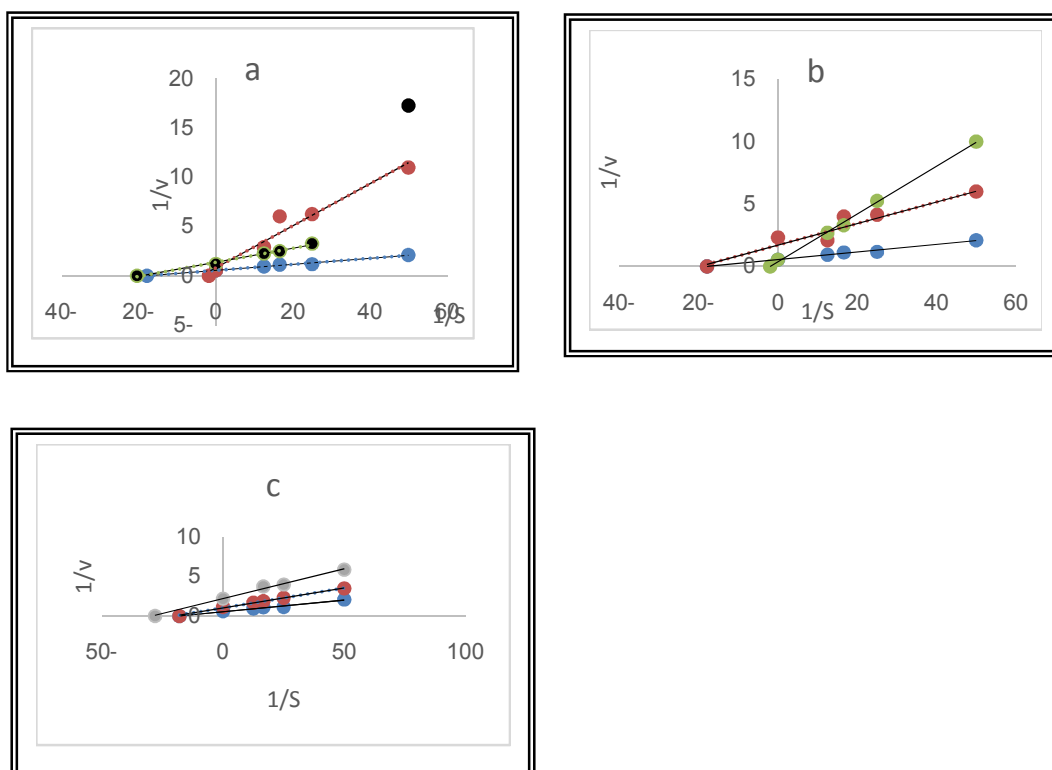


Figure (5): Lineweaver-Burk Plots of AChE with each Concentrations (10^{-4} , 10^{-10})

¹⁰)M of compounds a – compound A b – compound B c – compound D

Studies by Forget *et al.*⁽³⁴⁾, Tomanet *al.*⁽³⁵⁾, and Cacciatore *et al.*⁽³⁶⁾ showed that the combination of metal/pesticides, cadmium/diazinon, and azinphos-methyl oxon/chlorpyrifosoxon caused greater enzyme inhibitions compared to the individual effects. Synergistic effect has two main concepts, concentration effect and independent action, which enhance the adverse effect of the biological system⁽³⁷⁾. Other nerve agents such as carbamate and organophosphate inhibit ChE activity by binding through the process of carbamylation and phosphorylation at the active site and by blocking the binding of substrate^(38,39). In comparison, inhibition by metal ions is related to the binding affinity towards the amino acid side chain. Proteins containing the histidine residue is the most vulnerable to the metal binding such as by zinc and copper^(40,41). The imidazole group of histidine provides the strongest cation- π attraction that may interact with nitrogenous cations of substrates or free metal ions^(42,43). Copper, cadmium, and zinc have been reported to display noncompetitive inhibition behaviour towards ChE activity, while mercury has been reported to act as an irreversible inhibitor⁽⁴⁴⁾.

As well as, the results demonstrated that (A,B and D) exhibit different types of inhibition at concentrations (10^{-4} , 10^{-10} M). The competitive inhibition by A and B can explain in order to inhibitors structure that make a conformational changes after binding to –SH, –COOH, imidazole group of Ser, His, Glu in AChE, which are either localized in the active center or are important in determining the active conformation of enzyme molecule. On the other hand, non and uncompetitive inhibition can be explained according to the classical models described that the inhibitor binds to another site that causes conformational change, locks the enzyme and prevents the substrate binding or decreasing substrate affinity to AChE.

In order to understand the action of A,B and D as inhibitors to cholinesterase enzyme, the following proposed mechanism has been studied:

- a) Molecular interactions between the R groups-PhNO₂, -PhOH of the Phenobarbital moieties with the active site –SH, –COOH, imidazole groups.
- b) The Phenobarbital compounds containing moieties of substitution N-benzylidene derivatives bond to the acetyl enzyme and was proven to be correct by demonstrating that inhibition increases the steady-state concentration of acetyl enzyme. In contrast, the inhibitor may bond to the enzyme-substrate complex and free enzyme has predicted that the amount of acetyl enzyme will be drastically reduced when the inhibition is high.

Conclusion: It can be concluded that aromatic Phenobarbital derivatives showed an inhibitory effect on AChE with different types of inhibition and had proved the capability of N-benzylidene derivatives to inhibit the AChE activity. These findings may be related to (1) blockage of the enzyme active site, (2) alteration of AChE structure, and (3) amino acid sequence variety which tend to be affected differently by the N-benzylidene derivatives, thus preventing the formation of enzyme-substrate complex.

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