Kinetics for the Inhibition of Serum Acetylthiocholin Esterase Activity by Some Prepared Phenobarbital Derivatives

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

Article Information

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ABSTRACT

This work addresses the kinetic analysis on the interaction of some prepared Phenobarbital derivatives (A, B, C and D) with human serum acetylcholinesterase. It was found that these compound (A, B and 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) of concentrations for both compounds A and B, elevated inhibition with decrease concentration from 10^{-4} to 10^{-10}M for D. The effects of each A, B and D were reversible in nature. All of the results for C compound were neglected. Michaelis- Menten constant and maximum velocity for hydrolysis of acetylthiocholine iodide by AChE was determined in control and treated systems. Line weaver-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}, and 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.

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1. INTRODUCTION

Cholinesterase (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 acylcholineacylhydrolase. 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 measurablein 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, orpharmacological 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 ofeffect 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 the 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. The examples are: reversible inhibitor compounds that act as the reversible competitive or non-competitive inhibitors of cholinesterase that 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 use 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 thymitag and their activity asthymidylate enzyme inhibitors [10,11]. Heterocyclic of barbituric acid represent as a new class of antitumor r drugs, it was found to inhibit theepidermal growth factor receptor (EGFR) [12,13]. Furthermore, barbituric acid exert their antitumor activitythrough inhibition of DNA repair enzyme system, enzyme-mediated repair of strand lesion in DNA is anestablished mechanism for resistance toward antitumor DNA damagingdrugs and radiotherapy [14-16]. Schiff's bases can be considered as usefultools to prepare many compounds from barbituric acid.

The present study involves the preparation of four compounds which derivatives from Phenobarbital that have a biological importances through the anti bacterial activity for three type of bacteria (*Esherichia 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 values [17]. These derivatives were obtained from the preparation of ester by the reaction of 5-ethyl-5-phenylbarbituric acid with ethylbromoacetate. The ester was then reacts with hydrazine hydrate which utilize it's derivatives to prepare Schiff's bases.

Phenolbarbital derivatives required a largetension 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-benzyldine derivative requires this type of compounds 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 bases molecules that are useful in life, such as (Rhodopin) which curtained from the combination of vitamin A with macro protein (opsin). In addition, Schiff's bases have medical properties such as diuretic and cordiotonic, and these compounds were derivatived from furfural [21].

2. MATERIALS AND METHODS

2.1 Materials

Acetylcholinesterase activity was assayed by Ellman Method [22]. The principle of the method is the measurement of the production rate of thiocholine as acetylthiocholine was hydrolyzed. This was accomplished by the continuous reaction of the thiol with 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) to produce 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 the mixture of (6.9g)(5-ethyl-5-phenyl barbiutric acid) with (10 g) (bromoethyle acetate) and (8 g) of sodium carbonate for (6h) [23,24]. The product was then vaporized under vacuum pressure until the appearance of white crystal (ester), then (4.5 g) ester was treated with (2.5 g) of hydrazine hydrate and (25 ml) of benzen and refluxing the mixture for another (6 h) to produce pure hydrazine derivative, (0.5 g) of the mixture was thenrefluxed with (0.3 g) of aldehydesor ketons in (25mlabsolut ethanol) for (4 h). The mixture was cooled, dried and recrystallized with a suitable solvent as shown in Table (1).

2.1.1 Blood sampling

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

2.2 Methods

2.2.1 Determination of AChE activity

Human serum AChE activity was determined using Ellman et al. [22] method as follows:

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

2.2.2 Determination of biologicalactivity of phenobarbital derivatives [25]

A stock concentration solution (0.01M) concentration of each compounds in Table (1) has been prepared and then different concentrations (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, and 10^{-10}M) of each compound were prepared by diluting it with dimethyl sulfoxide (DMSO) as solvent. ChE activity is measured in human serum as follows:

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

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

2.2.3 Determination the type of inhibition

A constant concentration of inhibitors (10^{-4}, 10^{-10}µL) (higher and lower). (10^{-2}M was excluded in the study due to precipitation during experiment) were being used with different concentrations of substrate (0.02, 0.04, 0.06, 0.08M) to study the type of inhibition. These concentrations were prepared using the stock solution (0.1M) of AChI.

The enzyme activity was determined with and without the inhibitors using the lineweaver –Burk equation by ploting 1/V vs. 1/[s] [26]. The following values were then calculated as follwos* 1) K_i,, 2) Apparent V_{max} (V_{mapp}) , 3) Apparent K_m(K_{mapp}) , 4) type of inhibition.

3. RESULTS AND DISCUSSION

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

AChE activity in present study has been assayed in the absence and presence of Phenobarbital derivatives under different substrate concentrations. A series of compounds in Table

(1) were designed to investigate the effects of the Phenobarbital derivatives of on in vitro AChE activity. First experiment tried to study the effect of solvent DMSO which did not show any inhibitory effect as found and as Jaffer et 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).

Before each set of inhibition experiments were conducted, the AChE activity was measured by using four different concentrations of acetyl thiocholineiodide (substrate) (0.02, 0.04, 0.06, 0.08) Mas in Fig. (1).

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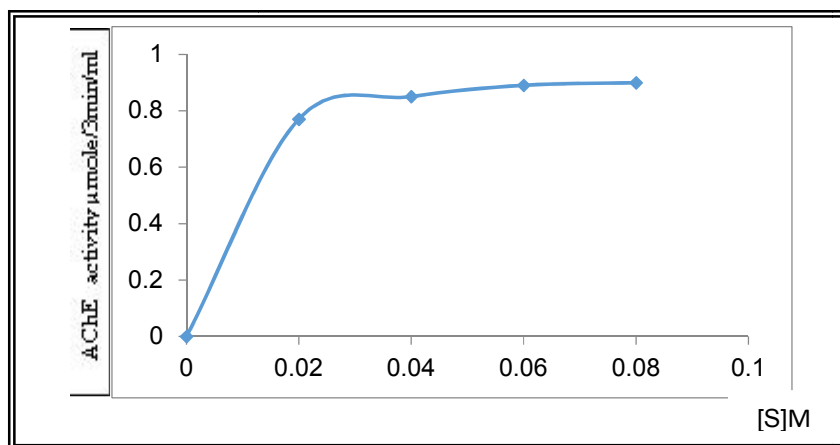

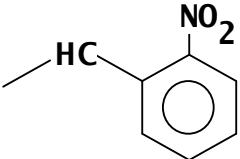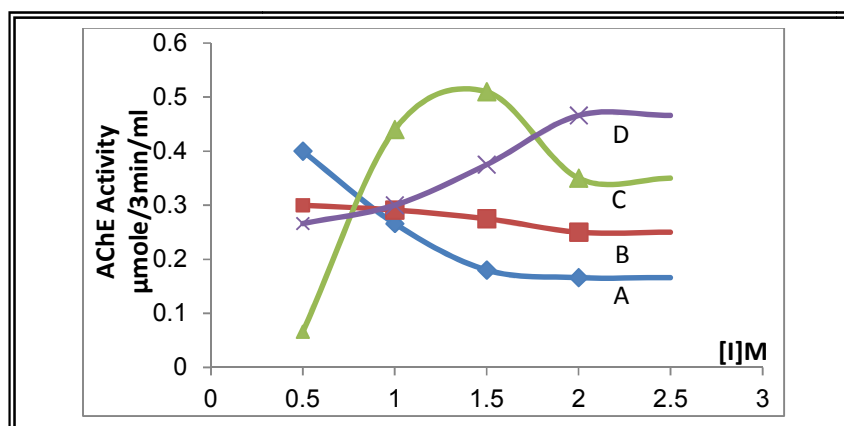**Fig. 1. The Michaelis-Menten plot of AChE with different concentrations of substrate without inhibitor**
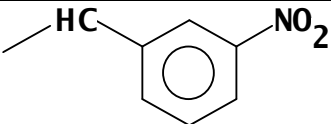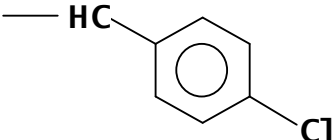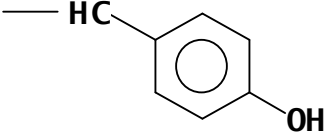

Fig. 2. The relationship between concentrations of compound A, B, C and D on AChE activity

Table 1. The derivatives of phenobarbital used to inhibit AChE

Comp. no.	Comp. name	R	Color	Yield%	m.p.c^o	M. formula	Purification solvents
A	5-ethyl-5-phenyl-1,3-di[N(2-nitrobenzilidin hydrazinyl)]barbituric acid		Yellow	50	(200-203)	$C_{30}H_{27}N_8O_9$	Ethanol + Water
B	5-ethyl-5-phenyl-1,3-di[N(3-nitrobenzilidin hydrazinyl)]barbituric acid		Light yellow	40	(185-188)	$C_{30}H_{27}N_8O_9$	Ethanol + Water
C	5-ethyl-5-phenyl-1,3-di[N(4-chlorobenzilidin hydrazinyl)]barbituric acid		Yellow crystal	45	(201-203)	$C_{30}H_{25}Cl_2N_6O_7$	Acetone + Water
D	5-ethyl-5-phenyl-1,3-di[N(4-hydroxybenzilidin hydrazinyl)]barbituric acid		Dark yellow	55	(166-168)	$C_{30}H_{28}N_6O_7$	Acetone + Water

We paid a little attention to compound C, which showed unclear inhibition to enzyme activity Fig. (2) as a result of incomplete dissolvent of these compound in DMSO. From the above figures we noticed 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. Fig. (3) Showed the effect of maximum and minimum concentrations of inhibitors at different acetylcholine concentrations on AChE activity.

(a) 10^{-4}M of A

(b) 10^{-10}M of A

(c) 10^{-4}M of B

(d) 10^{-10}M of B

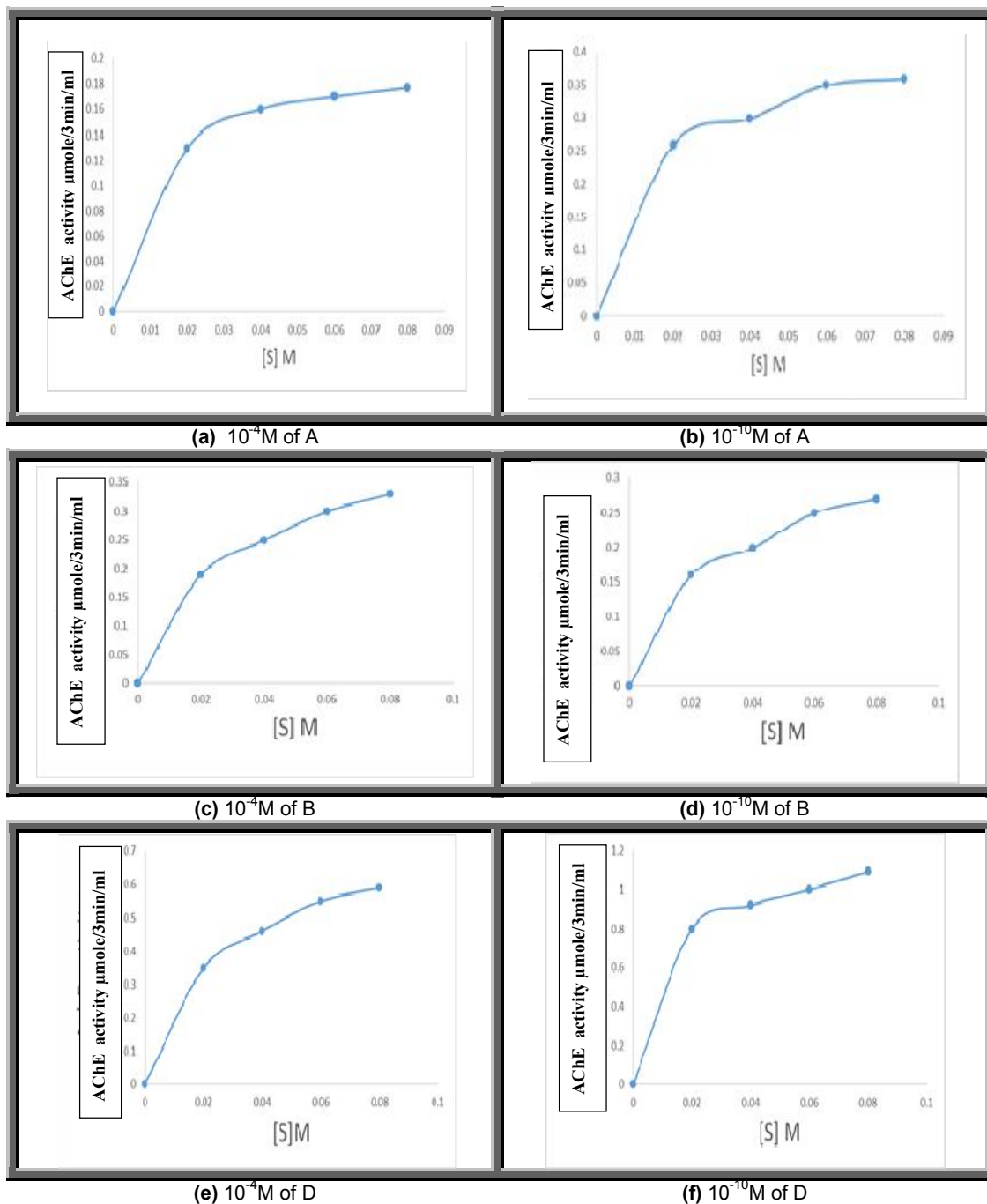**(e)** 10^{-4}M of D

(f) 10^{-10}M of D

Fig. 3. The Michaelis-Menten plots of AChE with different concentrations of substrate in presence of inhibitors

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

Table (2) showed that the highest significant inhibition percent was caused by compound A than others, these can be attributed to NO_2 group on ortho position of phenyl group which may led to good orient to active site gorge compared to rest compounds.

The greatest significant inhibition was found at concentration $(10^{-4}M)$ in A & Binversely, compound D showed greater significant inhibition at $(10^{-10}$ M) as cleared in Fig. (4).

It has been observed that the substituted of 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 NO_2 group in A and 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, polycyclicaromatic hydrocarbons are able to inhibit AChE activity in an additive manner together with organophosphate, being noncompetitive inhibitors of AChE [30].

In the same line there are other studies that are referring to the inhibitory effect of some classes of compounds on enzyme activity such as and carbamates [31], tacrine [32] and prophin [33].

3.1 Study Type of Inhibition

The second part of this study is to 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 Linweaver-Burk equation as shown in Fig. (5) and Table (3).

It is very clear from this presentation that Km 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 noncovalently 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 cleft of the enzyme. A consequence of such a good fit could bean orientation in space such that the covalent bonding to the serine residue is facilitated.

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

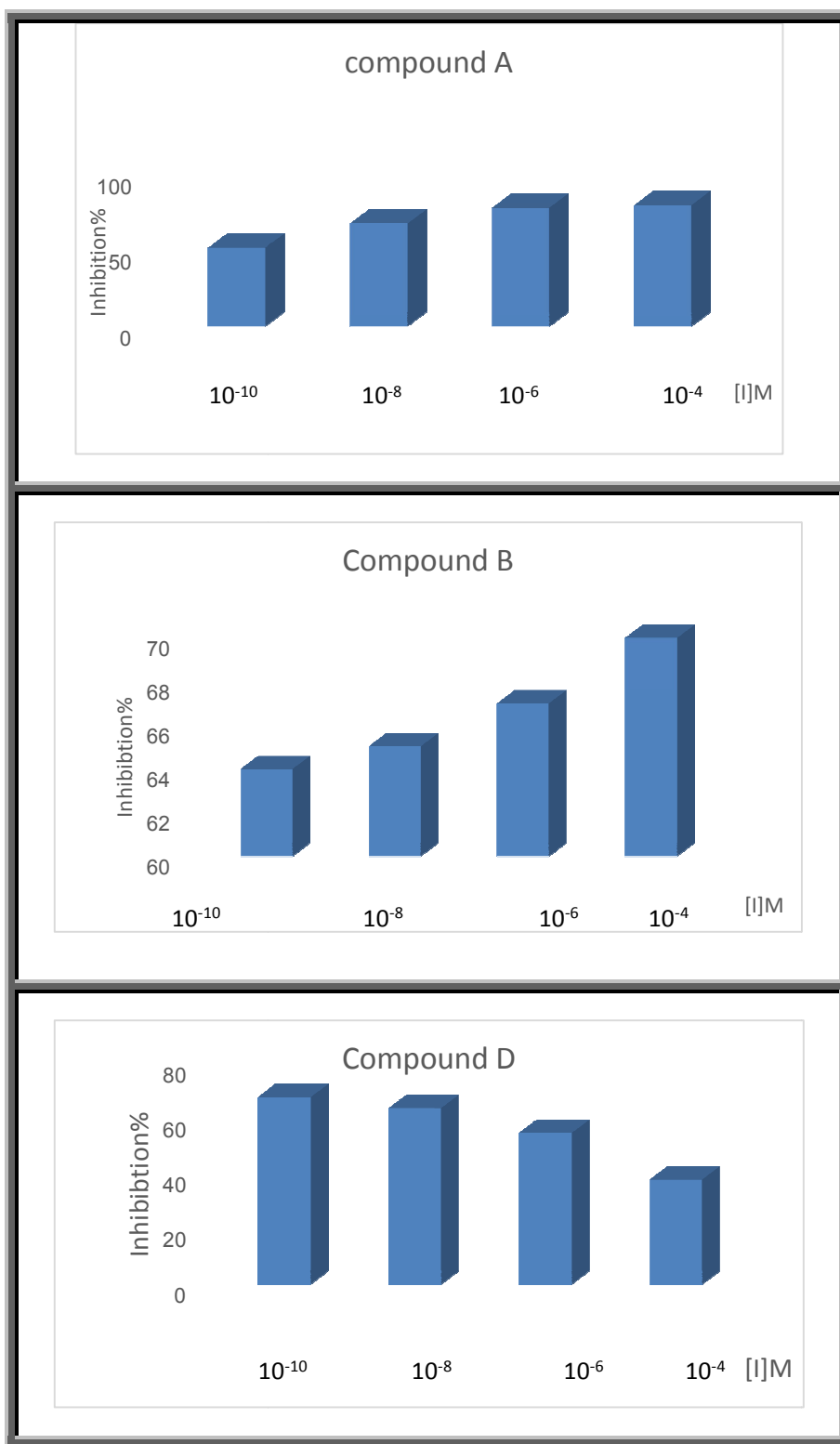|	Inhibition con. (M)	AChE activity (μmole/3 min/ml) mean±SD	% inhibition
Control	zero	2.4±0.300	-
A	10^{-4}	0.166±0.002	80.08*
	10^{-6}	0.180±0.002	78.4*
	10^{-8}	0.266±0.0025	68.07*
	10^{-10}	0.4±0.033	51.99*
B	10^{-4}	0.25±0.022	69.99*
	10^{-6}	0.275±0.004	66.99*
	10^{-8}	0.291±0.002	65.07*
	10^{-10}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

Results with (p≤0.05) considered significant

**Fig. 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)**

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}(µmole/3 min/ml) 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	$4.615×10^{-5}±9.1×10^{-7}$	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±0.01	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.
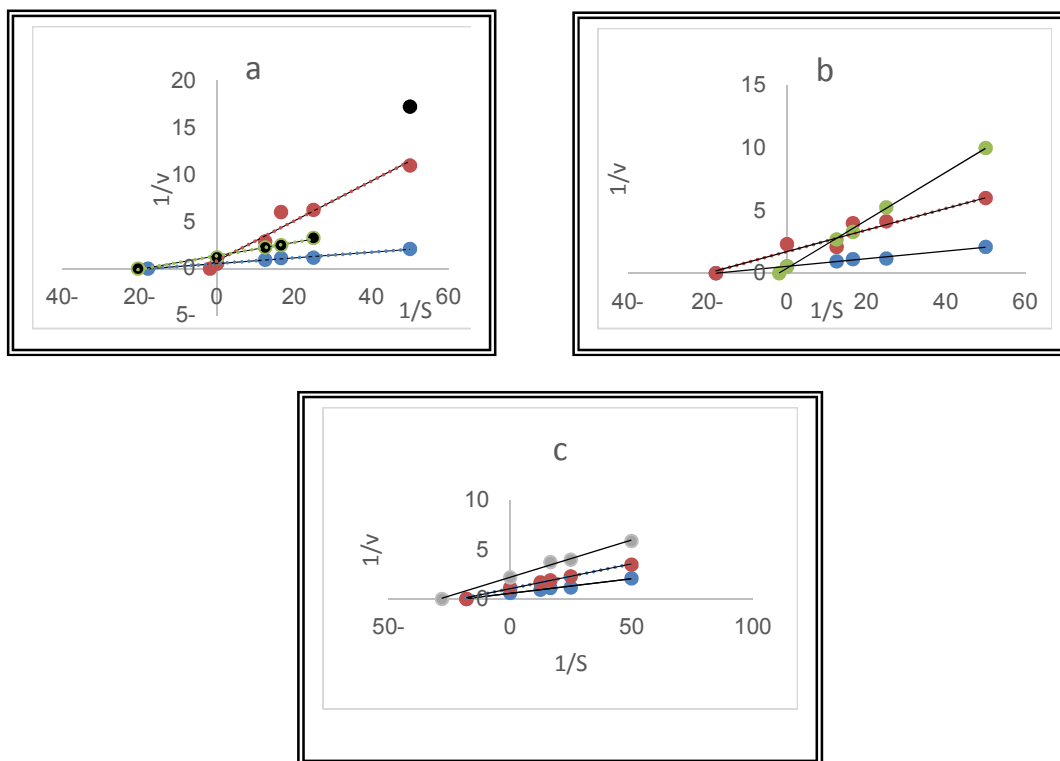
Fig. 5. Lineweaver-Burk Plots of AChE with each concentrations (10^{-4}, 10^{-10}) M of compounds a – compound A b – compound B   c – compound D

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 Ki (the binding affinity of the inhibitor) at 10^{-4} is higher than 10^{-10} for all studied compounds.

This difference in Ki 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.

Studies by Forget et al. [34], Toman et 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 explain according to the classical models described that the inhibitor bind to another site that cause conformational change lock the enzyme and prevent 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 have been studied:

a) Molecular interactions between the R groups-$PhNO_2$, -PhOH of the Phenobarbital moieties with the active site –SH,-COOH, imidazole groups.

b) The Phenobarbital compounds containing moieties of substitution N-benzylidine derivatives bond to the acetyl enzyme and was proven to be correct by demonstrating that inhibition ion 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.

Finally, the study should refer to the phenobarbital therapeutic blood concentration is between 10 and 40 mg/dm^3; the toxic concentration is 40-60 mg/dm^3 [45]. The mean lethal serum concentration (data from several handbooks) is 136 mg/dm^3, as long as the patient is not a drug addict [46]. Assuming examined compounds A which have molecular weigh about 634, the concentration 10^{-4} means that we have 64 mg/dm3. Wheres in concentration 10^{-10} we have 63.4*10^{-6} mg/dm^3, so practically the lethal dose may consider is 10^{-4}, and10^{-10} is possible regarding the typical pharmacological doses of barbituric acid derivatives.

4. 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-benzylidine derivatives to inhibit the AChE activity, these findings may 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-benzylidine derivatives, thus preventing the formation of enzyme-substrate complex.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

REFERENCES

1. Wilson B, Philip W. Encyclopaedia of Toxicology. New York: Elsevier; Cholinesterase inhibition. 2005;588–599.
2. Wilson BW, Robert IK, William CK: Handbook of Pesticide Toxicology. 2nd ed. San Diego: Academic Pr. Cholinesterases. 2001;967–985.
3. Chatonnet A, Lockridge O. Comparison of butyrylcholinesterase and acetylcholinesterase. Biochem J. 1989; 260:625–634.
4. Silver A. The biology of cholinesterase. In: Neuberger A, Tatum EL, editors. Frontiers of Biology. Amsterdam: North Holland. 1974;1–596.

5.   Hulka SB. Overview of biological markers, in biological markers in epidemiology, Hulka BS, Griffith JD, Wilcosky TC, Eds, Oxford University Press, New York, NY, USA. 1990;3–15.

6.   Naylor S. Biomarkers: Current perspectives and future prospects. Expert Review of Molecular Diagnostics. 2003; 3(5):525–529.

7.   Getman D, Eubanks J, Camp S, Evans G, Taylor P. The human gene encoding acetyl cholinesterase is located on the long arm of chromosome 7. American Journal of Human Genetics.1992;51(1):170–177.

8.   Herkert NM, Freude G, Kunz U. Thiermann H, Worek F. Comparative kinetics of organophosphates and oximes with erythrocyte muscle and brain acetylcho - linesterase. Toxicol. Lett. 2012;209:173–178.

9.   Nathan H, Sandra E, Abby L, Krista L. Discontinuing cholinesterase inhibitors: Results of a survey of Canadian dementia experts. Intern. Psychoger. 2010;1-7C.

10. Michel J, Cedric L, Alexandra V, Besson T. Synthesis of novel pyrimidine containing pyrazolinone, pyrazole and pyridinone moieties. Eur. J. Mid. Chem. 2008;43: 1469-1477.

11. Kalil A, Hamide S, HIEl-Sabbagh. Synthesis of some noveloxadiazole and barbituric acid analogues for their biological activity. Arch. Pharm. Chem. 2003;2:95-103.

12. Smaill J, Rewcastle G, Loo A. Synthesis and *In-vitro* anticancer evaluation of new 2-substituted mercapto- pyrimidineanalogs. J. Med. Chem. 2000;43:1380-1926.

13. Wissner W, Berger D, Boshe D, Zhang N. Synthesis and antitumor evalution of 4(3N)-Quinazolinone derivatives. J. Med. Chem. 2000;43:3244-3256.

14. Hayes J, Walf C. Bromo purine nucleosides asreagents for nucleoside analogue synthesis. Biochem, J. 1999;272: 281-295.

15. Griffin A, Srivivasan S, Gurtin N. RNA cleavage by a DNA enzyme with extended chemical functionality. J. Med. Chem. 2004;41:5247-5256.

16. Abdel Hamid S, El-Obid H, Rashood A. Synthesis antitumor activity of Quinazolin-4-onederivatives. Sci. Pharm. 2001;69: 351-366.

17. Redah IH, AL-Bayati, Raad M, Moslih, Nagam M. Synthesis of new s-ethyl-5-phenyl barbituric acid derivatives. Iraqi

18. National J. of Chemistry. 2005;(17):122-137.

18. Arun K, Ajit K, Arti S. Design and synthesis of novel schiff's bases having N-(4H-1, 2,4-triazole-4-yl) benzamido moiety as antimicrobial and anti-inflammatory agents. Der Pharma Chemica. 2011;3(5):146-154.

19. Sunny J, Anil J, Avneet G, Hemra J. Synthesis, biological activities and chemistry of thiadiazole derivatives and Schiff bases. Asian Jou of Pharm. and Clini. Rese. 2012;5(3).

20. Mithun R, Biplab V. Chemistry and Biological Importance of heterocy-clic Schiff's Bases. Intern. Res. Jou. of Pure & Appl. Chem. 2010;3(3):232-249.

21. Behl P, Lanctˆot KL, Streiner DL, Black SE. The effect of cholinesterase inhibitors on decline in multiple functional domains in Alzheimer's disease: Atwo-year observational study in the Sunnybrook dementiav cohort. International Psychogeriatrics. 2008;20:1141–1159.

22. Ellman GL, Courtney KP, Andres V, Feather Stone RM. Biochem. Pharmacol. 1961;(7):88-95.

23. Odd R. Gautun, Crlson JP.: Regioselectivity in the Thermal Rearrangement of Unsymmetrical 4-Methyl-4H-1,2,4-triazoles to 1-Methyl-1H-1,2,4-triazoles.molecules. 2001;6:969-978.

24. Hussein FA, Ali IT, Hassa DF: Preparation of 5-ethyl-5-phenyl barbituric acid derivatives. Iraqi J. of Chem. 2001;27(2): 445

25. Falah SD. Inhibition AChE activity by new derivatives of phenindione. Al-Taqani J. 2005;18(1):44-57.

26. Linweaver H, Burke D. The determination of enzyme dissociation constants. J. Am. Chem. Soc. 1934;56:658.27.

27. Colovic MB, Danijela Z, Lazarevic P, Tamara D, Aleksandra M, Vesna M. Acetylcho - linesterase Inhibitors: Pharmacology and Toxicology. Current Neuropharmacology. 2013;11(3):315–335.

28. Jaffer H, Mahond M, Al-Azzawi M. J. Biol. Sci. Res. 1988;19:793 .

29. Kang JJ, FangH W. Polycyclic aromatic hydrocarbons inhibit the activity of acetyl cholinesterase purified from electriceel . Biochemical and Biophysical Research Communications. 1997;2:367–369.

30. Jett D, Navoa R, Lyons Jr M. Additive inhibitory action of chlorpyrifos and polycyclic aromatic hydrocarbons on acetyl

cholinesterase activity *In vitro*. Toxicology Letters.1999;105(3):223–229,.

31. Pohanka M. Acetylcholinesterase inhibitors; a patent review. Expert Opinion on Therapeutic Patents. 2012;22(8):871–886.

32. Abdulaziz A, Mohammed A, Bdullah S. Sensitivity of bovinre final AChE toward tacrine: Kinetic Characterization. J. Biochem. Mol. Toxi. 1998;12(4):245-251.

33. Lee B, Park M, Yu B. Inhibition of elective eel AChE by porhin compounds. Bio org. Med. Chem. Left. 1998;16:8.

34. Forget J, Livet S, Leboulenger F. Partial purification and characterization of acetylcholinesterase (AChE) from the estuarine copepod Eurytemora affinis (Poppe). Comparative Biochemistry and Physiology C: Toxicology and Pharmacology. 2002;132(1):85–92.

35. Toman R, Hluch S, Golian J, et al. Diazinon and cadmium neurotoxicity in rats after an experimental administration, Scientific Papers. Animal Science and Biotechnologies. 2012;45:137–141.

36. Cacciatore L, Kristoff G, Verrengia Guerrero N, Cochón A. Binary mixtures of azinphos-methyl oxon and chlorpyrifosoxon produce *In vitro* synergistic cholinesterase inhibition in *Planorbarius corneus*. Chemosphere. 2012;88(4):450–458.

37. Zhu B, Wu Z, Li J, Wang G. Single and joint action toxicity of heavy metals on early developmental stages of Chinese rare minnow (*Gobiocypris rarus*). Ecotoxicology and Environmental Safety. 2011;74(8):2193–2202.

38. Weinbroum A. Pathophysiological and clinical aspects of combat anticholinesterase poisoning. British Medical Bulletin. 2004;72(1):119–133.

39. Rosenberry T, Johnson J, Cusack B, Thomas J, Emani S, Venkatasubban K. Interactions between the peripheral site and the acylation site in acetyl cholinesterase. Chemico-Biological Interactions. 2005;157-158:181–189.

40. Abdelhamid R, Obara Y, Uchida Y, et al. π-π interaction between aromatic ring and copper-coordinated His81 imidazole regulates the blue copper active-site structure. Journal of Biological Inorganic Chemistry. 2007;12(2):165–173.

41. Rajesh R, Balasubramania A, Boopathy R. Evidence for presence of Zn^{+2}-binding site in acetylcholinesterase. Biochimie. 2009; 91(4):526–532.

42. Stellato F, Menestrina G, Serra M, et al. Metal binding in amyloid β-peptides shows intra- and inter-peptide coordination modes. European Biophysics Journal. 2006;35(4):340–351.

43. Dvir H, Silman I, Harel M, Rosenberry T, Sussman J. Acetyl cholinesterase: from 3D structure to function, Chemico-Biological Interactions. 2010;187(1–3):10–22.

44. Armentrout P, Yang B, Rodgers M. Metal cation dependence of interactions with amino acids: bond energies of Rb+ and Cs+ to Met, Phe, Tyr, and Trp, Journal of Physical Chemistry B. 2013;117(14):3771–3781.

45. Baselt RC, Cravey RH. Disposition of Toxic Drugs and Chemicals in Man, 4th edn. Foster City, CA, USA: Chemical Toxicology Institute. 1995;612-614.

46. Ekwall B, Clemedson C, Crafoord B, Ekwall B, Hallander S, Walum E, Bondesson I. MEIC evaluation of acute systemic toxicity. Part V. Rodent and human toxicity data for the 50 reference chemicals. ATLA. 1998;26:571-616.

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