

Kinetics For The Inhibition of Serum Acetylthiocholin Esterase Activity by Some New Phenobarbital Derivatives.

Abstract:

This work addresses the kinetic analysis ~~of-on~~ the interaction of some new 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 ~~that-to have~~ elevated inhibition with increasing concentrations. ~~From-(10^{-10} to 10^{-4} M of concentration)-~~ for both compounds A and B ~~and~~ elevated inhibition 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 ~~about-for~~ C compound ~~was-were~~ neglected. Michaelis- Menten constant and maximum velocity for hydrolysis of acetyl thiocholine iodide by Ach E ~~were-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-non-competitive at 10^{-10} for D.)~~ respectively. The value of k_i was also estimated ~~also~~. The action mechanism ~~of-action~~ of these types of compounds acting as inhibitors to the Ach E is suggested.

Introduction:

Acetylcholinesterase (AChE, E.C. 3.1.1.7) is found in many types of conducting tissue: nerves s and muscles s, central and peripheral tissues, motor and sensory fibers, and cholinergic and noncholinergic fibers. The activity of Ach E is higher in motor neurons than in sensory neurons [1-3]. AChE is also found in the red blood cell membranes, where it constitutes the Yt blood group antigen. ~~The-This~~ enzyme exists in multiple molecular forms, which possess similar catalytic properties, but differ in their oligomeric assembly and mode of attachment to the cell surface. In ~~the-mammalian~~ brain, the majority of AChE ~~occurs-exists~~ as a tetrameric, a G4 form (10) with much smaller amounts of a monomeric G1 (4S) form [4].

The catalytic triad (Ser-203, His-447, and Glu-334)(all numbers refer to the

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amino acid position of human acetylcholinesterase) ~~has~~ have been shown to be ~~located~~ 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 ~~described as~~ found to be working in tandem, where ~~occupation the role of the~~ peripheral anionic site can decrease or increase ~~the~~ activity at ~~the~~ catalytic site. For example, at high concentrations, both acetylcholine and acetylthiocholine have been shown to bind ~~to~~ 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⁽⁵⁾.

A ~~cholinesterase~~ inhibitor (or "anticholinesterase") suppresses the action of the enzyme because ~~of the~~ essential function chemicals that interfere with the action of AChE are potent neurotoxins, ~~causing which caused~~ excessive salivation and eye-watering in low doses, followed by muscle spasms and ultimately death. ~~The~~ examples are: reversible inhibitor compounds ~~which function that act~~ as ~~the~~ reversible competitive or non-competitive inhibitors of cholinesterase ~~are those that~~ most likely to have therapeutic uses, ~~these~~ These include (~~organophosphates, carbamates, phenanthrene derivatives and~~ Tacrine). Quasi-irreversible inhibitor compounds ~~which function that act~~ as quasi-irreversible inhibitors of cholinesterase are ~~those~~ most likely to ~~have be~~ use as chemical weapons or pesticides, ~~and~~ these include (~~sarin, soman, and~~ phenmedipham)⁽⁶⁾.

Phenobarbital or phenobarbitone is a barbiturate, ~~which~~ first marketed as luminal, ~~it's~~ It is the most widely used anticonvulsant worldwide and ~~the~~ oldest ~~that is~~ still commonly used⁽⁷⁾. It also has sedative and hypnotic properties, but, ~~as with~~ compared to other barbiturates, ~~has been it was~~ superseded by the benzodiazepines for these indications. The World Health Organization ~~recommends~~ recommended its use ~~ge~~ as ~~the~~ first-line for partial and generalized tonic-clonic seizures in developing countries. It is a core medicine in the WHO Model List of Essential Medicines, which is a list of minimum medical needs for a ~~baic~~ basic healthcare system⁽⁸⁾. In ~~more~~ affluent countries, it ~~is was~~ no longer recommended as a first or second-line choice ~~of~~ anticonvulsant for most seizure types though it is still commonly used to treat neonatal seizures⁽⁹⁾.

Our study involves ~~the~~ preparation of many new compounds, which derivatives from Phenobarbital ~~that which~~ have biochemical ~~important importances~~. They are studies

and characterization by spectrum methods (U.V, IR, and C.H.N) with the determination of RF values ~~of RF~~. ~~This~~ ~~These~~ derivatives were obtained from the preparation of ester by the reaction of 5-ethyl-5- phenylbarbituric acid with ethylbromoacetate, ~~then~~ ~~The~~ ester ~~will be~~ ~~was~~ ~~then~~ reacts with aqueous hydrazine and utilize it's derivatives to prepare Schiff's bases. Phenolbarbital derivatives required a ~~big~~ ~~large~~ tension according to biological activities ~~which~~ ~~that~~ proved it, for example: these compounds were mostly utilized as anti spasmodic, ~~and the other~~ with some of them as anti microbial and antifungal^(10,11). Biological activity of Schiff's due to the presence of substitutes N-benzylidine derivative ~~which~~ requires this type of compounds good biological activity due to some ~~then~~ group ~~which is~~ that represent the ~~core of~~ Schiff buses core, such as ³salicylidene aniline and its derivatives, and the last is very useful as antimicrobial, fungi static agents and exhibit antitubercular activity⁽¹²⁾. There are many Schiff's bases molecules that are useful in life, such as (Rhodopin)-which curtained from the combine combination of vitamin A with macroprotein (opsin), ~~in~~ In addition, Schiff's bases have medical ~~property~~ ~~properties~~ such as diuretic and cordiotonic, and these compounds were derivative derivatived from furfural⁽¹³⁾. Cholinesterase is found in all excitable tissues whether in the nerve or muscle, central, or peripheral, cholinergic or adrenergic, motor or sensory, in most erythrocyte and in placental tissue-plasma, ~~cholinesterase~~ Cholinesterase is mostly found much more widely in the central and peripheral nervous system, in ~~most~~ tissues especially the liver and, ~~as its name implies,~~ in the plasma⁽¹⁴⁾.

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Materials and Methods:

Materials:

Acetylcholinesterase activity was assayed by Ellman Method⁽¹⁵⁾. The principle of the methode is the measurement of the ~~rate of~~ production rate of thiocholine as acetylthiocholine ~~is was~~ 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 was~~ measured at 430nm.

All compounds studied were prepared as following: By refluxing the mixture of (6.9gm, ~~)(5-ethyl-5-phenyl barbiutric acid)~~ with (10gm)(~~)(bromoethyle acetate)~~ for

(6hours.)^(16,17) ~~the~~ The product was then vaporized under vacuum pressure until the appearance of white crystal (ester), then ~~(4.5gm.) from~~ ester was treated with ~~(2.5gm)~~ of aqueous hydrazine and refluxing the mixture for another (6hours.) ~~a gain~~ to produce hydrazine derivative, ~~then (0.5gm)~~ of ~~it the mixture was then refluxing~~ refluxed with ~~(0.3gm)~~ of aldehydes and ketons in ~~(25ml Etanol)~~ for ~~(4hour.) the~~ The mixture was cooled, dried and recrystallized with a suitable solvent.

Comment [u3]: What is suitable solvent?

Blood sampling:

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

Methods:

1) AChE activity was determined in human serum using the method of Ellman et al as follows:

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~~(50μL)~~ of DTNB solution (0.001M) is added to ~~(2.25ml)~~ of sodium phosphate buffer solution (~~PHpH~~ = 7.3, 0.2M), then ~~(10μL) of serum is~~ added by 10μL of serum is, mixed well and ~~(2ml)~~ of the mixture is transferred to a measuring cell (1cm) after that, then ~~Then~~ (34μL) of acetyl thiocholine iodide (ASChI 0.06M) is added, ~~the~~ The changes 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 samples s in ~~(3min.)~~ and expressed as ~~(μmole/3min./ml).~~ □

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

~~(50μL)~~ of DTNB solution (0.001M) is added to ~~(2.25ml)~~ of sodium phosphate buffer solution (~~PHpH~~ = 7.3, 0.2M), 0.25ml of inhibitor ~~is was~~ mixed with 2ml of the same buffer, then ~~(10μL)~~ of serum is added, mixed well and ~~(2ml)~~ of the mixture is was transferred to a measuring cell (1cm), then ~~(34μl)~~ of ~~(AChI~~ (0.06M) ~~is was~~ added, the

changes in absorbency is measured after adding the substrate at 430nm for 3min. The inhibition percentage ~~is~~ was calculated by comparing the activity ~~between~~ with and without inhibitor ~~and~~ under the same conditions according to the equation:

$$\% \text{Inhibition} = 100 - \frac{\text{the activity in the presence of inhibitor}}{\text{The activity in the absence of}}$$

100 - The activity in the absence of

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3) A constant concentration of inhibitors (10^{-4} , 10^{-10} μL) (higher and lower) ~~concentration of inhibitors~~ (10^{-2}M ~~is~~ was excluded in ~~the~~ this study due to precipitation during experiment) ~~is~~ were being used with different concentrations of substrate (0.02, 0.04, 0.06, 0.08M) to study the type of inhibition. These ~~different~~ concentrations ~~are~~ were prepared from using the stock solution (0.1M) of AChI. The enzyme activity ~~is~~ was determined with and without the ~~inhibitions~~ inhibitors using the lineweaver –Burk equation by plotting $1/V$ vs. $1/[s]$ ⁽¹⁸⁾. The following values were ~~then~~ calculated ~~as follows*~~ 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, ~~and~~ increasing both ~~the~~ level and duration of action of the acetylcholine neurotransmitter ~~acetylcholine~~⁽¹⁹⁾.

AChE activity in present study ~~was~~ has been assayed in the absence and presence of new Phenobarbital derivatives under different substrate concentrations. A series of compounds in table (1) ~~is~~ were designed to investigate the effects of these new derivatives of Phenobarbital on in vitro AChE activity ~~in vitro~~.

Before each set of inhibition experiments ~~were conducted~~, the K_m and V_{max} were determined by using four different concentrations of acetyl thiocholineiodide (0.02, 0.04, 0.06, 0.08)M as in figure (1).

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

We paid a little attention to compound C, which ~~shows~~ showed unclear inhibition

to enzyme activity in figure(2)—as a result of ~~uncomplete-incomplete dissolving~~ ~~dissolvent~~ of these compound in DMSO. From ~~the~~ above figures, we noticed that the changes of AChE activity with [I] ~~is-was~~very ~~wide-significant~~in A ~~&and~~B ~~and-as~~it increased from concentration 10^{-4} to 10^{-10} in contrast, shows little inhibition in D but from concentration 10^{-10} to 10^{-4} . The Michaelis – Menten constant (K_m) and maximal Velocity (V_{max}) of AChE with different concentrations of substrate with inhibitor A,B, ~~and~~ D)) were determined from plots in figure (3), and are summarized in table (2):

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

The biochemical tests revealed that all compounds ~~s have~~ caused good inhibitory effects on enzyme activity ~~in~~ table (3). The normal value of ~~the~~ enzyme activity ~~range~~ ~~ranging~~between ~~(1.5 – 2.7)~~ $\mu\text{mol}/3\text{min}/\text{ml}$.

~~That-This~~means ~~thatthe~~ greater inhibition of A ~~&and~~B ~~is-was~~demonstrated at concentration ~~(10⁻⁴)M~~and in compound D at ~~(10⁻¹⁰ M)~~ as shown in figure (4):

~~It's-It has been~~observed that the substituted ~~with-of~~2-nitrobenzil (A) and 4-hydroxy benzyl (B) and 3-nitro benzyl (D) derivatives of Phenobarbital compound exhibits the main cause of inhibition and different position of NO₂ group in A ~~&and~~D, ~~which~~ also shows that the inhibitory effects of D inversely to A. In ~~the~~ same line, there ~~are-were also~~other studies that ~~are refer-referring~~to the inhibitory effect of some classes of compounds on enzyme activity such as phenindione, organophosphates⁽²⁰⁾, ~~—and~~ carbamates⁽²¹⁾, tacrine⁽²²⁾, ~~and~~ prophin⁽²³⁾.

Study Type of Inhibition:

The second part of this study ~~include-is to~~determine the type of inhibition and kinetic parameters (K_{mapp} , V_{mapp} , 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 ~~and~~), table (4).):

The results demonstrated that (A,B, ~~and~~ D) exhibit different types of inhibition at concentrations ~~(10⁻⁴, and 10⁻¹⁰)~~. Unlike the situation of irreversible inhibition, ~~however~~ the binding ~~is-was~~~~weak-weak~~ and the enzyme activity ~~is-has been~~restored when the

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inhibitor dissociates from the enzyme inhibitor complex. The hydrolysis of acetylcholine by the acetylcholinesterase involves initial attack of hydroxyl group of the amino acid serine residues to form covalent bond with the carbonyl carbon of acetylcholine and the attractive attraction of the acetylcholine cationic heads that heading to the enzyme anionic site, which leads to the formation of acetylcholinesterase-acetyl choline complex(ES), afterwards After that, the acetyl group is was catalytically transferred to a serine residue that present at the esteratic site while choline molecule is was lost and later, the hydrolysis takes its place to produce acetic acid and regenerated enzyme⁽²⁴⁾.

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

- . a) Molecular interactions between the atoms N and O of the Phenobarbital moieties with the active site.
- . b) The Phenobarbital compounds containing moieties of substitution N-benzylidenederivatives. Froed et al ⁽²⁵⁾, suggest suggested the theory of non competitive inhibition based on the binding of inhibitor to the acetyl enzyme and the free enzyme was proven to be correct by demonstrating that inhibition ion increases the steady-state concentration of acetyl enzyme, as predicted by the theory. by In contrast, the traditional theory stated that the inhibitor bind that is bonded to the enzyme-substrate complex and free enzyme has predicts predicted that the amount of acetyl enzyme will be drastically reduced when the inhibition is high. A third theory involving all these three types of binding remains possible.

Conclusion: The present study concludes that aromatic Phenobarbital derivatives showed an inhibitory effect on AChE with different types of inhibition.