Study of Alkaline phosphatase Inhibition by theophylline and trifluoperazine *in vitro*

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الخلاصة

الهدف من البحث هو دراسة تاثير التثبيط لعقاري الثيوفللين والترافلوبيرازين على انزيم الفوسفاتيز القاعدي في المختبر وتحديد نوع اتثبيط. تم تحضير سلسلة من تراكيز كل من العقارين وتمت دراسة تاثير التثبيط باضافة التراكيز المحضرة ضمن محلول البفر في طريقة تقدير فعالية انزيم الفوسفاتيز القاعدي. وم بعد تحويل قيم الامتصاصية الى سرعة التفاعل الانزيمي تم رسم سرعة التفاعل ضد تراكيز كل خظوة واستخدمت طريقة لينويفر – بيرك البيانية. اظهرتالنتائج ان كلا العقارين لهما تاثير تثبيطي لانزيم الفوسفاتيز القاعدي وبطريقة غير تنافسية. ان استخدام طريقة دكسن اشارت الى ان قيم Ki للثيوفللين هي 4.55ملي مولاري بينما القيمة للترافلوبيرازين هي 1.07ملي مولاري.

ABSTRACT

The aim of this research is to study the inhibition effect of theophylline and trifluoperazine in vitro on alkaline phosphatase and determine the type of inhibitions. A series of concentrations of both theophylline and trifluoperazine have been prepared and test their effect by adding them with the alkaline phosphatase assay.

* ***Presented at the second conference on Chemistry, University of Mosul, college of Education, 17-18 Novamber-2013.***

After converting the data from absorbance into enzymatic rate and test these velocities against concentrations of substrates using Linweaver – Burk equation, the results showed that both of theophylline and trifluoperazine have inhibition effect and the type of inhibition is non competitive. Using of Dixon methods indicate that the Ki of theophylline is 4.55 mM while for trifluoperazine Ki and is 1.07 mM.

INTRODUCTION

Alkaline phosphatase (ALP) (EC: 3.1.3.1) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids [(1)]. The process of removing phosphate group is called dephosphorylation. As the name suggest, alkaline phosphatases are the most effective in alkaline environment [(2)]. All mammalian alkaline phosphatase isoenzymes except placental (PALP) are inhipited by homoarginine, and, in similar manner, all except the intestinal and placental are blocked by levamisole [(3)]. Phosphatase enzymes are metalloenzymes that widely distributed in nature, from bacteria to plants to humans and are characterized by their ability to catalyze the hydrolysis of phosphoric acid esters [(4)]. The cofactors of alkaline phosphatase are zinc and magnesium. In human plasma the level is raised in the last trimester of pregnancy. Plasma levels may also be greatly elevated in cases of Paget's disease of bone, osteomalacia, and cirrhosis or biliary obstruction; levels may become moderately elevated in other types of bone disease [(5)]. The normal range in human plasma is 40 – 155 $IU.L^{-1}$ [(6)]. Alkaline phosphatase, which is present on membranes between liver cells and the bile duct, is also elevated in the blood in acute viral hepatitis [(6)].

Theophylline is a drug used in therapy for respiratory disease such as asthma under a variety of brand name. Theophylline, also known as

dimethylxanthine, is a methylxanthine drug. As a member of the xanthine family, it bears structural and pharmacological similarity to caffeine [7]. Trifluoperazine is a typical antipsychotic drug. The primary application of trifluoperazine is for schizophrenia. Trifluoperazine is of the phenothiazine chemical class [8].

Theophylline and trifluoperazine are wide prescribed recently to treat respiratory disease and psychiatric disorders, respectively, and there are some symptoms of these patients related to the bone problems of the patients who continuously take these drugs [9]. The signs and symptoms of bone problems are probably because the inhibition of alkaline phosphatase. So the hypothesis of this research is theophylline and trifluoperazine have inhibition effect on alkaline phosphatase activity. Accordingly, the aim of this paper is to study the inhibition effect, and if so the type of inhibition, of theophylline and trifluoperazine on alkaline phosphatase.

MATERIALS AND METHODS

Enzyme: bovine and calf intestinal alkaline phosphatase (phosphoric monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) (Sigma) diluted 1:10,000 with reaction buffer containing 1 mg/ml bovine serum albumin (Sigma).

Substrate: phosphatase substrate (p-nitrophenyl phosphate, disodium, hexahydrate) (Sigma), different concentrations 6.25,mM, 0.862mM, and 0.424mM.

Product: p-nitrophenol (Sigma),. Reaction buffer: Tris-HCl buffer . $MgCl_2.6H_2O$ (Fluka).

Procedure:

To measure the activity of these enzymes, one can follow the liberation of phosphate or of the other product released by hydrolysis. The assay can be simplified by using a substrate whose phosphate-free product is

highly colored. In this experiment, we will utilize 4-nitrophenylphosphate as the substrate, which upon hydrolysis releases phosphate to genterate 4-nitrophenolate under alkaline conditions.

4-nitrophenolate has a high molar absorptivity at 405 nm (ϵ_{405} = 18.8 x 10^3 $M^{-1}cm^{-1}$). The enzyme assay involve preparing a concentration of 6.25 mM of substrate.

Then, after adding the enzyme to the reaction mixture, the increasing in absorbance at 405 nm has been observed with time intervals of 5 min. the same thing has been carried out with different concentration of substrate (0.862mM and 0.424mM). From converting the (changing in absorbance) A to enzymatic velocity using the equation 1 we can plot the 1/v versus 1/[s]. The experiment should repeated again but this time with adding a concentration of drug to each set of substrate concentration. The different concentrations of theophylline and trifluoperazine were (1, 2,3,and 4 mM). The drugs dissolved in the assay buffer.

$$\text{enzyme velocity (v)} = \frac{d[P]}{dt} = \frac{\Delta =}{\epsilon l \Delta t} \quad \left(\frac{mM}{min}\right) \quad \text{.....(1)}$$

Where: $\Delta A/\Delta t$ = the slope from plotting absorbance of product versus time l: the optical bath length = 1 cm

ϵ= molar absrpitivity or molar extinction coefficient (ϵ_{405} = 18.8 x 10^3 $M^{-1}cm^{-1}$).

RESULTS

Plotting of Lineweaver – Burk equation of 1/v versus 1/[s] give a straight line. Figure 1 shows the effect of increasing the concentration of theophylline on the enzyme assay using Lineweaver – Burk methods of plotting enzyme reaction data. From the plot it can be calculated that Maximum velocity (V_{max}) = 0.31 $mM.min^{-1}$ while Michaelis constant which represents the substrate concentration giving half maximum velocity or the affinity of enzyme to bind to substrate K_m= 0.22 mM.

Intercept of each line in figure 1 ($1/V_{max}$)can be used to calculate K_i by plotting intercept value against inhibitor concentration. K_i is a constant represents the dissociation constant of inhibitor

$$K_i = \frac{[I][E]}{[EI]}$$

K_i of theophylline = 4.55, figure 2. This way of estimation is according to Dixon [10]. There is another important constant in this type of inhibition, noncompetitive inhibition, that is, αK_i which represents the dissociation constant of inhibitor from the complex ESI. When $\alpha = 1$ the αK_i will be equal K_i. Dixon calculated the αK_i by plooting the slope of each line in Lineweaver – Burk plot against inhibitor concentration. For theophylline αK_i equal to 1.5 mM (figure 3).

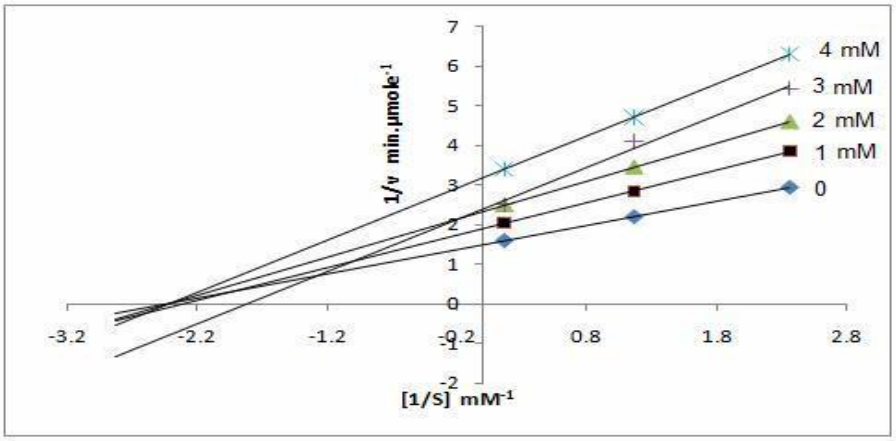


Figure 1: Lineweaver – Burk double reciprocal plot of enzyme reaction data in the absence and presence of four theophylline concentrations

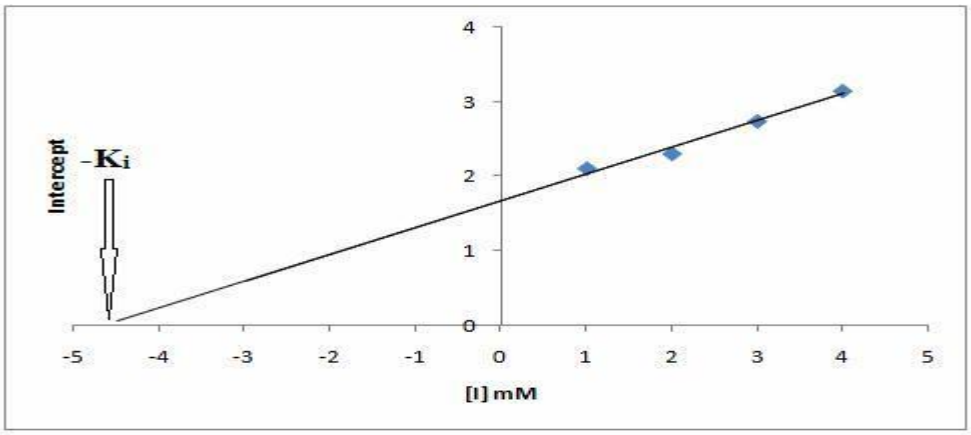


Figure 2: Dixon plot of intercept versus inhibitor concentration to estimate K_i of theophylline inhibition.

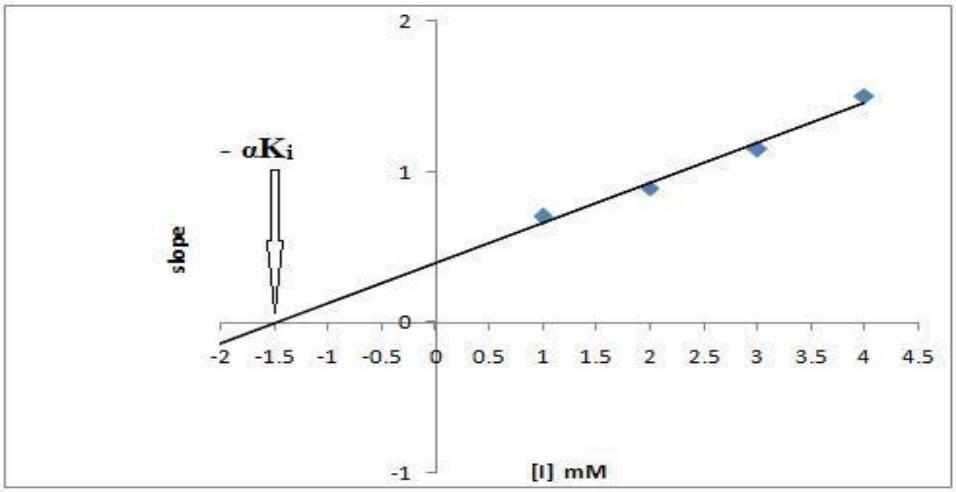


Figure 3: Dixon plot of slope versus inhibitor concentration to estimate αK_i of theophylline inhibition.

Figure 4 shows the effect of increasing the concentration of trifluoperazine on the enzyme assay using Lineweaver – Burk methods of plotting enzyme reaction data. From the plot it can be calculated that Maximum velocity (V_{max}) = 0.93 mM.min^{-1} while Michaelis constant K_m= 0.887 mM. Estimation of both K_i and αK_i has been done by plotting the intercept of figure 4 and slope versus inhibitor concentration, repectively, as shown in figure 5 and 6.

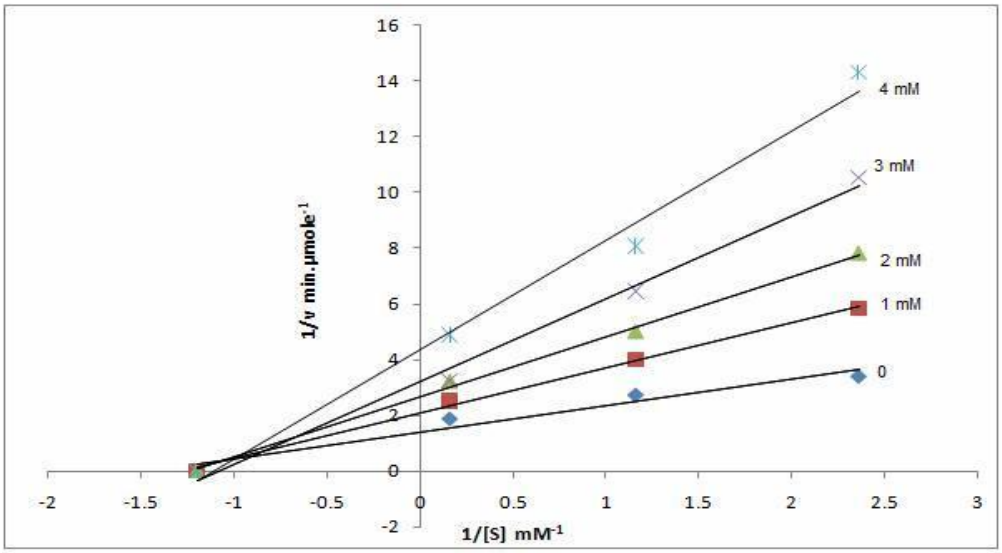


Figure 4: Lineweaver – Burk double reciprocal plot of enzyme reaction data in the absence and presence of four trifluoperazine concentrations

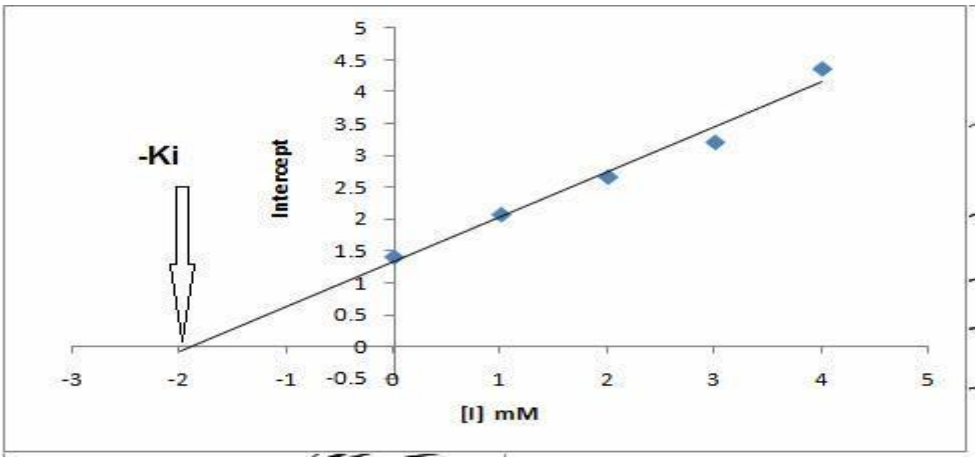


Figure 5: Dixon plot of intercept versus inhibitor concentration to estimate K_i of trifluoperazine inhibition.

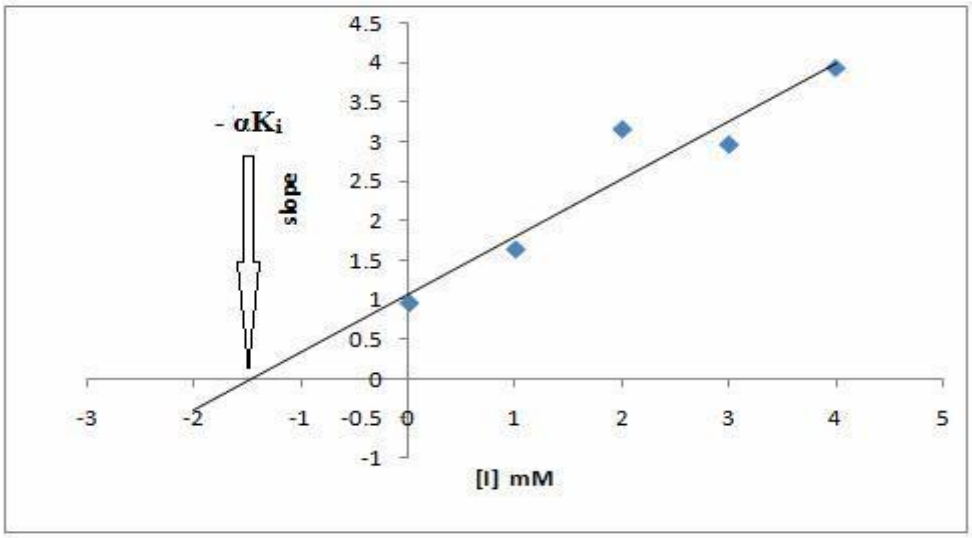
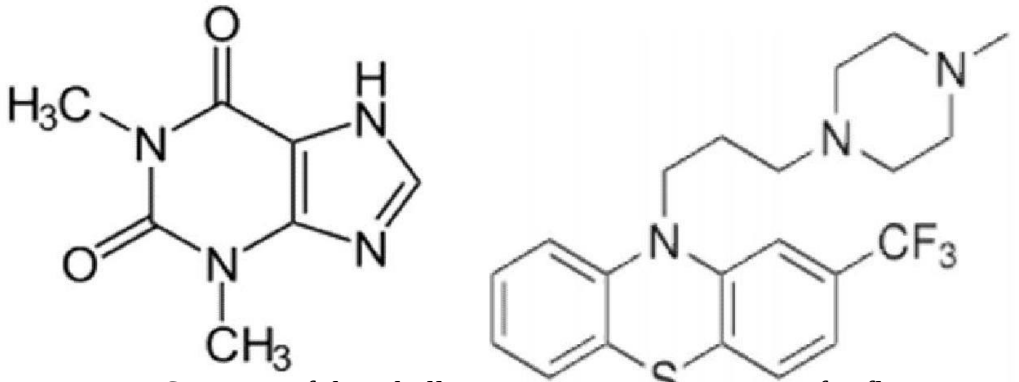


Figure 6: Dixon plot of slope versus inhibitor concentration to estimate αK_i of trifluoperazine inhibition.

DISCUSSION

The results indicated that the both types of inhibition of theophylline and trifluoperazine are noncompetitive inhibitions, or kinds of noncompetitive inhibitions. That reflects that the binding of both drugs with alkaline phosaphatae is in a site not the active site, so the drug would not compete with substrate, which lead accordingly to reduce the enzyme velocity. As mentioned in introduction, the alkaline phosphatase contains a cofactor in its structure "divalent ion either Mg or Zn" to do the activity. Probably the drugs "theophylline and trifluoperazine" scavenge the ion and make a complex, which finally affacts the enzyme activity. Complex formation probability may be ensured from the structure, because both drugs have the ability to make complexes with divalents [(11)]. The structure of drugs are shown in figure 7.



Structure of theophyllin structure of trifluoperazine

Figure 7: **structures of theophylline and trifluoperazine**

Kinetics concerns the study of reaction rates and the conditions that affect reaction rates. One of the primary concerns of kinetics deals with the study of the changes in the rate of a reaction as a function of the reactant (substrate) concentrations. Such

measurements can lead to the calculation of rate constants and, where appropriate, of equilibrium constants. Kinetics can since an observed reaction rate must satisfy the proposed mechanism. Studying enzyme inhibition gives a good picture about the study of pharmacological imagination and the decision of some drug concentrations.

To understand the molecular basis of reversible inhibition, it is useful to reflect upon the equilibria between the enzyme, its substrate, and the inhibitor that can occur in solution. Figure 8 provides a generalized scheme for the potential interactions between these molecules. In this scheme, K_s equilibrium constant for dissociation of the ES complex to the free enzyme and the free substrate, K_i is the dissociation constant for the EI complex, and k_p the forward rate constant for product formation from the ES or ESI complexes. The factor α reflects the effect of inhibitor on the affinity of the enzyme for its substrate, and likewise the effect of the substrate on the affinity of the enzyme for the inhibitor. The factor β reflects the modification of the rate of product formation by the enzyme that is caused by the inhibitor. An inhibitor that is the is completely blocks enzyme activity will have β equal to zero. An inhibitor that only partially blocks product formation will be characterized by a value of β between 0 and 1. An enzyme activator, on the other hand, will provide a value of β greater than 1 [(12)].

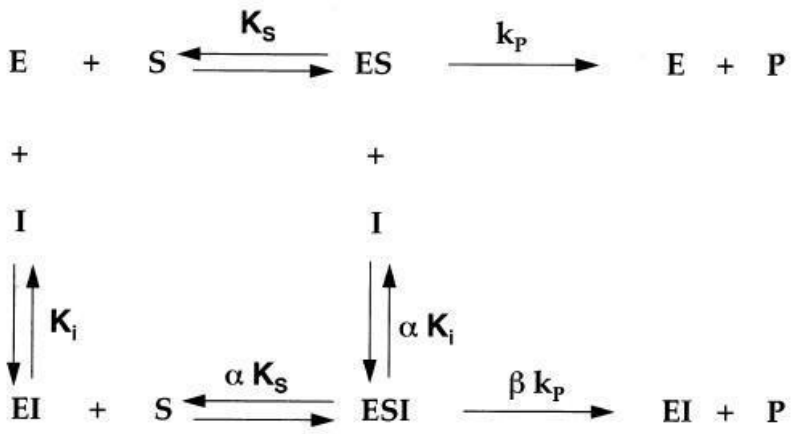


Figure 8: **Equilibrium scheme for enzyme turnover in the presence and absence of an inhibitor.**

The most The most general velocity equation for an enzymatic reaction in the presence of an inhibitor is:

$$v = \frac{V_{max}[S]}{[S]\left(1 + \frac{[I]}{\alpha K_i}\right) + K_m\left(1 + \frac{[I]}{K_i}\right)}$$

From this equation we can calculate the enzyme velocity with different concentration of inhibitor to compare with the rate without inhibitor, that is to estimate the IC_{50} which is the inhibitor concentration giving 50 % inhibition. Also from the values of IC_{50} one can compare between the two drugs inhibition. The comparison of two inhibitors indicates the trifluperazine has higher percentage of inhibition than theophylline, that is, probably to the affinity of trifluoperazine to complex formation – with Zn and Mg the enzyme cofactor- which is higher than that of theophylline. As noted above the type of inhibition is noncompetitive, the binding in this type of inhibition is in the a site not the enzyme active site. So the binding of drug which has high inhibition percentage probably change the affinity of enzyme to react with substrate. This phenomenon can be visualized by the value of α [13].

Another way for estimation of inhibition percentage is as the following

equation:

$$percentage\ of\ inhibition\ \% = 100 - 100\frac{the\ velocity\ in\ the\ prescence\ of\ inhibitor}{the\ velocity\ in\ the\ absence\ of\ inhibitor}$$

By applying the equation, the percentage of inhibition of drugs using the same substrate concentrations are as followes: using scbstrate conc. 6.25 mM the inhibition percentage of theophylline was 17% while for trifluoperazine 24%.

CONCLUSION

Theophylline and trifluoperazine have inhibition effect on alkaline phosphatase and the inhibition percentage of trifluperazine s higher than that of theophylline. The apparent medical disorders of those taking the above drugs related to the activity of alkaline phosphatase are probably because the inhibition effect of drugs on enzyme. So it is necessary to note that by physicians to improve the patients managements.

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