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Inhibition of Serum Peroxidase in The Iraq is Patients with Thalassemia by Four Newly Sulfonamide Derivatives

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Abstract

This study was aimed to determine the *in vitro* effects of four newly synthesized sulfonamide derivatives (Sulfacetamid ,Sulfanilamid, sulfadiazine, sulphamethoxazole) on human serum peroxidase activity in patients with thalassemia compared to healthy subjects. Total protein, the results indicated that there was non-significant decrease in STP, while a significant increase in peroxidase activity. Also, there was a significant increase in specific activity in thalassemia patients as compared to healthy subjects in the sera of thalassemia patients compared to healthy subjects. The results revealed that all used compounds caused inhibitory affection the peroxidase activity and the highest inhibition percent were obtained at (0.18 gm/ml from Sulphacetamide, 0.01 gm/ml from Sulphamethaxazole, 0.05 gm/ml from Sulphadizine and 0.02gm/mlfrom Sulphamide). This study also determined the kinetic parameters (K_m, K_i, V_{max} and $V_{max\ i}$) at different concentrations from substrate and each inhibitor under the same conditions by using Line weaver-Burk equation and the results indicated that the level of K_m was not affected by adding the inhibitor to the enzyme reaction and equal to the level of K_i, while the levels of V_{max} were decreased when the reaction of enzyme include the inhibitor. Finally, the type of inhibition was found as non-competitive inhibition to the all used sulfonamide derivatives, this type of inhibition is characterized by its effect on the maximum velocity and is obtained when the inhibitor and the substrate were linked to different sites at enzyme.

Keywords: Peroxidase, Sulfonamidederivatives, Thalassemia, Inhibition.

الخلاصة

ان الهدف من هذا البحث هو دراسة تأثير اربع مركبات من مشتقات السيلفا المحضرة حديثاً والتي تتضمن (Sulfacetamid,sulfanilamid,sulfadiazine,sulphamethaxazole)على فعالية انزيم البيروكسيديز في مصل مرضى الثلاسيميا ومقارنة بالأصحاء.ولقد تبين انخفاض ملحوض في تركيز البروتين الكلي في حين تكون هناك زيادة معنوية في فعالية الانزيم وكذلك زيادة معنوية في الفعالية النوعية للانزيم ، وتبين من خلال النتائج بأن كل المركبات المستخدمة كمثبط لفعالية انزيم البيروكسيديز وتم الحصول على اعلى نسبة تثبيط عند التراكيز الاتية (0.18 gm/ml sulfacetamide, 0.02 gm/mlsulphamethaxazole, 0.05 gm/ml sulphadizine and 0.02 gm/ml sulfanilamide). وفي هذه الدراسة تم تحديد الحركيات وقياس (K_m, K_i, V_{max} and V_{maxi}) عند تراكيز مختلفة من الركيزة والمثبط وتحت نفس الظروف وباستخدام معادلة لينوفر-بيرك وقد لوحظ من النتائج المستحصلة ان قيمة K_m لاتتأثر عند اضافة المثبط على فعالية الانزيم وتكون مساوية لقيمة Ki, اما السرعة القصوى V_{max} فانها تقل عند استخدام المثبط . واخيراً تم ايجاد نوع التثبيط والتي يكون تثبيط غير تنافسي في كل مشتقات مركبات السيلفا المستخدمة, وان هذا النوع من التثبيط يتميز بتأثيره على السرعة القصوى ويحصل هذا النوع من التثبيط عندما ترتبط الركيزة والمثبط في مواقع مختلفة من الانزيم.

Introduction

Peroxidases are large family of enzymes (EC number 1.11.1.x [10]), they are heme-containing enzymes in their active sites that catalyze one-electron oxidation of a variety of oxidizable xenobiotics and biomolecules[1][2]. The cycle of reactions are involved in the oxidation of xenobiotics and biomolecules by peroxidase were as in the (Equations 1–3)[3]**:**

$$\text{Peroxidase} + \text{ROOH} \rightarrow \text{Compound I} + \text{ROH} \quad (1)$$

$$\text{Compound I} + \text{XOH} \rightarrow \text{Compound II} + \text{XO}^{*} + \text{H}^{+} \quad (2)$$

$$\text{Compound II} + XOH \rightarrow \text{Peroxidase} + XO^{*} + H_2O \quad (3)$$

peroxidases utilize H_2O_2 to catalyze the oxidation of variety of organic and inorganic compounds that typically catalyze and other peroxidases are more active with organic hydroperoxides such as lipid peroxides. The nature of the electron donor is very dependent on the structure of the enzyme. Their Molecular weight ranges from 35 -100 KD. Peroxidases are widely distributed in nature especially in animal, plant, and microorganisms[4][6], they have great potential applications, as they can be used in a diagnostic kit for hydrogen peroxide, glucose and oxidase enzyme determination[4]. The commercial production of peroxidase has increased due to it is analytical diagnostics particularly biosensing in immunosensors and nucleic acid detection .Various factors are authentic for the regulation of peroxidase activity in the cell. Pathogens like bacteria also stimulate or suppress peroxidase mRNA levels in different organisms[5]. In mammalian cells, various peroxidases are distributed in the cytosol, nucleus and mitochondria[7]. Increased or decreased activity of peroxidase has been defined by many mechanisms in different for diseases, but these mechanisms do not favorable explain the role of these enzymes. Peroxidases are directly or indirectly correlated with some leading diseases of mankind like Parkinson disease, coronary artery disease, skin disease, and cancer, these diseases can also arise due to different agents like auto-antibodies, flavonoids and thiocyanates, which involve the metabolic pathway of peroxidase action[8]. This paper reports the inhibitory effects of four newly synthesized sulfonamide derivatives on peroxidase activity in human serum patients with thalassemia, these compounds have important roles in the field of medicinal chemistry. Sulfonamides are important class of drugs, with several types of pharmacological agents possessing antibacterial [9], antitumor [10], diuretic [11], hyperglycemic[12], antithyroid [13] or protease inhibitory activity[14]. The high therapeutic properties of the sulfonamide related drugs have encouraged the medicinal chemists to synthesize a large number of novel chemotherapeutic agents. The aim of this study was to evaluate the activity of human serum peroxidase in thalassemia patients compared to healthy subjects, and then determine the *in vitro* effects of four newly synthesized sulfonamide derivatives on human serumperoxidase activity.

Material and Methods

Subjects

Seventy thalassemia patients (40 male and 30 female) with age ranged between (3-40) years were selected in this study. Those patients visited Azadi hospital/Kirkuk city during the period from September 2016 to April 2017 .All patients were subjected to a personal interview using especially designed questionnaire format of full history with detailed information. Healthy subjects as control group includes 40 subjects (25 male and 25 female) with the same age range as patients group.

Collection of blood

The separated serum was used for measurements of serum total protein (STP), peroxidase assay, and enzyme inhibitors.

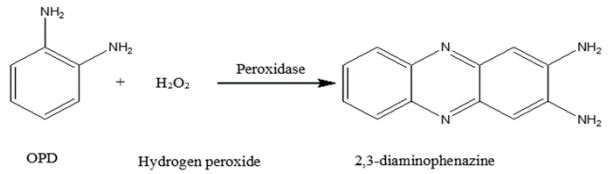
Total protein determination

Quantitative (STP) determination was achieved by absorbance measurements at 660 nm according to Lowry method 1951[15], with bovine serum albumin as a standard**.**

Peroxidase assay

Peroxidase activity was estimated according to Modified Sumer method, 1943[16].

The reaction of peroxidase with the substrate "O-phenylenediamine" (OPD, benzene-1,2-diamine) as electron donor and (H_2O_2) as oxidant as in the formula below:



The assay mixture (3mL) contained 0.15M of Phosphate buffer (pH=5), 0.1 M O-phynelenediamine, 30% H_2O_2 and 100 µL of serum .The peroxidase activity was calculated

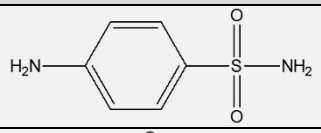
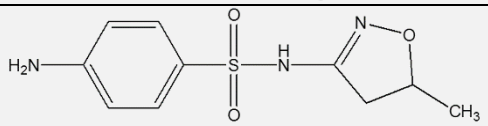
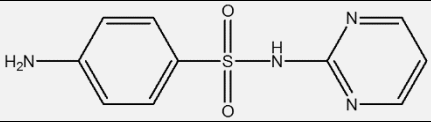
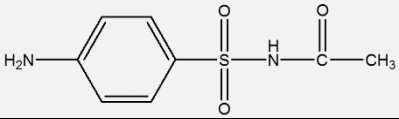
using an extinction coefficient of O-phynelenediamine (6.81 m M^{-1} cm^{-1}) at 420 nm and expressed as Unit of peroxidase/ml. Results were the average of at least three separate experiments and were expressed as mean ± standard deviation (mean±SD).

Enzyme inhibition

Preparation of inhibitors

The studied inhibitors were prepared as (0.4 M Sulphacetamide, 0.08 M Sulfomethoxazol and 0.08M Sulfanilamid) as stock solution by dissolving with ethanol and 0.4 M Sulphadiazine dissolved with 1NaOH, then prepared different concentrations from each compound:Sulfacetamide (0.18, 0.2 and 0.25 M), sulfamethoxazol (0.01, 0.02 and 0.03 M), sulfanilamid (0.02, 0.04 and 0.06 M) andsulfadiazine (0.05, 0.1 and 0.15 M).

Table 1: sulfa compounds were used as inhibitors to peroxidase activity.

NO.	Compound name	Structure of compound
1	Sulfanilamid 4-aminobenzensulfonamid	
2	Sulfomethoxazol [4-amino-N-(5-methylisoxazol-3yl)benzenesulfonamide]	
3	Sulfadiazine 4-amino-N-(pyrimidin-2-yl)benzenesulfinamide	
4	Sulfacetamid N-[(4-aminophenyl)sulfonylacetamid]	

Statistical Analysis

Statistical analysis was done using graph pad prism version 6 and values were expressed as (mean ±SD) .The comparison of mean ± SD was performed using Student t – test. Statistical significance was defined as $P \leq 0.05$.

and this study aimed to elucidate the kinetic parameters for O-phenyl diamine as substrate and four sulfonamide derivatives as inhibitors to the peroxidase activity, as well as determine the inhibition type.

Results

The serum STP and activity and specific activity of the peroxidase were determined of thalassemia patients and compared to healthy subjects, the results were mentioned as (mean ± SD) as present in Table 2:

Table 2: The STP and the activity with specific activity of peroxidase in the sera of thalassemia patients and healthy, (mean ±SD).

Groups	Total protein concentration mg/ml	Peroxidase activity U/ml	Specific activity U/mg
Patients	53.87 ± 1.318	37.7 ± 0.689	0.7019 ± 0.0206
Healthy subjects	71.03 ± 2.24	10.26 ±0.823	0.1533 ± 0.0149
P value	P ≤ 0.0001	P ≤ 0.0001	P ≤ 0.0001

The results indicated non–significant decrease in STP, while a significant increase in peroxidase activity. In addition, there was a significant increase in specific activity of thalassemia patients as compared to healthy subjects in the sera of thalassemia patients compared to healthy subjects. This study examined the *in vitro* inhibitory effect of four sulfoamide derivatives with different

concentrations on the serum peroxidase activity reaction in thalassemia patients and healthy subjects. Peroxidase activity without a sulphoamide compound was accepted as 100% activity, as in Tables1and 3.

The inhibition percent were represented in Figure 1.

Table 3: The effect of different concentrations of sulfoamidederivatives on serum peroxidase activity in the thalassemia patients and healthy subjects.

Compound concentration gm/ml		Enzyme activity U/ml	Enzyme activity+Inhibitor (U/ml)	Inhibition %
(A) Patients				
Sulphacetamide	0.18	50	25	50
	0.2		27	46
	0.25		30	40
Sulphamethaxazole	0.01	55	24	56
	0.02		26	52
	0.03		29	47
Sulphadizine	0.05	55	20	63
	0.1		22	60
	0.15		24	46
Sulphamide	0.02	55	20	63
	0.04		23	58
	0.06		25	54
(B) Healthy subjects				
Sulphacetamide	0.18	40	19	52.5
	0.2		22	45
	0.25		24	40
Sulphamethaxazole	0.01	45	18	60
	0.02		19	57.8
	0.03		21	53.3
Sulphadizine	0.05	40	20	50
	0.1		22	45
	0.15		24	40
Sulphamide	0.02	50	20	60
	0.04		21	58
	0.06		23	54

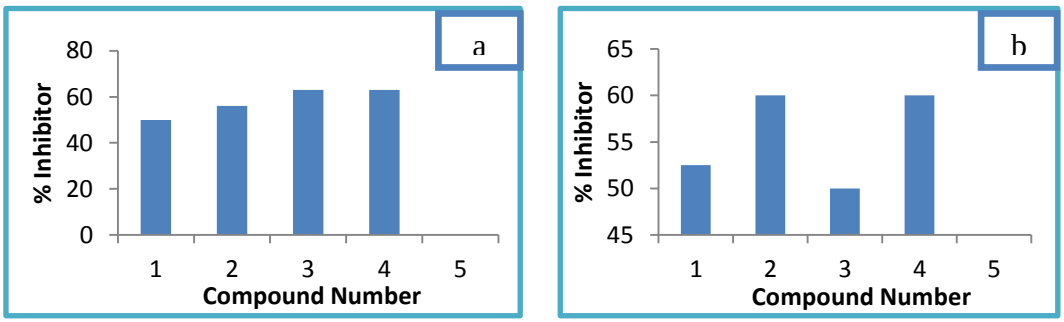


Figure1: The inhibition % of peroxidase activity, a) in thalassemia patients and b) in healthy subjects.

It was observed that the highest percentage of inhibition were obtained by use sulfacetamid with 50% for thalassemia and 52% for healthy subjects, sulfanilamid with 63% for thalassemia and 60% for healthy subjects, sulphamethoxazole with 56% for thalassemia and 60% for healthy subjects ,the sulfadiazine with 63% for thalassemia and 50% for healthy subjects. It was also observed that with increase the concentration of inhibitor, less percentage of inhibition obtained as shown in Figures 2 and 3.

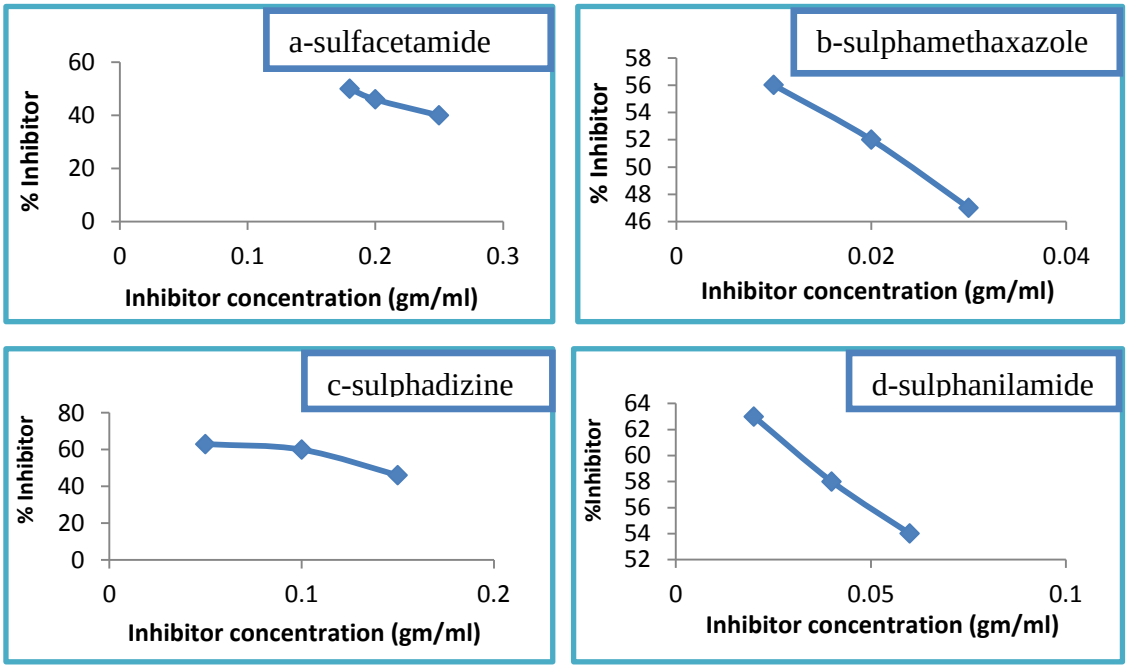


Figure 2: The relationship between the percentage of inhibition and the inhibitor concentration in thalassemia patients.

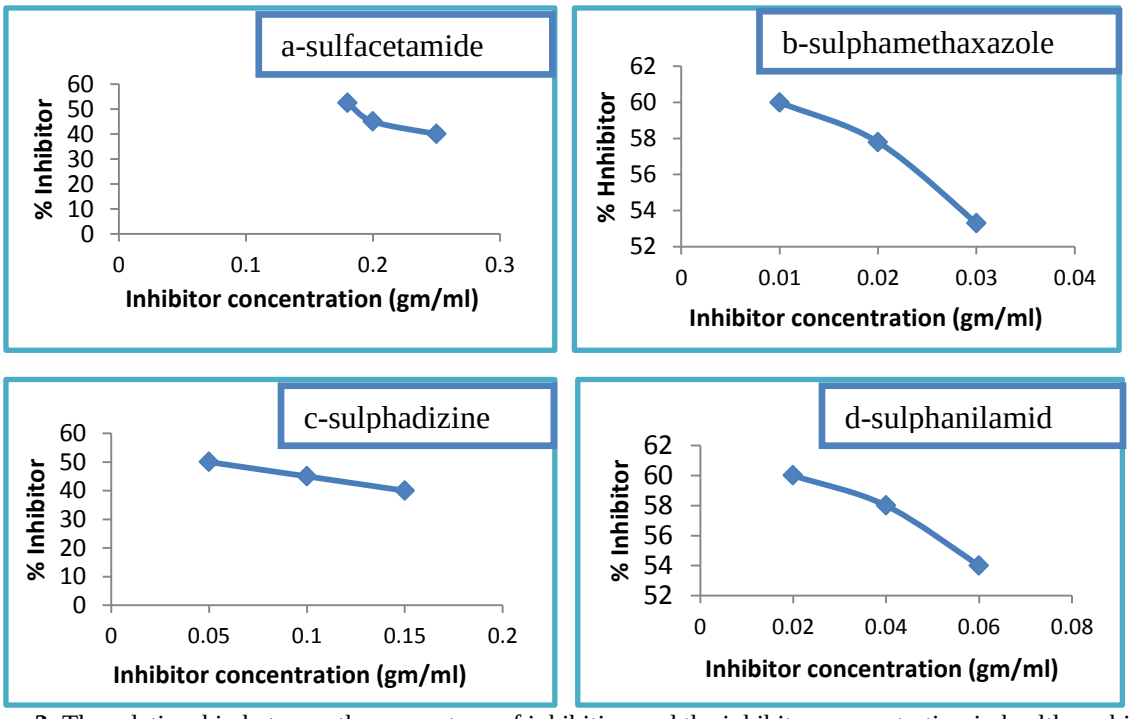


Figure 3: The relationship between the percentage of inhibition and the inhibitor concentration in healthy subjects.

The peroxidase kinetic parameters (with and without inhibitor) has been calculated from Line weaver - Burk plot as shown in Figure 4 and 5 which shown that all inhibitors used in this study were non-competitive inhibitor to the peroxidase activity.

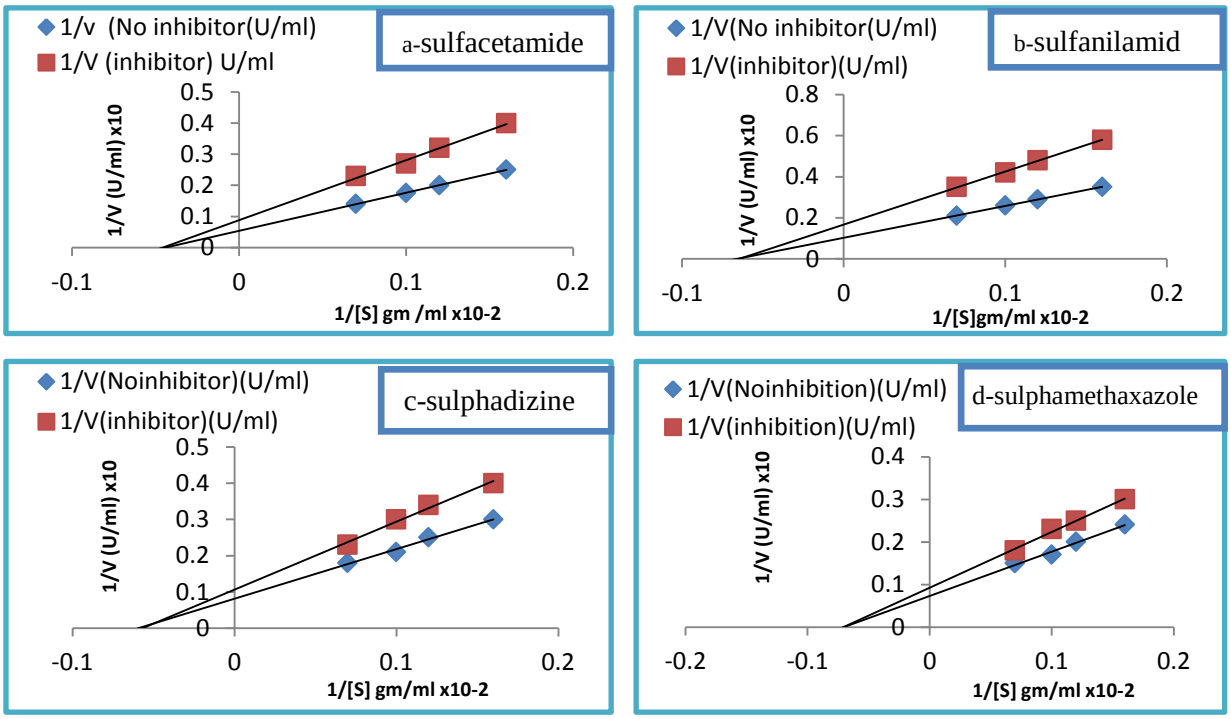


Figure 4: Line weaver-Burk plots for the studied inhibitors effects on peroxidase activity in thalassemia patients.

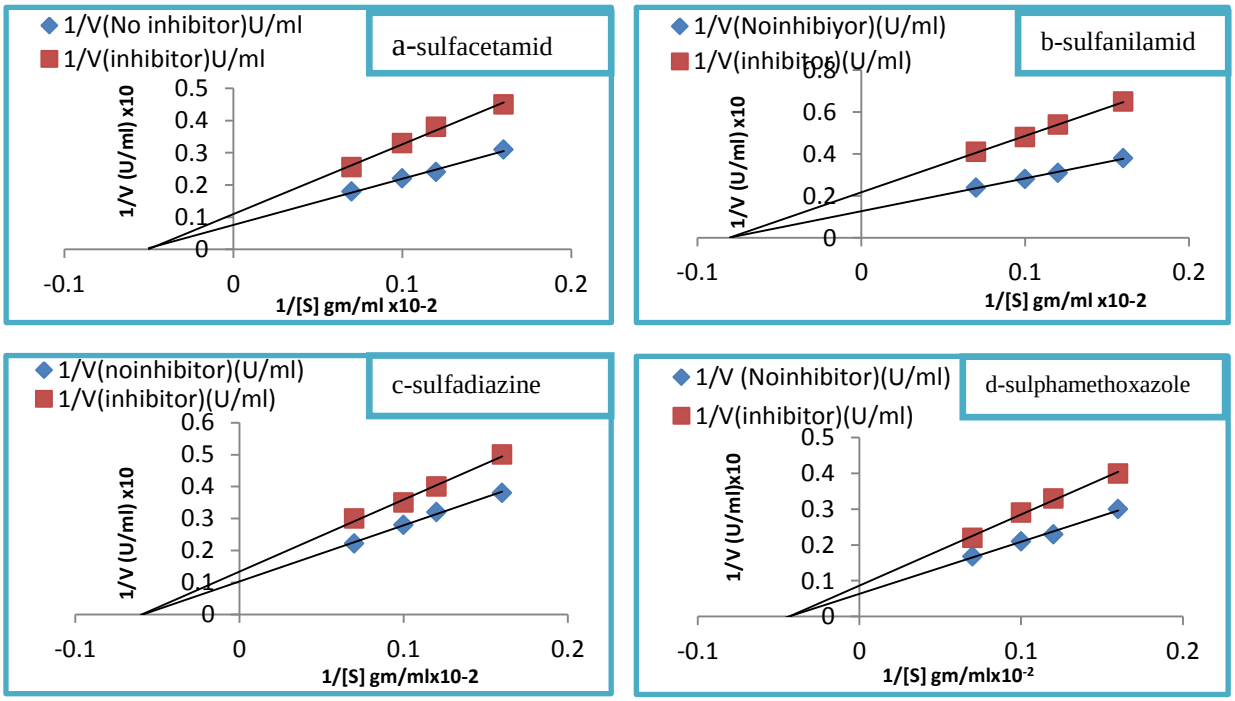


Figure 5: Line weaver-Burk plots for the studied inhibitors to the peroxidase activity in healthy subjects.

The levels of the kinetic parameters (K_m, K_i, V_{max} and V_{maxi}) at different concentrations from substrate and inhibitors under the same conditions were shown in Tables 4 and 5.

The type of inhibition caused by all studied inhibitors in this study were non- competitive inhibitoion type. The values of K_i and V_{max} were summarized in Tables 4 and 5. The

results indicated that with increasing the concentration of each inhibitor the percentage of inhibition decrease.

Table 4: The kinetic properties of peroxidase with sulfa compounds in thalassemia patients.

Parameter	Sulfadiazin	Sulfomethoxazol	Sulfanilamid	Sulfacetamid
K_i (gm/ml)*	1.53	0.142	0.142	0.25
V_{max} (U/ml)	125	142 .857	100	200
V_{maxi} (U/ml)	90	111.111	12.5	125

*The value of K_m which is fixed in the case of the presence of inhibitory and absence of inhibitory.

Table 5: The kinetic properties of peroxidase with sulfa compounds in healthy subjects.

Parameter	Sulfadiazin	Sulfomethoxazol	Sulfanilamid	Sulfacetamid
K_i (gm/ml)	0.166	0.25	0.125	0.2
V_{max} (U/ml)	100	166.666	83.333	125
V_{maxi} (U/ml)	71.428	111.111	47.619	100

Discussion

Peroxidases are large family of enzymes involved in oxidizing reactive oxygen species, innate immunity , hormone biosynthesis and pathogenesis of several diseases ,several researches are going on to understand peroxidase deficiency, over-expression and malfunction in relation with different diseases .Peroxidases have direct or indirect role in cancer, cardiovascular diseases and diabetes. So the status of peroxidase activity may also function as a marker of different diseases[17]. The results indicated that there were non-significant decrease in STP, while a significant increase in peroxidase activity .Also, there was a significant increase in specific activity in thalassemia patients as compared to healthy subjects in the sera of thalassemia patients compared to healthy subjects.

The significant increase in the specific activity may be attributed to the removing the effect of some compounds present as impurities by divided the activity of peroxidase on the concentration of serum TP ,by other words specific activity considered as a measure of enzyme purity[18].

The inhibitor is known as the substance that reduces the speed of the enzymatic reaction ,the structure of inhibitor may be similar to the structure of the substrate or differs from it, some inhibitors affect the substrate itself others combine with the active site on the surface of the enzyme and thus reduce the tendency of the enzyme to its substrate, the interactions with other sites on the enzyme and this type of inhibition may not affect the tendency of the enzyme but affects the rate of conversion of the enzyme - substrate to the product[19].Sulfoamides are group of synthetic pharmaceutical antibiotics[20][21].

Three main processes have been considered to be involved in the inactivation of peroxidase which include[22]:

1-Dissociation of prosthetic (heme) group from the holoenzyme (active enzyme system)

2-Conformational change in the apoenzyme (protein part of the enzyme); and/or:

3-Modification or degradation of the prosthetic group.

Although sulfonamide being major component of some drugs and causes positive effect on the treatment of most illness, it dramatically inhibited many human serum enzymes[23]. A large number of structurally novel sulfonamide derivatives have been recently reported to show inhibitory effect towards different enzymes of mammalian origin, and hence substantial antitumor, anti-inflammatory and antiviral activity. Although they have a common chemical motif of aromatic/heterocyclic sulfonamide there are a variety of mechanisms of their biological action some of them poorly understood till today[24].

The findings of the current study provide evidence based information about the impacts of four newly synthesized sulfonamide derivatives on the peroxidase activity and revealed that all of these compounds have the same type of inhibition(non-competitive)this type of inhibition is recognized by its characteristic effect on V_{max}, it occurs when the inhibitor and substrate bind at different sites on enzyme. The non-competitive inhibitor can bind either free enzyme or ES complex, thereby preventing the reaction from occurring[25].

Also the results indicated that any increase in the concentration of each compound causes decrease in the inhibition% of peroxidase.

The rote of inhibitory was computed by comparing the enzymatic efficacy with the presence and absence of inhibitor as in the equation below.

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

The results of this study also could be suggested that non-competitive inhibition can be explained according to the classical models described that the inhibitor bind to another conformational change that lock the enzyme & prevent the substrate binding or decreasing substrate affinity to enzyme[25][26]. A non-competitive mechanism of inhibition implies that the above compounds binding does not compete with O-phenyl diamine substrate but decreases the rate of catalytic turnover[27]. Line weaver - Burk graph showed the type of inhibition for each inhibitor and inhibition constant Ki was estimated as presented in Tables 4 and 5.Results indicated that the Ki value for sulfadiazin was more than for sulfomethoxazol, sulfanilamid and sulfacetamid in all studied groups, which reflects a better binding affinity (lower Ki) of(sulfomethoxazol, sulfanilamid) and sulfacetamide thansulfacetamid – substrate-based designs on peroxidase activity for patients group and sulfanilamid, sulfacetamid, sulfomethoxazol thansulfacetamid - substrate-based designs on peroxidase activity for healthy subjects. V_{max} was evaluated from the y-intercept of Line weaver - Burk graph, the data from tables 4 and 5, which reflected that V_{max} value for control sample (without inhibitor) was higher than in inhibited samples, So it is clear that the amount of active enzyme (V_{max}) present in non-inhibited which I in agreement with the study of Zayzafon and Nasif[26].

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