Facile Synthesis of Valsartan and Ribose Prodrug and Studying its Influence on the Pancreatic Lipase

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ABSTRACT:
Valsartan is a widely used drug that belongs to the type of angiotensin receptor blockers generally specified to control high blood pressure (hypertension) and heart failure. In the present work, a new ester prodrug utilizes ribose sugar as a carrier for the valsartan through an esterification reaction. The synthesized compound was characterized by the FT-IR and 1H NMR spectroscopic. The investigation furthermore involved measuring the activity of the lipase enzyme in the blood serum of individuals with high blood pressure and healthy individuals and purifying the lipase enzyme utilizing gel filtration technology. Besides, the investigation studied the kinetics of the lipase enzyme, the impact of various substrate concentrations, the impact of pH and optimal temperature on the enzyme's activity, and the impact of reaction time on the enzyme's effectiveness. It was found that the effectiveness of the lipase enzyme reduced with raising the concentration of the synthesized compound, and the sort of inhibition was determined as competitive.

1. Introduction

Hypertension, a chronic medical condition characterized by higher-than-normal arterial blood pressure (BP), influences around one billion people around the world [1] and is the major cause of morbidity and mortality. In high blood pressure patients, around 7.5 million people die per year, representing 12.8% of all global deaths. Especially, the give rise to 90-95% of hypertension patients are unknown and categorized as primary or essential hypertension. The remaining 5 - 10% is attributed to secondary causes such as renal disease, endocrine disorders, or other identifiable factors. The probability of developing hypertension is approximately 1.7 - 3.4 times higher in obese individuals as compared to those with normal weight [2]. The term prodrug indicates compounds converted into active drugs with medical and pharmaceutical efficacy within the body of a living organism after their absorption by the target molecule. According to the International Union of Applied Chemistry's system, a concomitant drug is a compound that undergoes a life transformation before its pharmacist effect appears [3].

Valsartan's chemical name is N-(1-isopentyl)-N-[[2'-(1H-tetrazol-5-yl) [1,1'-biphenyl]-4-yl] methyl]-L-valine (Figure 1). It is commonly obtainable in tablet form with the inactive compound combined with active compounds like microcrystalline cellulose, crospovidone, iron oxides, magnesium stearate, colloidal silicon dioxide, hydroxypropyl methylcellulose, polyethylene glycol 8000, and titanium dioxide. Valsartan's solubility depends on pH; at pH 5-8, it has higher solubility, whereas at acidic pH reduced solubility [4]. D-ribose is a highly water-soluble 5-carbon sugar, also known as D-furanose, present in different RNA molecules, including mRNA, transfer RNA, and ribosomal RNA [5, 6]. D-ribose was initially identified as a vital molecule in humans in 1958; however, its roles in human physiology and pathology, particularly in diseases, are still under investigation [7]. Until 1970, D-ribose was only known to increase blood sugar levels in low-energy states [8]. Lipases are enzymes that break down fats, including triacylglycerol and phospholipases. Triacylglycerol lipase converts triacylglycerol into free fatty acids, while phospholipases break down phospholipids [9, 10]. Human lipases consist of lingual, gastric preduodenal, pancreatic extra duodenal, hepatic, lipoprotein, and endothelial lipases, as recently described [11, 12]. Pancreatic lipase, created by pancreatic acinar cells, is one of the exoenzymes found in pancreatic juice that is important for digesting dietary fats in the intestinal lumen. The hydrolysis of dietary triacylglycerols using gastric and pancreatic lipases is necessary for the absorption of fats by intestinal cells and facilitating the assimilation of dietary fats in the body. The substrate of pancreatic lipase is many molecules; however, a non-aqueous phase of aggregated lipids. This phase consists of aggregates of ester molecules, micelles, or monolayers interacting with an aqueous medium [13, 14]. Pancreatic lipase requires colipase, a pancreatic protein, as a cofactor for its enzymatic activity [15, 16]. The colipase-dependent pancreatic lipase involved in the intestinal digestion of dietary fat is bile salt-catalyzed lipase, a component of

pancreatic juice and human milk. While colipase-dependent pancreatic lipase facilitates fatty acid absorption, bile salt-stimulated lipase accommodates the absorption of free cholesterol from the intestinal lumen [17]

2. Methods

Preparation of prodrug (valsartan, ribose) 6 mg of valsartan powder was added to 10 mL of thionyl chloride in a round bottom flask equipped with a condenser. The flask was then placed in the water bath at 50 °C and stirred for two hours to complete the sublimation process. Afterward, the reaction mixture was allowed to cool, and the thionyl chloride evaporated. Then, 0.6 gm of ribose was dissolved in a small amount of pyridine and added drop by drop to the first reaction mixture. The resulting mixture was then stirred at room temperature for 24 hours. After 24 hours, 10 mL of cold distilled water was added. The organic layer was separated using chloroform (15 (ml) three times and dried using anhydrous magnesium sulfate. The resulting material was purified using a fractionation column, and the solvent ratio hexane: ethyl acetate (1:4) was used.

The activity of Lipase was determined by colorimetric method by Italian analysis kit (GIESSE DIAGNOSTICS)[18] .

Lipase was extracted from 10mL of serum obtained from patients with hypertension using the following steps:1. Adding 4g of 80% ammonium sulfate to the serum, followed by dissolution of the precipitate in a smaller phosphate buffer solution at pH 7.2. 2. Dialysis in Tris-HCl at pH 7.2. 3. Subjecting the sample to gel filtration chromatography using Sephadex G100 at pH 7.2 with a flow rate of 5 ml/min in a column measuring 30x1.5 cm. The kinetic study was conducted based on existing literature.

For used solutions

Tris-HCl 1.576 gm was dissolved in one liter of distilled water in order to prepare a 10 mM Tris-HCl pH 7.2 buffer solution. Sephadex G100 column filler 2.5 gm was dissolved in 200 ml of 10 mM Tris - HCl pH 7.2 for preparing Sephadex G100 gel filtration suspension. The prepared suspension was left overnight at four Celsius. During this period, alter the buffer solution five times to remove any fine particles because their existence reduces the velocity of the leachate flow through the column. Finally, 500 mM of sodium chloride solution was prepared by dissolving 29.25 g of sodium chloride in a liter of 10 mM Tris-HCl pH 7.2 buffer.

Determining the purity of the enzyme by High-Performance Liquid Chromatography (HPLC)

(HPLC) device from the British company (CECIL) was used to measure the purity of the enzyme.

Table (1) shows the operating conditions of the device.

Col. Manufacturer	Col. Type	Col. Length	Col. Diameter	Col. Particle Size
Thermo	C18	250 mm	4.6 mm	5 µm

Kinetic Study and Characterization of a Partially Purified Enzyme

Lipase enzyme kinetic characteristics were investigated after isolation from blood serum and partial purification using gel filtration.

Impact of substrate concentration

The substrate concentration was investigated utilizing various substrate concentrations and its impact on the activity of the partially purified lipase enzyme. Various concentrations of the matrix (mmol/l 0.1, 0.08, 0.06, 0.04, 0.02, 0.01) were utilized to determine the impact of altering the substrate concentration on enzyme action.

Optimal pH

The impact of the pH of the buffer solution (Tris-HCL) at a concentration of (10 mM) (pH = 7.2) on the lipase enzyme reaction rate was investigated in various pH solutions (2.4, 4.4, 6.4, 8.4, 10.4, 12.4). Were utilized in the existence of the substrate for the lipase enzyme at (0.1 mmol/L) concentration and a temperature of (37°C), where the enzyme effectiveness was calculated according to the procedure of measuring effectiveness and by drawing the relationship between the reaction rate and pH, the optimal pH was identified.

Impact of Optimal Temperature

The efficacy of the lipase enzyme was estimated according to the way utilized to estimate the enzyme activity in different temperatures (7, 17, 37, 47, 57) and using Tris-HCL buffer solution (10 Mm) pH of 7.2 and 0.1 M

concentration of substrate, and then the relationship was drawn. Which relates the temperature with the reaction rate to obtain the optimum temperature.

Study of the impact of prodrug concentration on lipase enzyme efficacy

Six different concentrations of the prepared compound (inhibitor) were prepared. (150mg) of the prepared compound was dissolved in 5ml of dimethyl sulfoxide (DMSO), and the prepared concentrations were as follows: (150mg\5ml, 15mg\5ml, 1.5mg\5ml, 0.15mg\5ml, 0.015mg\5ml, 0.0015mg\5ml). When 10 µL of the prepared compound was added to each concentration immediately after adding the enzyme, the percentage of inhibition was calculated using the following equation.

$$\% \text{ Inhibition} = 100 - \frac{\text{the activity with inhibitor}}{\text{the activity with out inhibitor}} \times 100$$

Study of the type of lipase inhibition

To study the type of inhibition of the lipase enzyme, six different concentrations of the base material for the lipase enzyme were prepared, and the prepared concentrations were as follows: (0.1,0.08,0.06,0.04,0.02,0.01) mg\L The procedure for determining the effectiveness of the lipase enzyme was followed. However, in each test, a different concentration of the diluted concentrations of the base material was added according to the working method. After adding the enzyme,10µL of the prepared compound was added directly. To know the type of inhibition, the Lineweaver-Burk relationship plot is calculated.

3. Results and discussion

In the first step of enzyme purification, Proteins are usually precipitated by extracting a large amount of water and a specific purity standard. Ammonium sulfate is often utilized for Salt precipitates because of its high solubility in water. This step is known as salting out, and it is one of the significant steps that was used by the earlier works, as the sedimentation process reduces the enzymatic extract volume [17]. Lipase purification is briefed in Table (2). Enzyme purification was performed in several steps. When the enzyme was precipitation, ammonium sulfate concentration was 85%. The product was 78.8 %, and the specific efficiency was 0.00167 (IU/mg). Dialysis was performed utilizing a Tris-HCl buffer at pH 7.2 to obtain 3.2-fold purification; the product was 65%, and the specific efficiency was 0.00326 (IU/mg). Gel filtration and Sephadex G100 showed a single band of purified enzyme; with 7.7-fold purification, the product percentage was 67.5%, and the specific efficiency was 0.00768 (IU/mg), as shown in Fig (1)

Table 2: Partial purification of Lipase in serum of hypertension patients using ammonium sulfate, gel filtration, and chromatography

Purification steps	Volume ml	Efficiency IU/ml	Total Efficiency	Concentration of Protein	Specific Efficiency U/mg	Recovery Yield %	Fold of purification	Total protein
Crud	10	0.0448	0.448	45	0.000996	100	1	450
Precipitation $(NH_4)_2SO_4$	8	0.0568	0.4544	34	0.00167	78.8	1.6	272
Dialysis	9	0.0684	0.6156	21	0.00326	65	3.2	189
Gel filtration Sephadex G-100	5	0.1228	0.614	16	0.00768	36	7.7	80

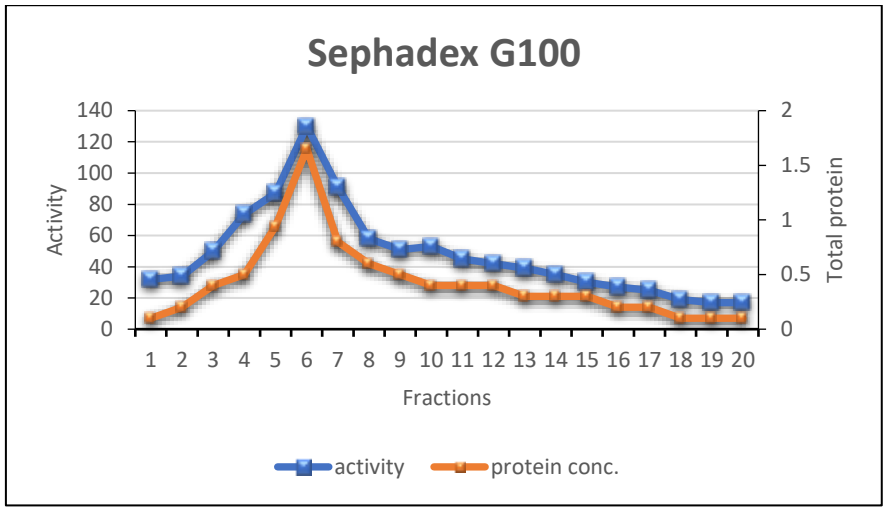

Fig 1: purification of lipase using Gel filtration

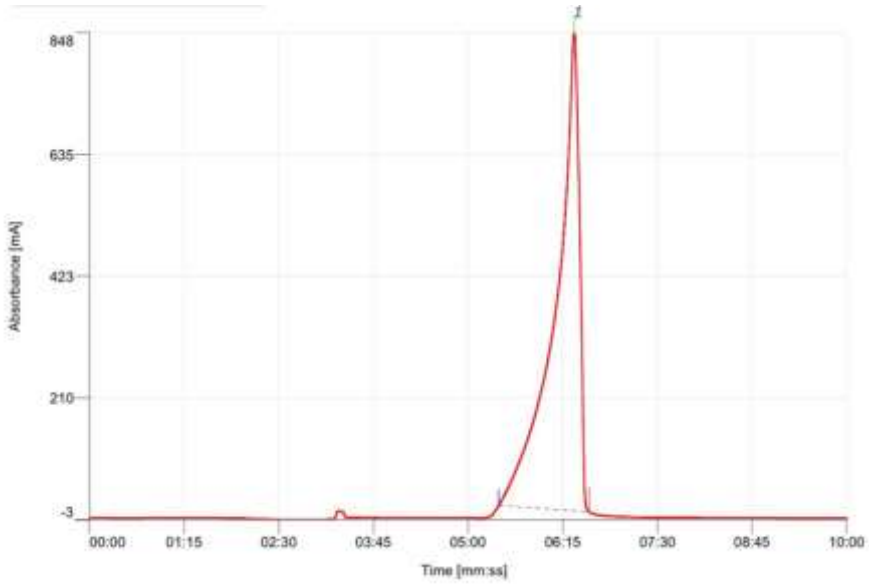

Fig 2: chromatography of lipase enzyme purified from hypertensive patients

Through Figure (2), which shows the relationship between absorbance and time, we will notice the appearance of a single band in the high-performance liquid chromatography device with a retention volume of (06:23.9), a height of (835.7), and an area of (18156.7) as shown in Table (3) This indicates the purity of the lipase enzyme purified from hypertensive patients.

Table 3

Ret.Time	Start Time	End Time	Area	Height	Area %
06:23.9	05:24.3	06:35.9	18156.7	835.7	100

Kinetic study of lipase enzyme:

The optimal concentration of substrate

The impact of the concentration of substrate was studied on the efficiency of the partially purified enzyme by measuring the efficiency of the enzyme with the change in the concentration of the substrate and at concentrations (0.001-0.1M, and as in Figure (2) which shows the effect of the substrate concentration with the effectiveness of the material. The enzyme as we observe a direct relationship with the substrate concentration increases, the effectiveness of the enzyme increases. When the enzyme concentration in the reaction mixture is constant, the increase in the base material concentration leads to a considerable increase in the rate of enzymatic reaction ,

and the most significant speed is achieved at concentration. (0.1 M), the speed at the highest material concentration is named the highest speed of the enzyme. For the enzyme, this is similar to what the researchers Michaels and Menten have confirmed, as they described that the use of low concentrations of the foundation material makes the effective sites of the enzyme unsaturated with the substrate. Utilized so much that the active sites of the enzyme become saturated with the substrate, the speed of enzyme reaction does not depend on the substrate concentration.

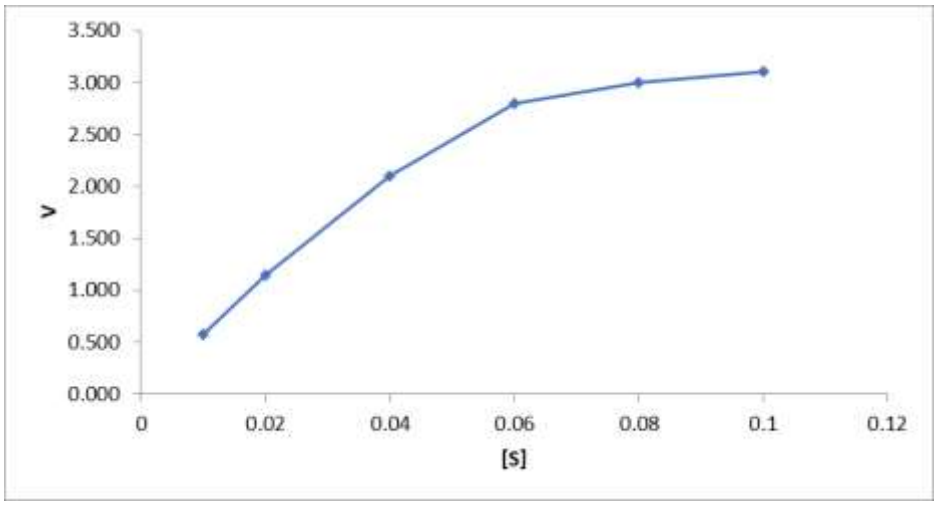

Fig 3: effect of substrate concentration on lipase activity

It is evident from Figure (3) that the enzyme follows the Michaelis-Menten equation, which shows the enzyme-substrate affinity. The Michaelis-Menten constant Km is measured in various ways. The affinity between the enzyme and the substrate decreases when the value of Michaelis-Menten constant Km is higher. Moreover, it describes the enzyme's activity to catalyze biological reactions and understand the enzyme's stability and the impact of activating and inhibitory substances on enzymatic efficiency or the base substance concentration when the speed rate is half the value of the highest speed. The Lineweaver-Burk way is considered considerably precise and best because of is not difficult to use, no plenty of mathematical operations, and is efficient in indicating the precision of the method. The above way was followed to measure the Km constant values for the purified enzyme, as shown in Figure (4). The Km value of the enzyme was equivalent to (0.016mM), and its highest speed at the identical base material was comparable to (4U/L).

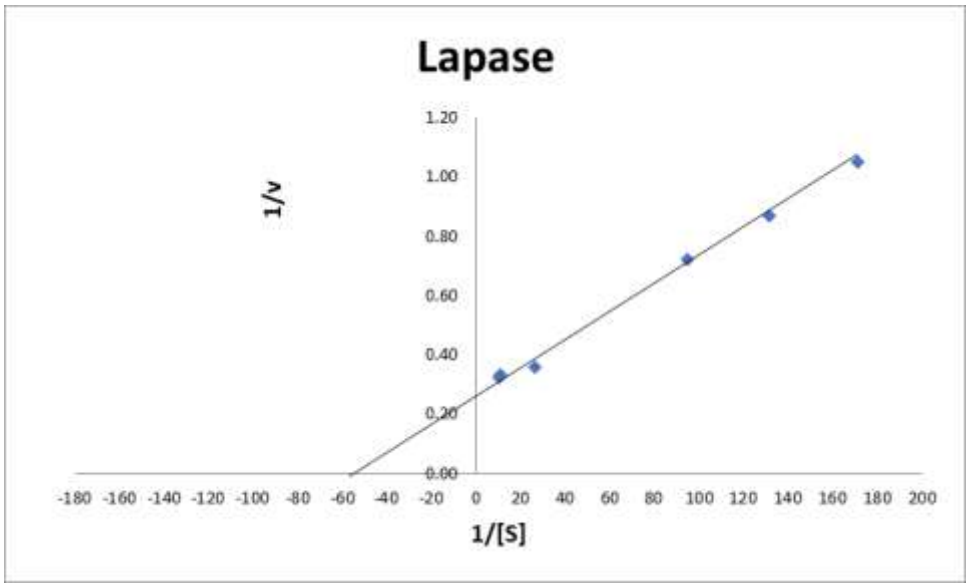

Fig 4: Lineweaver-Burke plot

The impact of temperature on the rate of reaction of the lipase enzyme was investigated within a specific temperature range of 7°C to 60°C. The results displayed those higher temperatures corresponded to an

acceleration in the reaction rate of the lipase enzyme, with the optimal temperature identified as 37°C, as depicted in Figure5.

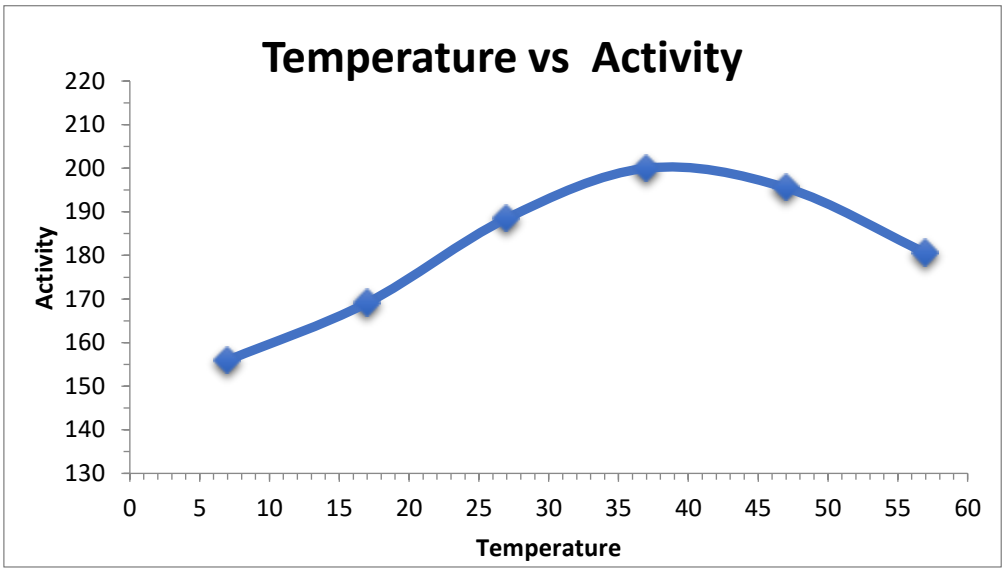

Fig 5: Effect of Temperature on lipase activity

The speed of enzymatic reactions generally rises with increasing temperature until the optimal level of reaction is achieved. The increase in temperature leads to increases in the kinetic energy of the molecules, consequently raising the proximity and collision between substrate and enzyme molecules. However, once the temperature exceeds the optimum level, the reaction speed gradually decreases as the enzyme undergoes denaturation. Denaturation occurs when the bond between the active amino acids in the enzyme molecule is disrupted, leading to a loss of enzymatic activity. The enzyme activity is affected by the ionization state of the groups on the surface of the enzyme and base material. Enzymes are complex protein molecules whose catalytic activity depends on their regular triangular structural shape. This shape is able to be changed using high temperatures, resulting in denaturation.

The effect of pH on the rate of lipase reaction

The pH affects the enzyme's effectiveness because of differences in its chemical composition and the existence of various ionic groups. Enzymes function best at an optimal pH because they are sensitive to hydrogen ion (H+) concentration changes. The rate of enzymatic reactions rises slowly with increasing pH until it achieves the highest rate at the optimal pH. After this point, the reaction speed starts to reduce. In a recent study, it was discovered that the optimal pH for the activity of the lipase enzyme is 8.5, as illustrated in Figure 6.

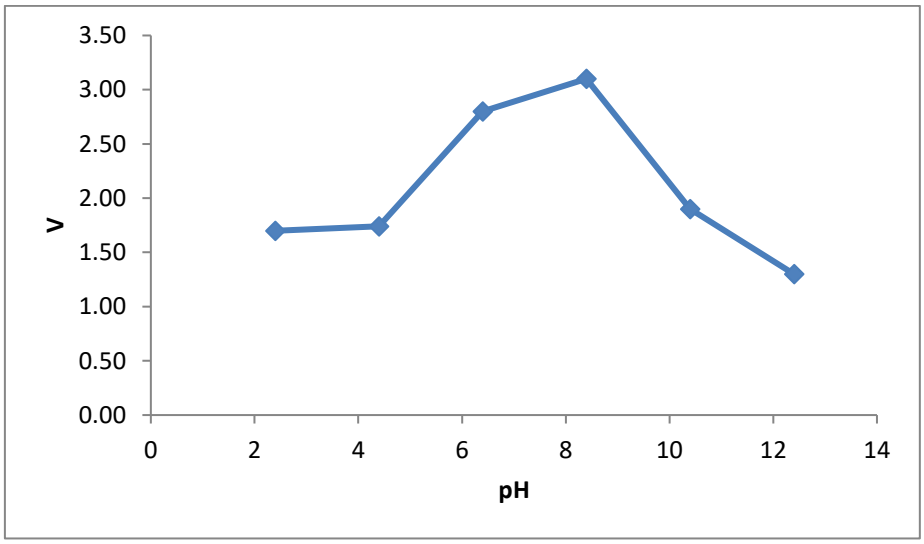

Fig 6: effect of pH on lipase activity

FT-IR spectrum of the prepared compound

FT-IR technology was used to identify effective functional groups. The FT-IR spectrum of the prepared compound exhibed the presence of a broad and strong band resulting from the merging of the NH and OH bands at a frequency of 3464 cm-1, as this frequency represents the stretching region belonging to the NH and OH groups. The spectrum also revealed the presence of a weak band at frequency 3016 cm-1 and a strong band at 756 cm-1 belonging to the Ar-H group. The spectrum showed the presence of peaks in the frequency range 2928-2962 cm-1 belonging to the CH3 group. And a weak band peak of the CH2 group at frequency 2856 cm-1. The spectrum also revealed a distinctive band with strong intensity at the frequency of 1676 cm-1, which is due to the merging of bands belonging to the groups N=N, C-COO-R, and C-CO-C. The appearance of strong double bands at 1400-1500 cm-1 is due to the stretching of the C=C bond in the aromatic groups. The spectrum also revealed the presence of a distinctive band with moderate to strong intensity at 1022 cm-1 resulting from the stretching of the C-O-C group. This band is strong evidence of the occurrence of the reaction between ribose and the compound.

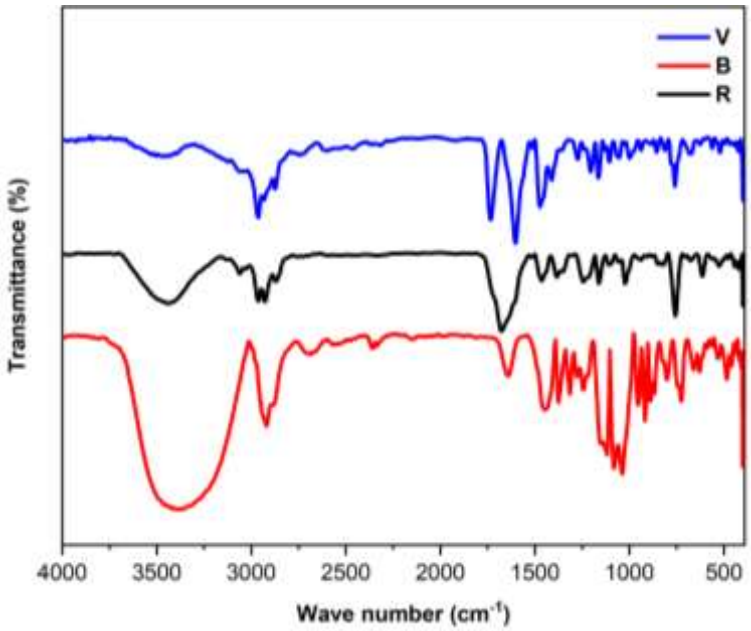

Fig 7: FT-IR spectrum of the prepared compound

^{1}H NMR spectrum of the prepared compound

The NMR spectrum of the prepared compound, shown in Figure (8), revealed a signal at the chemical shift = 0.9 ppm δ, which is due to the protons A shown in the figure, and their integration indicates that they correspond to 9 protons for the prepared compound. It also showed a signal at the chemical shift = 1.3 ppm δ belongs to protons b, and the two signs at chemical displacement = 1.7 and 1.8 ppm δ belong to protons c and d, respectively. While the signal at the chemical shift = 2.4 ppm δ is due to the f protons of the compound. Also, when the chemical displacement = 2.7 ppm, δ returns to the proton g of the compound. The signal at the chemical displacement = 3.5 ppm δ, which is due to the protons h of the compound. As for the proton e, it showed a signal at the chemical displacement = 5.2 ppm δ. Both protons k and i showed two signals at chemical displacement = 4 and 5.2 ppm δ, respectively. As for the protons j and l, their signals converged with the signals of the aromatic protons of the compound within the range = 7.4-8 ppm δ. The signal at the chemical shift = 9 ppm δ goes back to the s proton of the –NH group of the compound, as shown in Figure (8).

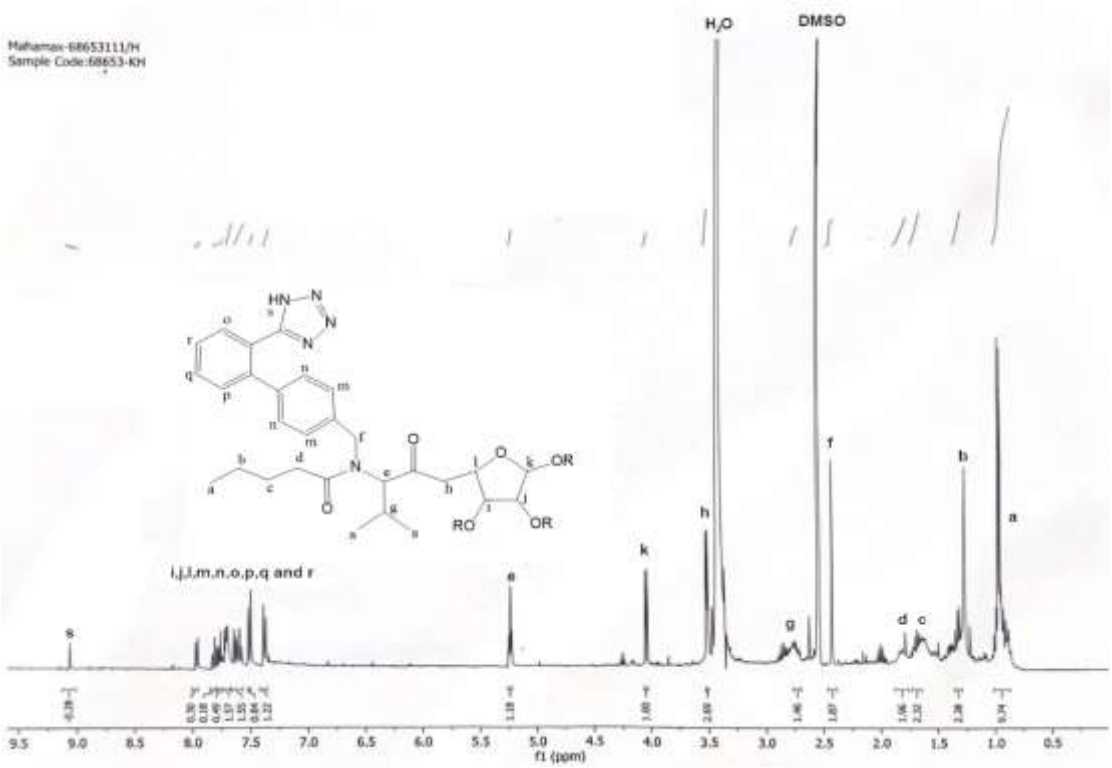

Fig 8: ^{1}H NMR spectrum of the prepared compound

Studying the effect of the prepared compound on the effectiveness of lipase enzyme partially purified from the sera of patients with high blood pressure

The effect of the prepared compound was studied according to the method of action, where multiple concentrations of the compound were used (150mg/5ml, 15mg/5ml, 1.5mg/5ml, 0.15m/5ml, 0.015mg/5ml, 0.0015mg/5ml). The findings exhibited a decrease in the purified lipase enzyme's activity. This attribute displays that the enzyme inhibition is directly proportional to the concentration of the prepared compound in comparison to the control group. The control group's activity was 73.7 U/L, while the activity of the enzyme and the percentage of inhibition were, respectively, as shown in Table 2

Table 4: Effect of prodrug on the activity of lipase enzyme

Conc.	Activity U/L	%inhibition
without Inhibitor	73.7	0
150mg/5ml	15	79.64
15mg/5ml	18.6	74.76
1.5mg/5ml	34	53.86
0.15mg/5ml	36	51.15
0.015mg/5ml	36.3	50.7
0.0015mg/5ml	69	6.37

Study the type of inhibition

The type of inhibition of the lipase enzyme was studied, as (6) different concentrations of the base material for the lipase enzyme and one concentration of the prepared compound were prepared. After drawing the Lineweaver-Burk equation, it was found that the type of inhibition is of the competitive type, as we note that the value of the highest speed Vmax has not changed. The Michaelis-Menten constant Km value increases, as in Figure (9)

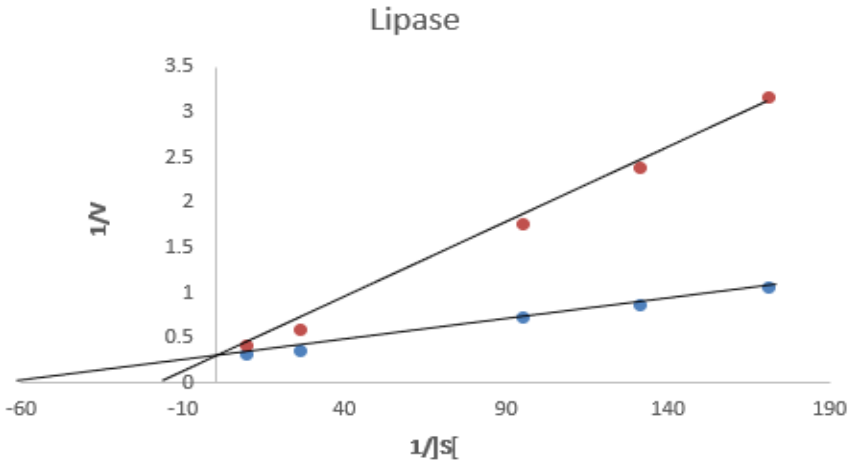

Fig 9: Lineweaver-Burke plot

4. Conclusion

This study developed a new ester prodrug for valsartan using ribose sugar as a carrier. The synthesized compound was characterized using FT-IR and 1H NMR spectroscopy. The activity of the lipase enzyme was measured in blood serum from hypertensive individuals. The study found that increasing the concentration of the synthesized compound reduced the lipase enzyme's effectiveness, indicating competitive inhibition.

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