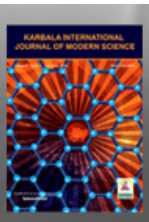

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Kinetic study for the effect of new inhibitors on the activity of purified GPT from blood of cardiovascular patients

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University of Kerbala

Kinetic study for the effect of new inhibitors on the activity of purified GPT from blood of cardiovascular patients

Abstract

In this study several biochemical parameters for cardiovascular patients have been measured CK-MB ,LDH ,GPT and GOT in addition to lipid profile , MDA ,Vitamin C and Vitamin E . New derivatives of ascorbic acid have been synthesized and tested to inhibit the purified GPT from the blood of cardiovascular patients Glutamate pyruvate transaminase (GPT) was purified from cardiovascular patients using ammonium sulphate salt for precipitation then dialysed using 0.1 M Tris-HCL buffer pH7.8 , then filtrated by gel filtration chromatography using Sephadex G-100 , followed by electrophoresis using poly acrylamide – bis acrylamide and sodium dodecyl sulphate (SDS). Derivatives of 3-(acetyl salicyloyl)-5,6 –O-isopropylidene-L-ascorbic acid (1) 2,3-di(acetyl salicyloyl)-5,6 –O-isopropylidene-L-ascorbic acid (2) and effect of 2,3,5,6 -tetra(acetyl salicyloyl)-L-ascorbic acid (3) which have been synthesized and characterized in this study , have been used in different concentrations to inhibit the purified enzyme activity using DMSO as a solvent .Results showed that these derivatives have inhibition effect on the enzyme activity with new value of kinetic parameters been detected using Linweaver-Burk plot and the type was un-competitive inhibition for the three derivatives been used in this study. Many biochemical parameters have been detected in this study including CK-MB, LDH, GPT and GOT as known marker for the disease in addition to lipid profile parameters Alb. UA, G6PDH, Vit.C and E as antioxidants as well as malondialdehyde (MDA). Results also showed significant differences in the levels of most parameters used in this study.

Keywords

cardiovascular disease, LDH, CK-MB, GPT, Inhibition

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1. Introduction

Glutamate pyruvate transaminase (GPT) [EC.2.6.1.2] which is also called Alanine aminotransferase (ALT) is normally found in the liver, and as well as in heart, muscle cells, kidneys, erythrocytes and pancreas. . When organ and their tissues such as that of the heart or liver became damaged, the enzyme leaks from tissues to the blood stream and that causes an increase in the circulating level of that enzyme [1]. Cardiovascular disease (CVD) as a collective term is describing a wide range of disorders which affect the conformational structure and the function of the heart. This includes various organs including the blood vessels that supply organs with blood and oxygen. Cardiovascular disease is the leading the cause of death for men and women. Common CVD includes ischemic heart disease, peripheral vascular disease rheumatic heart and chronic heart failure. Creatine kinase (CK-MB), lactate dehydrogenase (LDH) are the most important biomarkers in (CVD) in addition to troponin, lipid profile and others [2−4]. In this study several biochemical parameters for cardiovascular patients have been measured CK-MB, LDH, GPT and GOT in addition to lipid profile, MDA, Vitamin C and Vitamin E. New derivatives of ascorbic acid have been synthesized and tested to inhibit the purified GPT from the blood of cardiovascular patients.

2. Materials and methods

2.1. Patients

Blood samples were collected from 100 Cardiovascular patients and 100 apparently healthy individuals (50 males and 50 females) as control from Tikrit teaching hospital, (Tikrit, Iraq/This research was conducted with the approval of the Biochemistry Branch/Department of Chemistry/Faculty of Science. The research was registered on 20/9/2017). The ages of the participants were between 33 and 65 years. Disposable plastic syringes of 10 ml were used to draw venous blood from each patient and control then samples were left for 20−30 min at 37 °C. The serum was used for the analysis after separation by centrifugation. LDH, creatine kinase −MB,G6PDH, GOT, and GPT activity were measured. Cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol, VLDL-cholesterol albumin and uric acid were estimated using Creatine Kinase-MB, LDH, cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol, VLDL-cholesterol, GOT, GPT, G6PD and Alb. Kits from Biolabo company (France) using colorimetric method. MDA, Vitamin C and vitamin E were estimated according to the literature [5,6].

2.2. Purification of GPT

Glutamate pyruvate transaminase (GPT) was purified from the serum of cardiovascular patients (15 ml of serum) using the following steps: 1-addition ammonium sulfate (75%, 4.74 g, The precipitate was dissolved in a less amount of phosphate buffer solution pH 7.2); 2- Dialysis, phosphate buffer solution pH 7.2; 3-Gel filtration Chromatography (Sephadex G100, pH 7.2 flow rate was (2 ml/min) column (20 × 2 cm)); 4-Ion exchange: diethyl amino ethyl cellulose A-50 (DEAE) (column (20 × 2 cm)); concentrations of sodium chloride solution (0.1−0.4)M; The flow rate was (2ml/4 min) according to the literature [7].

2.3. Determination of molecular weight

The molecular weight of purified GPT was determined using Sodium dodecyl sulfate polyacrylamide gel electrophoresis [8,9].

Statistical analyses were performed using SPSS version 15.0 for Windows. Descriptive analysis was used to show the mean and standard deviation of variables. The significance of difference between mean values was estimated by Student T-Test. The probability $P < 0.05$ = significant, $P > 0.05$ = non-significant.

2.4. Synthesis of new inhibitors

Derivatives of 3-(acetyl salicyloyl)-5,6 −O-isopropylidene-L-ascorbic acid (1) 2,3-di(acetyl salicyloyl)-5,6 −O-isopropylidene-L-ascorbic acid (2) and effect of 2,3,5,6 -tetra(acetyl salicyloyl)-L-ascorbic acid (3) were synthesized and identified according to the literature [10].

2.4.1. Synthesis of 5,6-O-isopropylidene-L-ascorbic acid

A saturated solution of L-ascorbic acid ($C_6H_8O_6$) M. Wt = 176 g/mol (10.00 g,57.00 mmol) in 100 ml of freshly distilled acetone, (HCl) gas was bubbled at

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room temperature for 20 min, to this solution n-hexane (80 ml) was added, stirred, and decanted. The residue was washed with acetone – hexane (4:7) four times, then the solvent was removed under reduced pressure to give (10) (11.37 gm,92% yield) as a white crystalline residue, **m. p.** 219–220 °C, **IR(KBr disk)** (υ cm^{-1}) 3240 (O–H), 2995 (C–H) aliphatic 1755 (C═O) lactone.

2.4.2. Synthesis of O- acetyl salicyloyl chloride

To a dry powder acetyl salicylic acid (17) (19.5 mmol) in claisen flask was added redistilled thionyl chloride (1.74 ml) and the mixture was refluxed for 6 h or until evolution of hydrogen chloride ceases. The reaction mixture was left to cool, the condenser was removed and the flask was heated at 60 °C for 3 min with occasional shaking. Excess thionyl chloride was removed under reduced pressure to give (18). formula $C_9H_7O_3Cl$.(4.09 g, 95.94% yield) B.P. 135 °C. IR spectra (υ cm^{-1}) show stretching band at 1755 for (C═O) carboxylic acid chloride.

2.4.3. Synthesis of 3-(acetyl salicyloyl)-5,6 –O-isopropylidene-L-ascorbic acid (1)

To an ice – cooled solution (–12 °C) of 5,6-O-isopropelidine-*L*-ascorbic acid (10) (4.21 gm, 19.5 mmol) in pyridine, was added, drop wise, acetyl salicyloyl chloride (18) (4.37 gm,19.5 mmol). The resulting reaction mixture was kept at room temperature for 24 h, and then the cold distilled water (300 ml) and chloroform (400 ml) were added. The organic layer was separated, dried over anhydrous ($MgSO_4$), filtered, and the solvent was removed under reduced pressure, (trace of pyridine was removed under reduced pressure by co evaporation with toluene (3 × 50 ml)). The remaining syrup was purified on a silica gel column to give (19) (6.80 gm, 86.38% yield) , IR (υ cm^{-1}),3400-3082 (O–H), 2895 (C–H) aliphatic 1753 (C═O) ester, 1616,1485 (C═C) aromatic, 758 (C–H) out of plan.

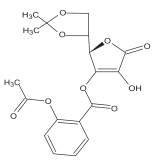
2.4.4. Synthesis of 2,3 - di (acetyl salicyloyl) - 5,6 – O –isopropylidene – L - ascorbic acid (2)

To a solution of 3-(acetyl salicyloyl)-5,6 –O-isopropylidene -*L*- ascorbic acid (19) (19.5 mmol) in pyridine, was added, drop-wise, acetyl salicyloyl chloride (18) (4.37 gm,19.5 mmol). The resulting mixture was kept at room temperature for 24 h, and then the cold distilled water (300 ml) and chloroform (400 ml) were added. The organic layer was separated, dried over anhydrous ($MgSO_4$), filtered, and the solvent was removed under reduced pressure, (trace of pyridine was removed under reduced pressure by co evaporation with toluene (3 × 50 ml).

The remaining semi solid was purified on a silica gel column to give (20) (8.60 gm, 81.6% yield) as semi solid, IR (υ cm^{-1}), 2925 (C–H) aliphatic 1741 (C═O) ester, 1608,1485 (C═C) aromatic, 756 (C–H) out of plan.

2.4.5. Synthesis of 2,3,5,6 -Tetra (acetyl salicyloyl)-L-ascorbic acid (3)

To a solution of *L*-ascorbic acid (8) (4.21 g, 24 mmol) in pyridine, was added, drop-wise, acetyl salicyloyl chloride (18) (17.0 g,96.0 mmol). The resulting reaction mixture was kept at room temperature for 24 h, and then the cold distilled water (300 ml) and chloroform (400 ml) were added. The organic layer was separated, dried over anhydrous ($MgSO_4$), filtered, and the solvent was removed under reduced pressure, (trace of pyridine was removed under reduced pressure by co evaporation with toluene (3 × 50 ml)). The remaining syrup was purified on a silica gel column to give (21) (18.51 gm, 84.38% yield), IR (υ cm^{-1}), 2858 (C–H) aliphatic 1730 (C═O) ester, 1568,1444 (C═C) aromatic, 758 (C–H) out of plan.



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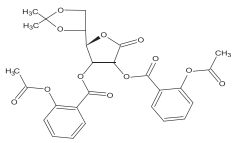

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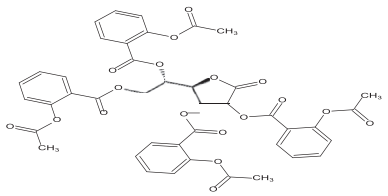

(3)

2.5. Effect of the new inhibitors (1), (2) and (3) on the purified GPT activity in serum

The effect of the new compounds were calculated at different concentrations (0.5 g/25 ml–1. 5 × 10^{-7}g/25 ml). The compounds which were prepared by serial dilutions in DMSO from the stock solution (0.5 gm/25 ml). An inhibition type study was done at different

Table 1
Mean ± SD of the biochemical parameters used in the research for control group (healthy individuals) and patients.

	Control	patients Male	patients Female	Patients Age (33–50 yrs)	patients Age (51–65 yrs)	p values
CK-MB U/L	110.43 ± 14.21	253.46 ± 31.66	268.56 ± 33.76	260.66 ± 34.16	263.56 ± 32.42	0.05
LDH U/L	77.41 ± 11.23	269.11 ± 35.21	280 ± 37.54	271 ± 36.64	275 ± 36.76	0.05
Alb mg/dl	4.1 ± 1.10	2.89 ± 1.05	3.1 ± 1.07	2.78 ± 1.06	3.0 ± 1.08	0.05
UA mg/dl	4.5 ± 1.71	6.74 ± 1.21	7.24 ± 1.23	6.58 ± 1.30	6.87 ± 1.31	0.05
Cholester ol mg/dl	125 ± 22.41	206 ± 25.11	210 ± 27.12	222 ± 29.02	217 ± 28.32	0.05
Cholester ol-HDL mg/dl	44.76 ± 5.21	28.63 ± 2.77	30.81 ± 2.98	29.56 ± 2.62	31.24 ± 3.01	0.05
Cholester ol-LDL mg/dl	101 ± 11.40	167 ± 21.36	174 ± 22.31	171 ± 21.38	170 ± 20.22	0.05
Cholester ol-VLDL mg/dl	24.10 ± 2.20	39.84 ± 2.50	41.54 ± 2.61	40.41 ± 2.58	42.03 ± 2.76	0.05
TG mg/dl	101 ± 24.51	215 ± 26.57	226 ± 27.31	221 ± 27.11	219 ± 26.46	0.05
Vitamin C µmol/L	29.2 ± 2.44	17.96 ± 1.86	19.70 ± 1.96	18.34 ± 1.91	19.61 ± 1.94	0.05
Vitamin E µmol/L	20.88 ± 1.78	15.85 ± 1.43	16.65 ± 1.57	15.57 ± 1.88	16.21 ± 1.59	0.05
MDA mg/dl	0.871 ± 0.743	1.75 ± 0.74	1.87 ± 0.75	1.78 ± 0.74	1.81 ± 0.75	0.05
GPT IU/L	34.68 ± 4.62	71.34 ± 10.62	74.67 ± 11.67	72.76 ± 10.91	75.21 ± 12.11	0.05
GOT IU/L	29.87 ± 3.85	67 ± 9.28	72.27 ± 10.54	70.51 ± 11.17	70.87 ± 10.43	0.05
G6PD IU/gHb	12.5 ± 2.1	19.3 ± 2.6	21.4 ± 2.7	23 ± 3.1	24 ± 3.1	0.05

concentrations of the substrate (0.5–5 mM) with fixed concentrations of the synthesized compounds 1, 2 and 3 (0.005 g/25 ml). The enzyme activity was measured in the presence and absence of inhibitor at the same conditions. Lineweaver-Burke plot was applied to obtain Km, Vmax and type of inhibition.

3. Results and discussion

The mean (±SD) of (CK-MB), LDH, GOT,GPT, G6PDH activity and levels of cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol, VLDL-cholesterol and Alb. in the serum of control group (healthy individuals) and patients are shown in Table 1.

There was a highly significant increase ($p < 0.05$) in the serum levels of CK-MB, LDH, cholesterol, triglyceride, HDL-cholesterol, GOT, GPT and MDA in comparison with the control group and significant decrease in Alb,HDL-Cholesterol, Vitamin C and vitamin E in comparison with the control. There were no significant differences between gender (males and females) and no significant differences between ages. These results were in agreement with Venkateswarlu. K et al. (2011) and Morris CJ, Morris P (1998) who showed an increase in plasmatic CK-MB activity in acute myocardial infarction (AMI) and acute coronary syndromes[11,12], This cardiac marker enzyme depends on its relatively high concentration in heart muscle (>20%) in comparison with skeletal muscle (1–2%). Other studies [13] showed that there was an increase in CK-MB concentration in ventricular myocardial tissues in animals and ischemia in humans in variety of conditions in young males with and without cardiac disease. The concentration of CK-MB in myocardial biopsy in human has been reported be a hundred times greater in heart of aortic stenosis

Table 2
Partial purification of GPT in serum of cardiovascular using ammonium sulfate, gel filtration chromatography and Ion exchange.

Step	Elute Ml	Activity (IU/mL)	Total activity (IU)	Protein conc (mg/mL)	Total protein (mg)	Specific activity (IU/mg)	Folds of Purification	Yield %
Crude proportion	15	30	450	9.32	139.8	3.21	1	100
Ammonium Sulfate	12	26	312	4.56	54.72	5.70	1.78	71
Dialysis	9	21	189	2.73	24.57	7.69	2.40	42
Gel filtration	5	16	80	1.81	9.05	8.83	2.75	17.7
Ionic exchange	5	12	60	1.1	5.5	10.90	3.40	13.4

patients and coronary artery disease with left ventricular hypertrophic patients compare with healthy individual. Moreover there was an increase in enzyme activity in progressive muscular dystrophy, toxic myopathy, polymyositis and severe scleroderma. This enzyme therefore was not a good marker for diagnosis of AMI or heart diseases [14,15]. The increase in the activity of CK-MB started for 3–6 h after starting symptom in MI and continue to elevate for 24–36 h. The results of this study were in agreement with Bhardwaj et al. [*16*], Ch sankeerthi et al. [17] Aharm Erez et al. [18] and srikrishna et al. [19] who found that the activity of some enzymes such as CK,CK-MB and LDH were increased in patients compare to healthy control. The activity of LDH level was significantly increased in the coronary artery (CAD) patients compare to control. The concentration of Albumin was decrease compare to healthy control. These results were in agreement with Nehal Rachit Shah [20], Sathi S., et al. [21] and Dick Zeeuw et al. [22]. There was a highly significant increase ($p < 0.005$) in serum levels of cholesterol, triglyceride, LDL,VLDL, GPT, GOT and G6PD. A significant decrease ($p < 0.05$) in serum levels of HDL when compared with the control group. Results were in agreement with Schwaiger et al.(2006) who studied the cardiovascular events and lipid abnormalities in CKD patients. In their study the lipid abnormalities were elevated serum total cholesterol, HDL, LDL and triglyceride levels [23]. While disagreeing with Mannangi N et al.(2014) who demonstrated a significantly decreased HDL level, they observed no differences in total cholesterol, LDL level [24] There was a highly significant decrease ($p < 0.005$) in serum levels of vitamin C and vitamin E and a significant increase ($p < 0.05$) in serum level of MDA when compared with the control group. These results were in agreement with Dennis, V & Parke, D. (1994) [25] and Velasco *etal* [26].

Purification of GPT was summarized in Table 2. It was purified in several steps; The first purification step used ammonium sulfate salt by saturation 65% to precipitate the protein and get a degree of purity to obtain 1.78 fold of purification, the yield was 71% and specific activity was (5.7 IU/mg). Dialysis was done by Tris-HCl buffer pH 7.2 to obtain 2.4 fold of purification, the yield was 42% and specific activity was (7.69 IU/mg). Gel filtration Sephadex G100 gave a single band of purified enzyme; with 2.75 folds of purification, yield percentage was 17.7% and specific activity was (8.83 IU/mg). Ion exchange chromatography was used to obtain 3.4 fold of purification and the yield was 13.4% and specific activity was (10.9 IU/mg).

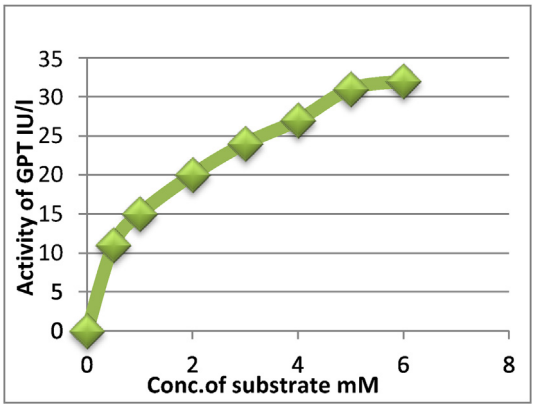

Fig. 1. Effect of substrate concentration on GPT activity.

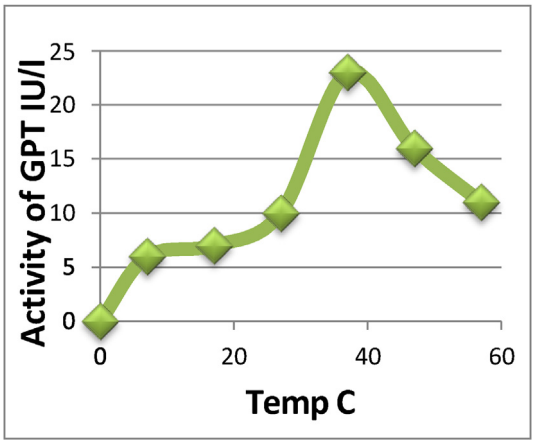

Fig. 2. Effect of temperature on GPT activity.

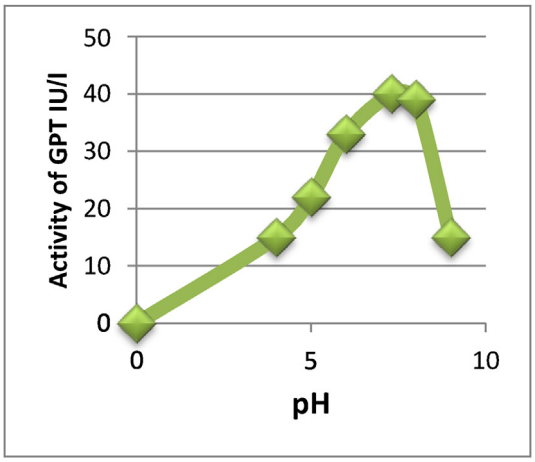

Fig. 3. Effect of pH on GPT activity.

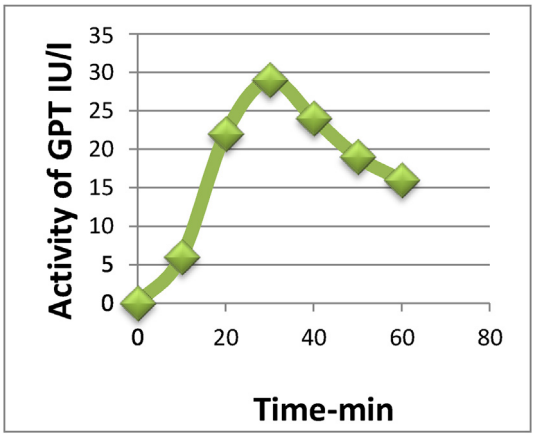

Fig. 4. Effect of Time on GPT activity at 25 °C and pH7.3.

3.1. Kinetic study of partial purified GPT

The effect of substrate concentration, pH, Time and temperature on the activity of purified GPT concluded the following results (all results were obtained in 25 °C, pH 7.3): Increased in the activity of GPT with increased substrate concentration to the maximum activity at (5) mmol Fig. 1. Velocity of enzyme versus substrate concentration is subjected to the equation of Michaels – Mentens equation and the curve was hyperbolic. The value of Km was 1.1 mM and Vmax was 33.9 IU/L. Increased in GPT activity with increasing temperature at the maximum degree of 37 °C Fig. 2. Increased in GPT activity with increasing pH with maximum velocity at pH 7.3 then began to decrease Fig. 3. Increase in the activity of enzyme with increasing time to maximum activity at 37 °C after a 40 min then the activity began to decrease Fig. 4. These results were in agreement with everaldo gon et al. [27], Fumio Mizutani et al. [28], J.-S. Shin et al. [29] and Akira Iwasaki et al. [30].

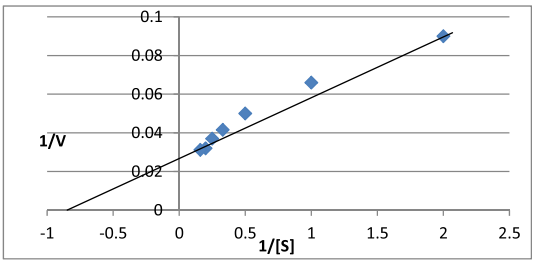

Fig. 5. Lineweaver-Burke plot.

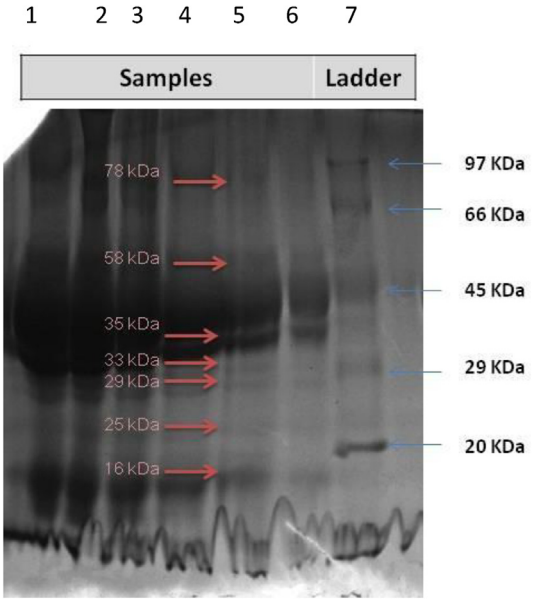

Fig. 6. The SDS-PAGE of GPT at different stages of purification: 1 and 2 crude protein,3-ammonium sulfate fraction (65%), 4- dialysis 5- Sephadex G100, 6- ion exchange chromatography, 7- 1, 5 proteins. Marker (molecular mass 97, 66, 45, 29, 20 kDa).

3.2. Determination of molecular weight of GPT

Molecular weight of enzyme was determined by SDS Polyacrylamide gel electrophoresis (PAGE) according to the literature. Fig. 6 showed the band of enzyme compared with standard molecular weight preparations. The molecular weight of GPT was found to be 58 KDa which was in agreement with [8]. Fig. 5 showed that the SDS-PAGE of GPT at different stages of purification: 1 and 2 crude protein,3-ammonium sulfate fraction (65%), 4- dialysis 5- Sephadex.

G100, 6- ion exchange, 7- 1, 5 proteins Marker (molecular mass 97, 66, 45, 29, 20 kDa).

3.3. Effect of the new inhibitors (1), (2) and (3) on the purified GPT activities

The effect of synthesized compounds (1), (2) and (3) on purified GPT activity in serum with the mean (±SD) was illustrated in Table 3.

The effect of the new compounds on the activity of the purified GPT *in vitro* of the body was tested. When increasing concentrations of the new compounds, Inhibition of the enzyme activity was increased. The activity of purified GPT was (34.68 ± 4.62U/L). The activity of purified GPT with the new compounds (Inhibitors) was ranged from

Table 3
The effect of compounds (1), (2) and (3) on purified GPT activity.

Concentrations	GPT Activity U/L Compounds 1	GPT Activity U/L Compounds 2	GPT Activity U/L Compounds 3
Normal/without any concentrations of compounds	34.68 ± 4.62.	34.68 ± 4.62	34.68 ± 4.62
0.5mg/25 ml	15.11 ± 1.19	12.22 ± 1.20	10.12 ± 1.30
0.5×10^{-1}mg/25 ml	17.23 ± 1.98	14.34 ± 1.31	12.24 ± 1.41
0.5×10^{-2}mg/25 ml	20.11 ± 1.78	17.44 ± 1.19	15.54 ± 1.2
0.5×10^{-3}mg/25 ml	23.43 ± 2.51	21.13 ± 2.42	16.33 ± 2.12
0.5×10^{-4}mg/25 ml	27.36 ± 2.18	23.36 ± 2.18	18.24 ± 2.44
0.5×10^{-5}mg/25 ml	30 ± 3.22	26.31 ± 3.11	23.31 ± 3.43
0.5×10^{-6}mg/25 ml	32.18 ± 4.61	28.68 ± 4.62	26.68 ± 4.57

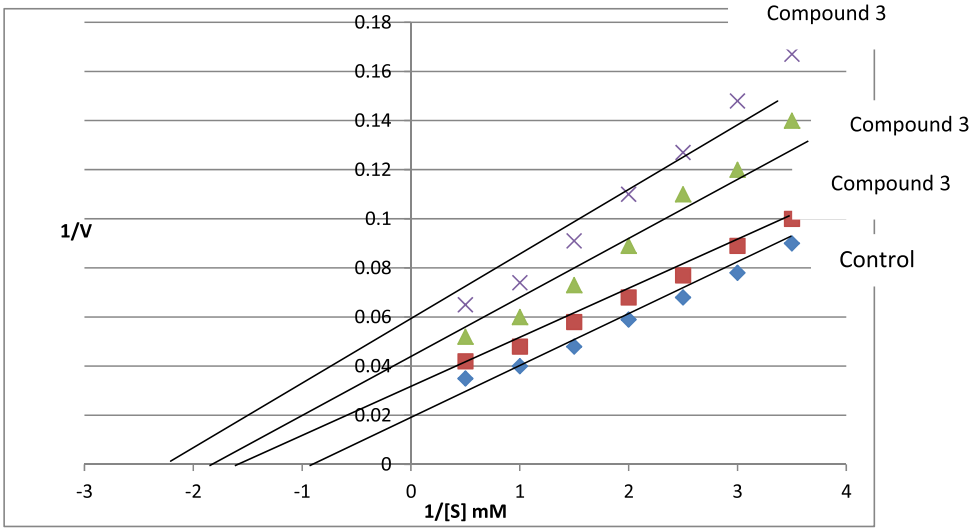

Fig. 7. Lineweaver-Burke plot with and without inhibitors (uncompetitive Inhibitors).

(15.11 ± 1.19–32.18 ± 4.61) U/L, (12.22 ± 1.20–28.68 ± 4.62) U/L and (10.12 ± 1.30–28.68 ± 4.62) U/L with compounds 1,2 and 3 respectively. In the concentration 0.5×10^{-5}mg/25 ml and 0.5×10^{-6}mg/25 ml it was found that the activity of the enzyme reached the normal value because of the multi dilutions of the concentration of compounds. Aspartate transaminase, as with all transaminases, operates via dual substrate recognition; that is, able to recognize and selectively bind two amino acids (Asp and Glu) with different side-chains In either case, the transaminase reaction consists of two similar half-reactions that constitute what is referred to as a ping-pong mechanism. In the first half-reaction, amino acid 1 (e.g., L-Asp) reacted with the enzyme-PLP complex to generate ketoacid 1 (oxaloacetate) and the modified enzyme-PMP. In the second half-reaction, ketoacid 2 (α-ketoglutarate) reacted with enzyme-PMP to produce amino acid 2 (L-Glu), regenerating the original enzyme-PLP in the process [31,32].

The effect of inhibition by compound 3 > compound 2 > compound 1 because compound 3 has more carbonyl groups in its structure compare to both compound (2) and compound 1. As a result, these carbonyl groups effect on the residual amino acid in the active site of GPT may transfer and Prevent the PLP(pyridoxal phosphate) reaction with enzyme (compounds 1,2 and 3 are similar structure with PLP). Synthesized compounds have functional group (ester groups) and the carbonyl group has dual electronic. The Lineweaver-Burke relation showed that the type of inhibition was un-competitive inhibition (Km value changed and the Vmaxi was less than Vmax observed in the absence of inhibitor) Fig. 7.

4. Conclusion

Results showed a highly significant increase ($p < 0.005$) in the serum levels of CK-MB, LDH, cholesterol, triglyceride, HDL-cholesterol, GOT,GPT, MDA in comparison with the control group and significant decrease in Alb,HDL-Cholesterol, Vitamin C and vitamin E in comparison with the control. No significant differences between gender (males and females) and no significant differences between ages. Results also showed that the new compounds had inhibition effect on the enzyme activity with new value of kinetic parameters been detected using Lineweaver-Burke plot and the type was un-competitive inhibition for the three derivatives which have been used in this study.

Authors' contributions

Firas taher maher designed the experiments and performed the experiments. Authors read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this articles.

Consent for publication

Applicable.

Ethics approval and consent to participate

Applicable.

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Competing interests

Applicable.

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