

Biochemical Variables and G6PD purification and activity in Viral Hepatitis A, B and C in Sample of Iraqi Patients

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Abstract

The results obtained from the measurement of some biochemical variables showed significant differences in patients with HCV in the analysis of the level of creatinine where the value of the mean \pm SD (1.35 \pm 0.52) mg/dl in (p<0.05), while no significant differences in the concentration of urea in (p<0.05), while significant differences found in the level of GPT, GOT, ALP, where the values of the mean \pm SD (179.35 \pm 74.65, 115.55 \pm 41.7, 245.70 \pm 167.46) U/L respectively and in the level of total Bilirubin, direct and indirect Bilirubin where the values of the mean \pm SD (13.64 \pm 10.13, 9.40 \pm 7.64, 4.23 \pm 2.74) mg/dl respectively and in the level of G6PD where the value of the mean \pm SD (7796.25 \pm 953.01) U/L in (p<0.05) in patients with HAV infected and in the purification of the enzyme G6PD for the four extracts showed a decrease in the activity of the enzyme for samples infected with hepatitis, especially for the enzyme extracted from the sample infected with HCV. The activity of enzymatic extracts control, HAV, HBV and HCV at the end of the purification process was (3.83, 3.709, 3.5 and 2.8) U/ml respectively.

Keywords : Glucose-6-phosphate dehydrogenase (G6PD), Hepatitis A, B, and C . purification .

1. INTRODUCTION

Glucose-6-phosphate dehydrogenase is a cytoplasmic enzyme widely distributed in all tissues and blood cells, and is one of the most important enzymes of metabolism as the first step of hexose monophosphate shunt⁽¹⁾⁽²⁾. It stimulates the first step in the pathway to oxidation of hexose monophosphate.⁽³⁾

Where this enzyme is working to oxidize a molecule glucose-6-phosphate (G-6-P) and converts it to a (6-Phosphogluconate- δ -lactone) molecule, and it reduce the electronic power transmission (NADP⁺) and converts it into a reduced form (NADPH)⁽⁴⁾ then the molecule(6-Phosphogluconate- δ -lactone) is degraded and Carboxylic group(-COOH) is remove from it to produce pentose monophosphate(D-Ribose-5-phosphate) needed to synthesize the DNA bar and cell growth as well as build the molecules of the : RNA, FAD⁺, NAD⁺, CoA and ATP.⁽⁵⁾

The production of a power carrier reduced by G6PD enzyme will protect cells against oxidative agents by the production of reduced glutathione (GSH)⁽⁶⁾. The NADPH also acts as an enzymatic assistant in the synthesis of many biomolecules such as fatty acids, steroids and some amino acids⁽⁷⁾. In the event of a defect in the production of the reduced power carrier (NADPH), this leads to a decrease in the concentration of reduced glutathione in the system and therefore the death of cells, and for this reason, G6PD can be defined as an antioxidant enzyme.⁽⁸⁾

Hepatitis is caused by viral infection and it may occur in a severe condition (recent infection, relatively rapid onset) or chronic forms, the liver functions firstly as a metabolic member and is in fixed exposure to dietary and microbial antigens, and this requires a state of relative immune tolerance, which evolutionarily has been exploited by pathogens such as hepatitis viruses, bacteria, and parasites.⁽⁹⁾

There are three main types of viral hepatitis are hepatitis A (HAV), B (HBV) and C (HCV), and all of which directly infect liver cells, causing a similar acute illness in patients with symptoms despite displaying different methods of infection and pathological levels.⁽¹⁰⁾

Viral hepatitis usually showed as a self-limiting disease. However, in the presence of G6PD deficiency, and the condition is more complicated with kidney failure, hemolysis, hepatic encephalopathy and may lead to death and a few cases of this type of hepatitis have described as involved by hepatitis B and hepatitis E.⁽¹¹⁾

Patients who infected with the hepatitis virus type A and they have a deficiency in the G6PD enzyme they have an acute hemolytic due to this infection, and the possible explanation was G6PD-deficient hepatocytes with reduced glutathione can't afford

viral insult cumulating free radicals resulting in delayed hepatocytes repair.⁽¹²⁾

Sundry reports have shown increased morbidity rate and fulminant clinical course of acute viral hepatitis when developing with underlying G6PD deficiency, and in the study of a number of patients with a lack of the enzyme and when they infected with hepatitis the type A and E it found that there is a significant increase in levels of bilirubin and prothrombin time compared with healthy persons⁽¹³⁾. The conditions of acute renal failure lead to hemodialysis secondary to severe intravascular hemolysis in hepatitis with preexisting G6PD deficiency have also been reported⁽¹⁴⁾. In another study, two children with a deficiency of the enzyme and autoimmune antibodies and have infected with hepatitis type A have developed into severe hemolytic anemia The condition of any of the patients has not evolved into renal failure or needed the blood transfusion, and there was no difference in hemoglobin level between patients with G6PD deficiency (7.2-14.8 gr/dl) and patients with normal G6PD level (7.6-15 gr/dl).⁽¹⁵⁾

Hepatitis C and B are working to increase the regulates of the G6PD⁽¹⁶⁾, and to find out the role and function of the G6PD enzyme in the hepatitis type B tissue microarray, quantitative real-time PCR, and western blot was performed to analyse the differences in G6PD expression levels in patients with hepatitis B and non-infected patients, HepG2.2.15 cells, and HepG2 cells, and the siRNA transfection technique was used to pick off G6PD gene in HepG2.2.15 cells for three days, Chemiluminescence was used to estimate HBV and HBeAg in floating matter, and real-time quantitative PCR was used to measure HBV DNA, type I interferon (IFN), and IFN-driven inducible genes and the t-test used for comparison between groups, and an increase was observed in the regulation of the G6PD enzyme in the tissues and cells of persons diagnosed with hepatitis compared to non-infected persons and the intensity of spots and immunological samples was 89.69 \pm 54.92 and 31.90 \pm 18.62, respectively (P <0.05), and after siRNA interfered G6PD expression in HepG2.2.15 cells, the quantitative levels of HBV DNA, HBV, and HBV in the supernatant reduced significantly, and the mRNA expression levels of IFN α 1, IFN β 1 and G6PD enzyme plays an essential role in the process of replication of hepatitis virus type B, and the working mechanism for the operation of copying or replicating a liver virus may be associated with type I IFN signaling pathway.⁽¹⁷⁾

Stimulation of pentose phosphate pathway by HBX up regulation of G6PD i may be explained by the fact that metabolic disorders such as the use of nucleoside isotopes can prevent the production

of hepatitis virus type B and on the other hand the primary role of HBx in HBV replication and it is easy to predict that the increased enzyme level due to the stimulation of the pentose cycle due to the virus. The host cell was able to use glucose for the bio-synthesis of large molecules to support its high-energy proliferation, which may be closely related to the development of HBV-associated HCC⁽¹⁸⁾. This study demonstrates increased G6PD secretion in HBV-associated HCC, which are well associated with the results that show that HBx excels G6PD in the cells of a transplanted liver, and as a primary initiator of the pentose phosphate pathway G6PD is highly regulated at copy level, and in many cases by the transcription factor SREBP.⁽¹⁹⁾

Aims of study

- 1- Determining the incidence and severity of blood degradation in patients with infectious hepatitis.
- 2- Know the extent of the effect of viral hepatitis at the level of the G6PD.
- 3- Measuring the activity of the G6PD enzyme quantitatively and qualitatively in Iraq.
- 4- Purification and labeling of G6PD in known purification methods.

2. METHODS

Sampling:

The study conducted between March and June in 2018 where samples were collected at the Gastroenterology and Liver Hospital by taking samples of patients with hepatitis A, B and C. The study included infected persons aged 10-40 years and diagnosed by the competent physician and not taking any treatment for infection where the number of samples was that collected 112, and five samples excluded for multiple reasons, and the number of samples became under study 100, with 67 males and 33 females, where part of the sample put in the gel tube, and its separation to get the serum of the sample to perform some tests that require it, and the rest of them put in the EDTA tube and the tubes placed in a closed, heat-insulated container containing ice so that the snow touches the entire surface of the tubes until they transferred to the laboratory. In the laboratory, the samples were carried out as soon as possible and in all cases 36 hours before the sampling time.

Estimation of G6PD activity:

The activity of the G6PD enzyme measured by the method used in the kit that was supplied by Randox by UV spectrum.

Biochemical Tests:

The activity of GPT, GOT, ALP, Bilirubin Total, direct & indirect, urea and creatinine enzymes measured by the method used in the kit that was supplied by Mindray by using Mindray BS 330.

Purification of G6PD enzyme

The enzyme purification of the healthy sample and samples of hepatitis A, B and C were performed by deposition of the enzyme using ammonium sulphate with concentrations of 20% -90% . The process of dialysis was then performed for enzymatic extracts and the ion exchange was then carried by using the carboxy methyl cellulose column. A process was then carried out Gel filtration chromatography by using the Sephacryl-S300 gel filter Supplied by sigma Aldrich Company. The process of assigning purity and estimation of the molecular weight of the enzyme was then carried out by the electrophoresis process using the Ladder solution, which contains standard proteins according to the following molecular weights: 10, 15, 25, 35, 50, 75, 100, 150, 225 KDa.^{(20) (21)}

3. RESULTS AND DISCUSSION

Biochemical study results.

A total of 100 samples were collected, with 40 samples of control and 20 samples with HAV and 20 samples with HBV and 20 samples with HCV. The results were taken and counted using SPSS version 22 as showing in the following.

The results of the present study showed that there was a difference in the mean values of creat between the group of patients with hepatitis type A,B,C and the control group. The values of the means \pm SD (0.60 \pm 0.21 , 0.78 \pm 0.21 , 1.35 \pm 0.52 , 0.76 \pm 0.15) mg/dl respectively, as shown in figure (3-1). There was an increase significant in creat values in HCV patients (p<0.05). In addition, non-significant differences between HAV, HBV patients and control groups(p<0.05).

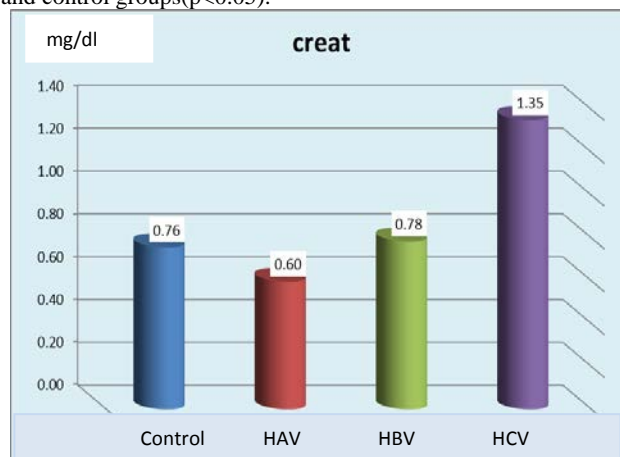


Fig 3-1 : level of creat in patients with HAV, HBV , HCV and control groups.

These results varied slightly with those obtained when calculating the level of creatinine in patients with HAV⁽²²⁾, where the value of the mean \pm SD (1.93 \pm 2.26). In another study of the same type for patients under the age of 28 years, the values of mean \pm SD (0.9 \pm 0.5), and in the same study, patients who were older than 28 years had the values of mean \pm SD (0.7 \pm 0.3)⁽²³⁾. In a study of patients⁽²⁴⁾, with hepatitis type B values were close to what was reached where the value of mean \pm SD (0.7 \pm 0.37), and in another study the values of mean \pm SD of patients with co-infected HBV and HCV is (0.72 \pm 0.61)⁽²⁵⁾. While there was a marked variation in the level of creatinine in the obtained values reached in another research on patients with HCV genotype 4⁽²⁶⁾, where the values of mean \pm SD (3.06 \pm 0.86), while the values of the mean \pm SD in the other research by a small percentage (0.78 \pm 0.154)⁽²⁷⁾. In another study, patients with hepatitis C genotype 1 who were over 65 years of age had the values of mean \pm SD (0.76 \pm 0.18).⁽²⁸⁾

The results of the present study showed that there was a difference in the mean values of urea between the group of patients with hepatitis type A,B,C and the control group. The values of the means \pm SD (32.27 \pm 5.99 , 33.76 \pm 6.83 , 37.41 \pm 25.76 , 32.18 \pm 5.97) mg/dl respectively, as shown in figure (3-2). There was an increase non-significant in urea values in patients and control group (p<0.05).

These results varied slightly with those obtained when calculating the level of urea in patients with HAV⁽²⁹⁾, where the value of the mean \pm SD (25.4 \pm 8.9). In a study of patients, with hepatitis type B values were close to what was the values of mean \pm SD (23.63 \pm 16.80), and in the same study the values of mean \pm SD of patients with co-infected HBV and HCV is (23.63 \pm 8.44)⁽²⁵⁾. While there was a marked variation in the level of urea in the obtained values reached in another research on patients with HCV, where the values of mean \pm SD (87.38 \pm 25.22).⁽²⁷⁾

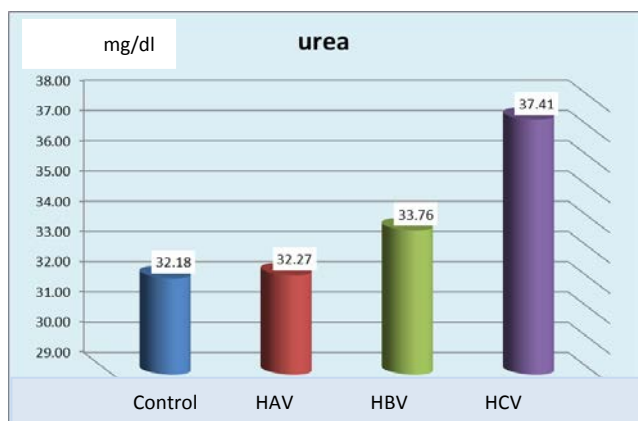


Fig 3-2 : level of urea in patients with HAV, HBV , HCV and control groups.

The results of the present study showed that there was a difference in the mean values of ALP between the group of patients with hepatitis type A,B,C and the control group. The values of the means \pm SD (245.70 \pm 167.46 , 118.25 \pm 35.62, 107.00 \pm 49.21, 108.49 \pm 46.82) U/L respectively, as shown in figure (3-3). There was an increase significant in ALP values in HAV patients ($p < 0.05$). In addition, non-significant differences between HCV, HBV patients and control groups ($p < 0.05$).

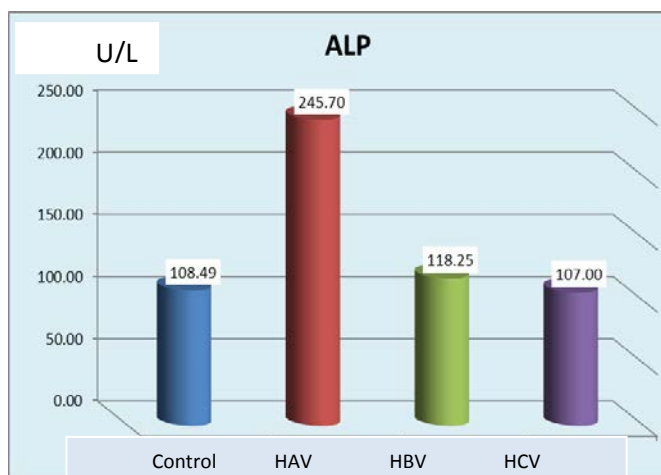


Fig 3-3 : level of ALP in patients with HAV, HBV , HCV and control groups.

These results varied slightly with those obtained when calculating the level of ALP in patients with HAV⁽³⁰⁾, where the value of the mean \pm SD (291 \pm 125). In another study, it observed that there was a significant difference in the values of the mean \pm SD with what obtained where the value of the mean \pm SD (817 \pm 439)⁽²⁹⁾ . In another study, it observes that there was a slight variation in the values of mean \pm SD with what obtained, where the value of mean \pm SD (318.3 \pm 167.0)⁽³¹⁾. In a study of patients with co-infection (HIV/HBV) values were close to what was reached where the value of mean \pm SD (92.51 \pm 25.91)⁽³²⁾, and in another study with same co-infection the value of mean \pm SD (262 \pm 118)⁽³³⁾, and in another study of patients with hepatitis type B the values of mean \pm SD (90.42 \pm 43.65)⁽³⁴⁾, and in another study the values of mean \pm SD (286.98 \pm 139.17) and in the same study the values of mean \pm SD of patients with co-infected HBV and HCV is (344.47 \pm 187.05)⁽²⁵⁾. These results varied slightly with those obtained when calculating the level of ALP in patients with HCV⁽²⁷⁾, where the values of mean \pm SD (109 \pm 43), while the values of the mean \pm SD in the other research by a small percentage (73.70 \pm 25.10)⁽³⁵⁾. In another study, patients with

hepatitis C genotype 1 the values of mean \pm SD (692.3 \pm 487.7).⁽³⁶⁾

The results of the present study showed that there was a difference in the mean values of GPT between the group of patients with hepatitis type A,B,C and the control group. The values of the means \pm SD (179.35 \pm 74.65 , 49.70 \pm 72.99 , 43.80 \pm 36.93 , 28.46 \pm 10.07) U/L respectively, as shown in figure (3-4). There was an increase significant in GPT values in HAV patients ($p < 0.05$). In addition, non-significant differences between HCV, HBV patients and control groups ($p < 0.05$).

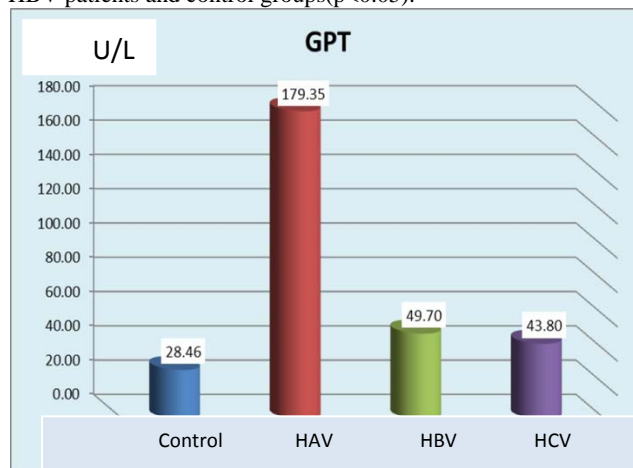


Fig 3-4 : level of GPT in patients with HAV, HBV , HCV and control groups.

These results varied slightly with those obtained when calculating the level of GPT in patients with HAV⁽³⁰⁾, where the value of the mean \pm SD (507 \pm 445). In another study, it observed that there was a significant difference in the values of the mean \pm SD with what obtained where the value of the mean \pm SD (1,172 \pm 743)⁽²⁹⁾ . In another study, it observes that there was a slight variation in the values of mean \pm SD with what obtained, where the value of mean \pm SD (514.7 \pm 394.1)⁽³¹⁾. In a study of patients with co-infection (HIV/HBV) values were close to what was reached where the value of mean \pm SD (36.10 \pm 45.55)⁽³²⁾, and in another study with HBV infection the value of mean \pm SD (23.97 \pm 7.54)⁽³⁷⁾, and in another study of patients with hepatitis type B the values of mean \pm SD (33.3 \pm 5.7)⁽³⁴⁾, and in another study the values of mean \pm SD of patients with co-infected HBV and HCV is (21.61 \pm 10.14)⁽²⁵⁾. These results varied slightly with those obtained when calculating the level of GPT in patients with HCV⁽²⁷⁾, where the values of mean \pm SD (90.7 \pm 53), while the values of the mean \pm SD in the other research by a small percentage (50.10 \pm 25.80)⁽³⁵⁾. In another study, patients with hepatitis C genotype 1 the values of mean \pm SD (274.0 \pm 230.1)⁽³⁶⁾. and in another study the values of mean \pm SD (40.91 \pm 31.81)⁽²⁶⁾ . In another study, patients with hepatitis C genotype 1 who were over 65 years of age had the values of mean \pm SD (87.6 \pm 55.3).⁽²⁸⁾ The results of the present study showed that there was a difference in the mean values of GOT between the group of patients with hepatitis type A,B,C and the control group. The values of the means \pm SD (115.55 \pm 41.7 , 36.95 \pm 27.20 , 42.65 \pm 33.45 , 24.93 \pm 5.49) U/L respectively, as shown in figure (3-5). There was an increase significant in GOT values in HAV patients ($p < 0.05$). In addition, non-significant differences between HCV, HBV patients and control groups ($p < 0.05$). These results varied slightly with those obtained when calculating the level of GOT in patients with HAV⁽²³⁾, where the value of the mean \pm SD (372.3 \pm 337.5). In another study, it observed that there was a significant difference in the values of the mean \pm SD with what obtained where the value of the mean \pm SD (1,053 \pm 809)⁽²⁹⁾ .

In another study, it observes that there was a slight variation in the values of mean±SD with what obtained, where the value of mean±SD (130.3±62.2) ⁽²³⁾. In a study of patients with co-infection (HIV/HBV) values were close to what was reached where the value of mean±SD (28.74±16.84) ⁽³²⁾, and in another study with HBV infection the value of mean±SD (21.75 ± 10.54) ⁽³⁷⁾, and in another study of patients with hepatitis type B the values of mean±SD (39.7 ± 8.5) ⁽³⁴⁾, and in another study with the same infection the value of mean±SD (47.9 ± 32.3) ⁽³⁸⁾. and in another study the values of mean±SD (28.06 ± 14.13) and in the same study the values of mean±SD of patients with co-infected HBV and HCV is (35.95 ± 20.44) ⁽²⁵⁾. These results varied slightly with those obtained when calculating the level of GOT in patients with HCV ⁽²⁷⁾, where the values of mean±SD (101±53), while the values of the mean±SD in the other research by a small percentage (35.4±24.5) ⁽³⁵⁾. In another study, patients with hepatitis C genotype 1 the values of mean±SD (261.3 ± 203.8), and in the same study with genotype 3 the values of mean±SD (217.3 ± 191.6) ⁽³⁶⁾, and in another study the values of mean±SD (36.02 ± 25.90) ⁽²⁶⁾.

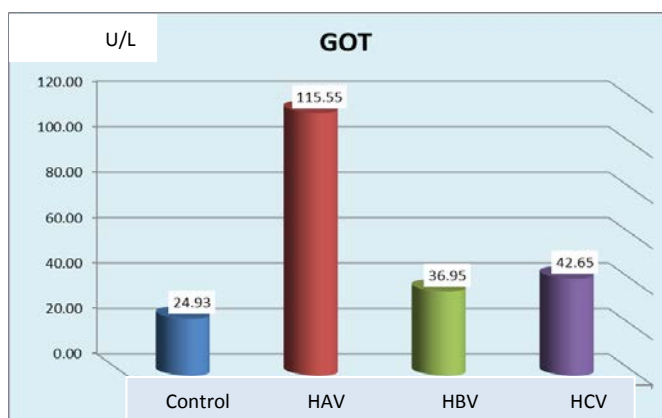


Fig 3-4 : level of GOT in patients with HAV, HBV , HCV and control groups.

The results of the present study showed that there was a difference in the mean values of IND Bilirubin between the group of patients with hepatitis type A,B,C and the control group. The values of the means ±SD (4.23± 2.74 , 0.57± 0.67 , 0.41± 0.28 , 0.54± 0.20) mg/dl respectively, as shown in figure (3-6). There was an increase significant in IND Bilirubin values in HAV patients (p<0.05). In addition, non-significant differences between HCV, HBV patients and control groups(p<0.05).

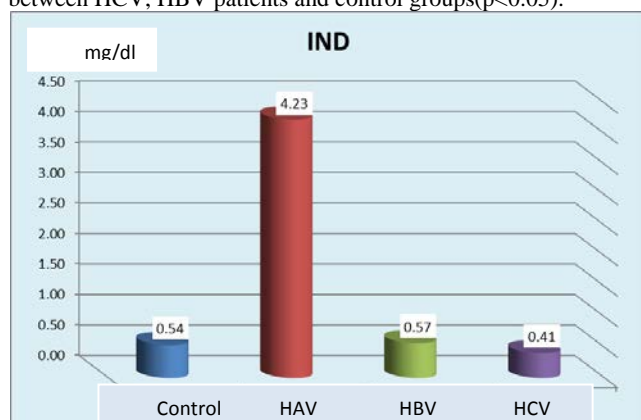


Fig 3-6 : level of IND Bilirubin in patients with HAV, HBV , HCV and control groups.

These results varied slightly with those obtained when calculating the level of Indirect Bilirubin in patients with HAV ⁽³⁹⁾, where the value of the mean±SD (6.75± 3.41). In another study, it observed

that there was a significant difference in the values of the mean±SD with what obtained where the value of the mean±SD (24.87 ± 8.84) ⁽²²⁾. In a study of patients with HBV infection the value of mean±SD (0.66 ± 0.30) ⁽⁴⁰⁾. while in patients with HCV the values of the mean±SD in the other research by a small percentage (0.44 ± 0.032). ⁽⁴¹⁾

The results of the present study showed that there was a difference in the mean values of Dir Bilirubin between the group of patients with hepatitis type A,B,C and the control group. The values of the means ±SD (9.40± 7.64 , 0.23± 0.12 , 0.33± 0.49 , 0.22± 0.07) mg/dl respectively, as shown in figure (3-7). There was an increase significant in IND Bilirubin values in HAV patients (p<0.05). In addition, non-significant differences between HCV, HBV patients and control groups(p<0.05).

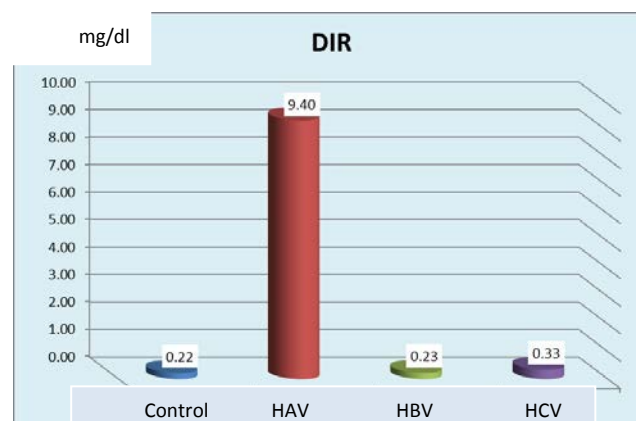


Fig 3-7 : level of Dir Bilirubin in patients with HAV, HBV , HCV and control groups.

These results varied slightly with those obtained when calculating the level of Dir Bilirubin in patients with HAV, where the value of the mean±SD (2.8 ± 1.5) ⁽⁴²⁾. In another study, it observed that there was a significant difference in the values of the mean±SD with what obtained where the value of the mean±SD (21.26 ± 14.51) ⁽³⁹⁾. In another study, it observes that there was a slight variation in the values of mean±SD with what obtained, where the value of mean±SD (3.7 ± 2.4) ⁽²⁹⁾. In a study of patients with co-infection (HIV/HBV) values were close to what was reached where the value of mean±SD (28.74±16.84) ⁽³²⁾, and in another study with HBV infection the value of mean±SD (21.75 ± 10.54) ⁽³⁷⁾, and in another study of patients with hepatitis type B the values of mean±SD (39.7 ± 8.5) ⁽³⁴⁾, and in another study with the same infection the value of mean±SD (47.9 ± 32.3) ⁽³⁸⁾. and in another study the values of mean±SD (28.06 ± 14.13) and in the same study the values of mean±SD of patients with co-infected HBV and HCV is (35.95 ± 20.44) ⁽²⁵⁾. These results varied slightly with those obtained when calculating the level of Dir Bilirubin in patients with HCV, where the values of mean±SD (0.07 ± 0.04) ⁽²⁷⁾, while the values of the mean±SD in the other research by a small percentage (0.23±.11) ⁽⁴³⁾. In another study, patients with hepatitis C the values of mean±SD (0.3 ± 0.02). ⁽⁴¹⁾

The results of the present study showed that there was a difference in the mean values of total bilirubin between the group of patients with hepatitis type A,B,C and the control group. The values of the means ±SD (13.64± 10.13 , 0.79± 0.76 , 0.74± 0.67 , 0.76± 0.24) mg/dl respectively, as shown in figure (3-8). There was an increase significant in total bilirubin values in HAV patients (p<0.05). In addition, non-significant differences between HCV, HBV patients and control groups(p<0.05).

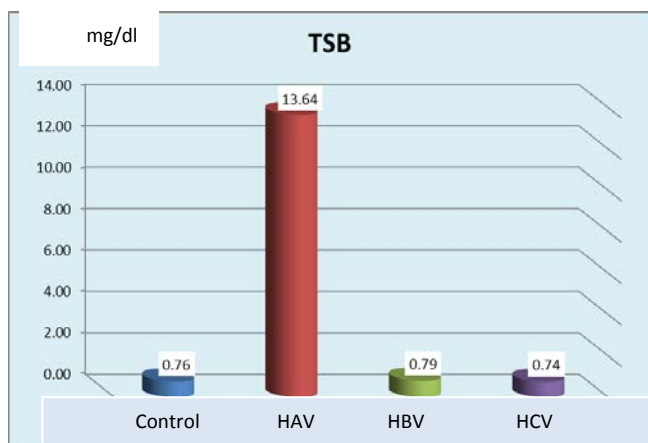


Fig 3-8 : level of total bilirubin in patients with HAV, HBV , HCV and control groups.

These results varied slightly with those obtained when calculating the level of Total Bilirubin in patients with HAV⁽³⁰⁾, where the value of the mean±SD (6.57 ± 3.7). In another study , it observed that there was a significant difference in the values of the mean±SD with what obtained where the value of the mean±SD (28.01± 7.25)⁽³⁹⁾ . In another study, it observes that there was a slight variation in the values of mean±SD with what obtained, where the value of mean±SD (5.1 ± 3.1)⁽²⁹⁾, and in another study the value of mean±SD(3.6± 2.36)⁽³⁰⁾ . In a study of patients with hepatitis type B the values of mean±SD (0.86 ± 0.36)⁽⁴¹⁾, and in another study the values of mean±SD (0.25± 0.18)⁽⁴²⁾, In a study of patients with HBV the values of mean±SD (0.73 ± 0.16)⁽³⁴⁾, and in another study the values of mean±SD (0.95 ± 4.70), and in the same study the values of mean±SD of patients with co-infected HBV and HCV is (0.72 ± 0.61)⁽²⁵⁾. These results varied slightly with those obtained when calculating the level of Total Bilirubin in patients with HCV⁽²⁷⁾, where the values of mean±SD (0.24 ± 0.10), while the values of the mean±SD in the other research by a small percentage (0.7 ± 0.2)⁽³⁵⁾. In another study, patients with hepatitis C the values of mean±SD (0.9±0.3)⁽⁴³⁾, in another study, patients with hepatitis C genotype 1 who were over 65 years of age had the values of mean±SD(1.12 ±0.59).⁽²⁸⁾

The results of the present study showed that there was a difference in the mean values of G6PD between the group of patients with hepatitis type A,B,C and the control group. The values of the means ±SD (7796.25 ± 953.01 , 6394.50 ± 670.08 , 6612.80 ± 1861.14 , 6940.44 ± 733.35) U/L respectively, as shown in figure (3-9). There was an increase significant in G6PD values in HAV patients (p<0.05). In addition, non-significant differences between HCV, HBV patients and control groups(p<0.05).

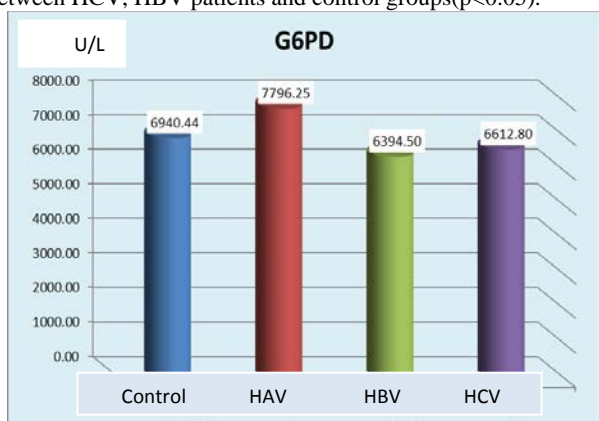


Fig 3-9 : level of G6PD in patients with HAV, HBV , HCV and control groups.

These results varied slightly with those obtained when calculating the level of G6PD in patients with HAV⁽⁴⁴⁾, where the value of mean±SD (6834±351). In another study, it observes that there was a slight variation in the values of mean±SD with what obtained, where the value of mean±SD (7123 ± 340)⁽⁴⁵⁾. In a study of patients with hepatitis type B the values of mean±SD (6570 ± 263)⁽⁴⁶⁾. These results varied slightly with those obtained when calculating the level of G6PD in patients with HCV, where the values of mean±SD (6783 ± 257).⁽⁴⁷⁾

G6PD Enzyme Purification Technique Concentration Using Ammonium Sulphate

Figure (3-10) and figure (3-11) shows the gradual precipitation for G6PD Enzyme from the crude enzyme extract of the control, A, B, and C sample. This control sample has the highest activity and is infected with hepatitis type A, B and C. The used saturation percentages of ammonium sulphate salts are ranged from 20% - 80%. The saturation percentages were calculated according to special table of saturation percentages for the ammonium sulphate salt. The figure shows a noticeable and gradual rise for the enzymatic specific activity in the resulted precipitate. Also we can notice that there is an obvious decrease in the enzyme specific activity of the resulted filtrate until reaching a saturation percentage of 60%.

This step showed a specific activity of 0.25 unit/mg with filtration of 1.4 times and enzymatic yield of 10% when using saturation percentages of 40%-80%. In the sample infected with hepatitis type A, this method showed a specific activity of 0.238 unit/mg with purification 1.32 times and enzymatic yield of 10% when using the same saturation percentages. While in the sample infected with hepatitis type B, the specific activity was 0.252 unit/mg using purification 1.3 times and the enzymatic yield 10.3% using the same saturation percentages. In the sample of hepatitis type C this method showed a specific activity of 0.24 unit/mg and purification 1.26 times and enzymatic yield 10% when using the same saturation percentages. The characteristics of this method is the elimination of G6PD enzyme of many effects that affects its activity such as hemoglobin and other enzymes and by using this method we can get a homogenous mixture of enzyme and this enables us from studying the enzyme after the purification. This method is considered the main idea in enzymes concentration and is summarized by the balancing of the charges on the protein surface and make a disruption in the aquatic layer around the protein and minimize the hydrolysis degree and reduce the protein solubility and precipitate it.⁽⁴⁸⁾

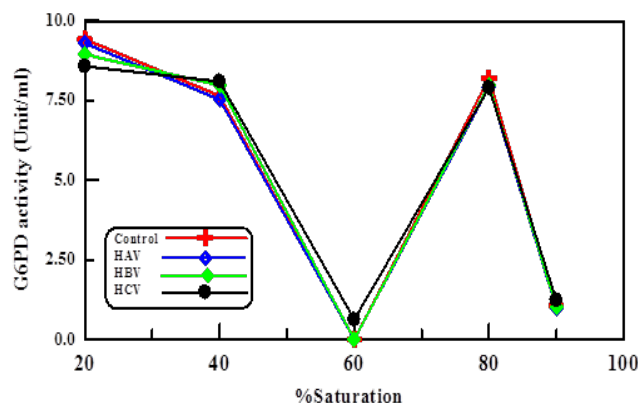


Figure (3-10) : the gradual filtration for G6PD enzyme from the crude enzyme extract of the control, A, B, and C sample

The concentration of ammonium sulphate salt is considered a very important method and is widely used in many previous studies. It should be applied before the next purification steps and this is due to the positive characteristics of it. The special feature of this

method is the reduction in the size of the enzymatic yield and the elimination of a great amount of proteins and water and increasing in purification efficiency. Also it is very useful to know the electrical dynamics and many other characteristics. The temperature was kept constant at 4°C in each step of the purification process⁽⁴⁹⁾. The significant feature of the ammonium sulphate salt is its high solubility in water and it has no negative effects on the enzyme structure and hence its efficiency without any damage to the protein content in general and this salt is available and cheap⁽⁵⁰⁾. This salt is added gradually in several steps in order to get rid of some proteins that are available with the enzymatic extract and increasing the salt concentration before using the appropriate concentration that is necessary for attaining the complete enzyme precipitation.⁽⁵¹⁾

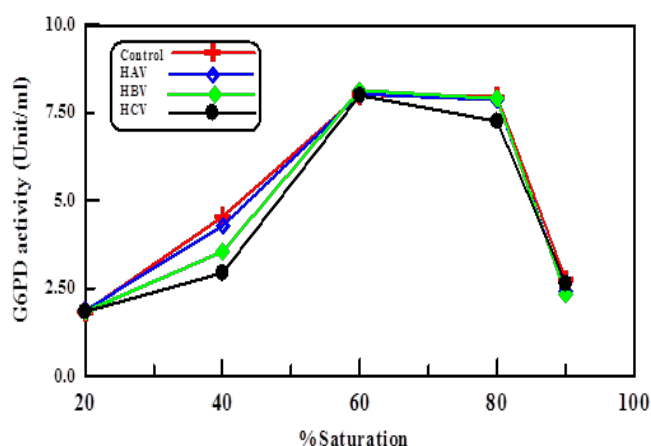


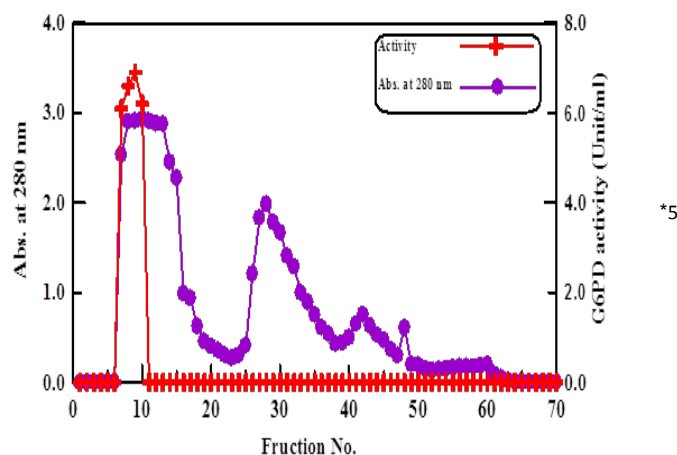
Figure (3-11) : the gradual precipitation for G6PD enzyme from the crude enzyme extract of the control, A, B, and C sample

The results were different from that in⁽⁵²⁾ when using the purification of G6PD enzyme from the lens of sheep's eyes. The addition of ammonium sulphate salts with saturation percentages of 0%-30% to precipitate the enzyme resulted in quantitative effectiveness of 0.017 unit/mg with purification 22 times and enzymatic yield 88.2%. In another study 1.251 unit/mg quantitative effectiveness and 53.8% enzymatic yield was obtained when using ammonium sulphate with saturation percentages ranged from 35%-65% to precipitate the G6PD enzyme from the human red blood cells⁽⁵³⁾. Other studies showed that the quantitative effectiveness is 0.370 unit/mg and enzymatic yield 79.18% after the addition of ammonium sulphate salt with saturation percentage of 40%-60% for the G6PD enzyme that is extracted from the red blood cells of geese⁽⁵⁴⁾. While for the G6PD precipitation from the adrenal gland of cows with saturation percentages 40% - 50% by using the ammonium sulphate salt. The results were as follows: quantitative effectiveness 13.30 unit/mg and 2.3 purification times, enzymatic yield 70.9%⁽⁵⁵⁾. In colored salmon fishes the G6PD enzyme was concentrated and precipitated using the ammonium sulphate salt with saturation percentages of 40%-65% and the resulting quantitative effectiveness is 0.044 unit/mg and purification 3.92 times and enzymatic yield 72.78%⁽⁵⁶⁾. For the enzyme extracted from the local isolate of *Saccharomyces cerevisiae* yeast by using the ammonium sulphate salt with saturation percentages of 40%-80%. The results are quantitative effectiveness 36.09 unit/mg and purification 1.62 times and enzymatic yield 82.56%⁽⁵⁷⁾. And for the precipitation of G6PD enzyme from the human red blood cells by using the ammonium sulphate salt with saturation percentages of 35%-65% and this

method resulted in quantitative effectiveness 0.882 unit/mg and enzymatic yield 39.4% and purification of 0.95 times.⁽⁵⁸⁾

Ion Exchange and Gel Filtration

The ion exchange (CM-Cellulose) was used in the purification of G6PD enzyme by applying the purification process for the enzymatic yield. It has been noticed that when using the negative ion exchange the appearance of one protein peak as shown in figure (3-12), (3-13), (3-14) and (3-15). This peak presents the activity in the washing stage. And also it has been noticed that there is an appearance of multiple protein peaks in the recovering stage. These proteins were recovered by the use of the linear salt gradient for the sodium chloride with concentration of (0-1) mol and this method showed a specific activity of 0.48 unit/mg and purification times 2.69 and enzymatic yield of 9.47% for the control sample. While in the sample infected with hepatitis type A, the specific activity is 0.184 unit/mg and purification times 1.02 and enzymatic yield 8.2%. The results for the sample infected with hepatitis type B, the results are as follows: specific activity 0.16 unit/mg, purification times 0.8, and enzymatic yield 6.7%. The specific activity is 0.172 unit/mg for the sample that is infected with hepatitis type C, and the purification times are 0.8 times with enzymatic yield 4.43%. 10.81%.



Figure(3-12) :Ion exchange chromatography for the purification of the G6PD enzyme from the human red blood cells using the CM-Cellulose ion exchange column for control

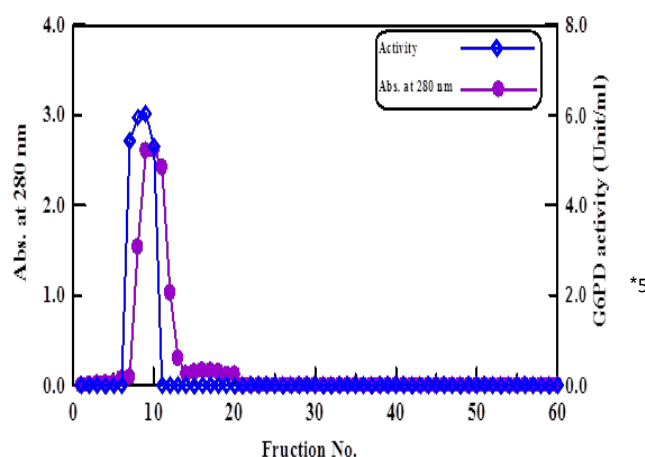
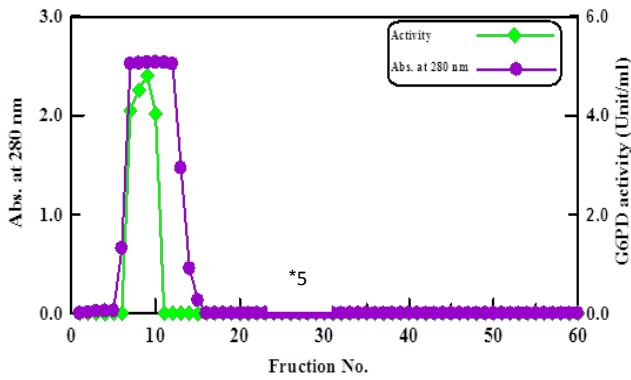
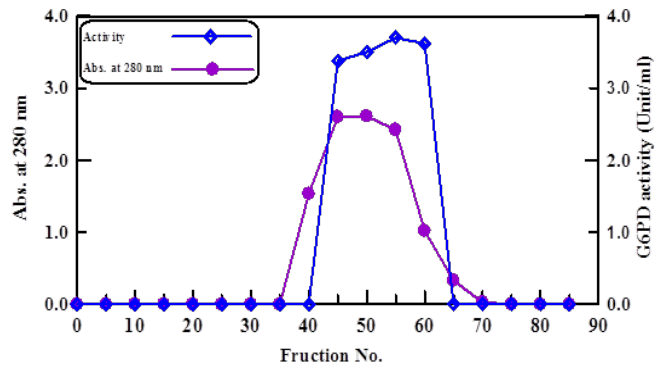


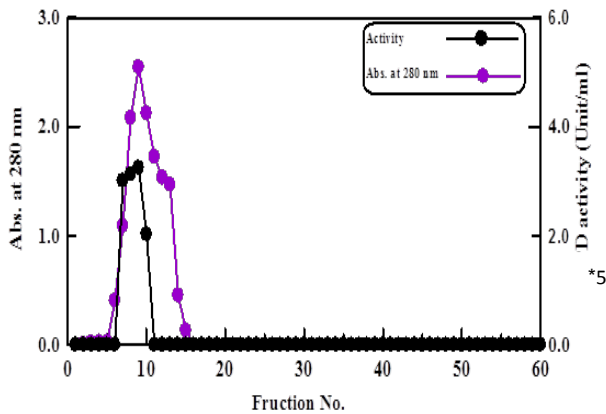
Figure (3-13) :Ion exchange chromatography for the purification of the G6PD enzyme from the human red blood cells using the CM-Cellulose ion exchange column for HAV



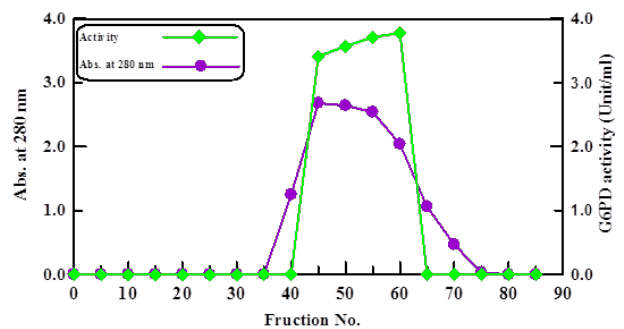
Figure(3-14) : Ion exchange chromatography for the purification of the G6PD enzyme from the human red blood cells using the CM-Cellulose ion exchange column for HBV



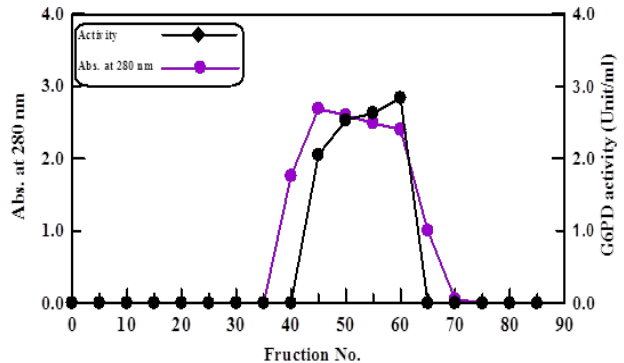
Figure(3-17) : Gel filtration chromatography for the purification of the G6PD enzyme from the human red blood cells using the CM-Cellulose ion exchange column for HAV



Figure(3-15) : Ion exchange chromatography for the purification of the G6PD enzyme from the human red blood cells using the CM-Cellulose ion exchange column for HCV

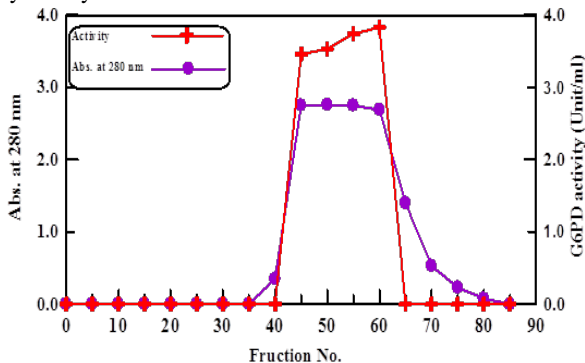


Figure(3-18) : Gel filtration chromatography for the purification of the G6PD enzyme from the human red blood cells using the CM-Cellulose ion exchange column for HBV



Figure(3-19) : Gel filtration chromatography for the purification of the G6PD enzyme from the human red blood cells using the CM-Cellulose ion exchange column for HCV

After all these steps, the concentrated enzymatic solution is added by PEG-1000 to the to the gel filtration Sephacryl S-300. It can be seen from figures (3-16), (3-17), (3-18) and (3-19) the appearance of a protein peak that exactly matches the enzymatic activity peak. This method resulted in specific activity of 0.25 unit/mg with purification times 1.4 times and enzymatic yield of 4.21% for the control sample. For the sample that is infected with hepatitis type A the specific activity is 0.19 unit/mg and purification times 1.06 and enzymatic yield of 4.6%. The specific activity for the sample that is infected with hepatitis type B is 0.13 unit/mg and purification times 0.72 and enzymatic yield 4.43% while for the sample that is infected with hepatitis type C the specific activity is 0.16 unit/mg and purification times 0.84 and enzymatic yield 3.5%.



Figure(3-16) : Gel filtration chromatography for the purification of the G6PD enzyme from the human red blood cells using the CM-Cellulose ion exchange column for control

Table (3-1), (3-2), (3-3) and (3-4) shows the steps for the purification process of the G6PD enzyme from the human red blood cells in the control, HAV, HBV and HCV units by using the ion exchange (column) CM-Cellulose. There is an obvious decrease in the samples that are infected with hepatitis and especially those of type C and this is because of the greater damage to the liver, taking into account that the level is responsible for producing this enzyme and this great damage to the producing source of the enzyme resulted in the lack of resistance to the applied conditions. Although there are many methods to purify the G6PD enzyme from the different sources such as vegetative, animal or microbial sources. All these methods participated in the extraction of G6PD enzyme with high purity. This ion exchange was used because it has many important characteristics such as high range of separation and the ease of

preparation and availability in addition to the possibility of multiple use after reactivating it⁽⁵⁹⁾. The results were varied with those found in the purification of G6PD from the human red blood cells using the sulphate salt and the ion exchange APD Sepharose 2.5. An enzymatic yield of 28% has been⁽⁵³⁾. In a similar study for the purification of G6PD from the human red blood cells using saturation percentages ranged from 35%-65% and after that using the ion exchange APD Sepharose 2.5. This step resulted in the obtaining of specific activity of 50 unit/mg and enzymatic yield of 34.2% and purification times 5435⁽⁵³⁾. It has been found that when using the sulphate salt with saturation percentage from 0%-30% and using the ion exchange APD Sepharose 2.5 it showed specific activity of 7.6 unit/mg with purification times of 10,000 times and enzymatic yield of 83.8% in the enzyme purified from the sheep's iris⁽⁵²⁾. In⁽⁵⁵⁾ an enzymatic yield of 19.9%, specific activity 326 unit/mg and purification times of 2.5, these results belong to the G6PD enzyme extracted from the Adrenal Gland of cows. The steps included the use of sulphate salt and the use of DEAE Sephadex column and then the gel filtration column Sephadex G-200. In another similar study on the colored salmon fishes that the purification process yielded a specific activity of 14.51 unit/mg and purification times 906 with an enzymatic yield of 70.38% when purifying G6PD enzyme using the concentration by the sulphate salt and the ion exchange column by APD Sepharose 2.5.⁽⁵⁶⁾

In a study applied by⁽⁵⁷⁾ about the G6PD enzyme that is extracted from local isolate of *Saccharomyces cerevisiae* yeast by using the ammonium sulphate salt with concentration of 40%-80% and then the use of ion exchange CM-Cellulose and after that the use of ion

exchange DEAE Cellulose in addition to the gel filtration Sephacryl S-200. The last step of purification showed a specific activity of 65.68 unit/mg with purification times 2.94 and enzymatic yield 20.62%. While the G6PD enzyme purified from the *Leuconostoc mesenteroides* using the sulphate salt and two mediums Matrex Gel Purple A and Matrex Gel Orange B a specific activity of 715 unit/mg is obtained in addition to an enzymatic yield of 45.9%.⁽⁶⁰⁾

When using several steps for the purification of G6PD from *Neurospora crassa* included passing the concentrated enzymatic yield in the column of calcium phosphate and then passing the yield in the ion exchange DEAE-Cellulose with pH 7.4. Then using the Hydroxyapatite column and double DEAE-Cellulose column with pH 8.3 and then applying the gel filtration process with Bio-Gel A-150 column. These steps showed specific activity of 470 unit/mg and purification times 2.42 and enzymatic yield of 10%⁽⁶¹⁾. The separation using the ion exchange is considered an early step and the common point in the enzyme purification and that is because those ion exchange have high separation and for the related proteins. The main idea of the separation process is based on the difference of protein charges at certain pH values based on the Isoelectric point for these proteins in addition to that, it could be implemented in commercial prospects⁽⁵⁹⁾. In a study for the purification of G6PD from the Coriander leaves⁽⁶²⁾, the purification process was at two steps: first step is the addition of ammonium salt and then the use of DEAE-Sephadex A50 column and then the gel filtration column Sephadex G-200 the results were : specific activity 1.82 unit/mg and enzymatic yield 26.4% and purification times 74.

Table (3-1): Purification of the enzyme G6PD extracted from human red blood cells-Control

No	Purification step	Volume (ml)	Enzyme Activity (U/ml)	Protein Con (mg/ml)	Specific Activity (U/mg)	Total Activity Unit	Purification factor	Yields %
1	crude enzymatic extract	8	8.012	45	0.178	64.09	1	7.9
2	Concentration with ammonium sulphate (20-80%) and then dialysis	10	8.015	31.5	0.25	80.15	1.4	10
3	Ion exchange	11	6.9	14.1	0.48	75.9	2.69	9.47
4	Gel filtration	10	3.83	15	0.25	33.8	1.4	4.21

Table (3-2): Purification of the enzyme G6PD extracted from human red blood cells- HAV

No	Purification step	Volume (ml)	Enzyme Activity (U/ml)	Protein Con (mg/ml)	Specific Activity (U/mg)	Total Activity Unit	Purification factor	Yields %
1	crude enzymatic extract	8	8.031	44.85	0.179	64.24	1	7.9
2	Concentration with ammonium sulphate (20-80%) and then dialysis	10	8.035	33.65	0.238	80.35	1.32	10
3	Ion exchange	11	5.99	31.7	0.184	65.9	1.02	8.2
4	Gel filtration	10	3.709	19.37	0.19	37.09	1.06	4.6

Table (3-3): Purification of the enzyme G6PD extracted from human red blood cells-HBV

No	Purification step	Volume (ml)	Enzyme Activity (U/ml)	Protein Con (mg/ml)	Specific Activity (U/mg)	Total Activity Unit	Purification factor	Yields %
1	crude enzymatic extract	8	7.9	43	0.18	63.2	1	8
2	Concentration with ammonium sulphate (20-80%) and then dialysis	10	8.14	32.3	0.252	81.4	1.3	10.3
3	Ion exchange	11	4.81	29.5	0.16	53	0.8	6.7
4	Gel filtration	10	3.5	25.4	0.13	35	0.72	4.43

Table (3-4): Purification of the enzyme G6PD extracted from human red blood cells-HCV

No	Purification step	Volume (ml)	Enzyme Activity (U/ml)	Protein Con (mg/ml)	Specific Activity (U/mg)	Total Activity Unit	Purification factor	Yields %
1	crude enzymatic extract	8	7.94	41.3	0.19	63.52	1	7.94
2	Concentration with ammonium sulphate (20-80%) and then dialysis	10	8.002	33	0.24	80.02	1.26	10
3	Ion exchange	11	3.24	18.79	0.172	35.64	0.905	4.48
4	Gel filtration	10	2.8	17.4	0.16	28	0.84	3.5

Set the purity and the molecular weight of the G6PD enzyme

Electrophoresis methods are one of the essential techniques that must be completed after the completion of all purification stages to verify the purity of the enzyme before beginning to end the rest of the enzyme description steps. From the observation of image (3-1), the emergence of a single protein pack in the acryl polyacrylamide is shown to represent the enzyme G6PD and obtained after the gel filtration process of the Sephacryl-S300 column for enzymatic extracts. The emergence of a single protein pack indicates the efficiency of the purification process as it shows the elimination of all proteins associated with the enzyme in the crude extract. S1 control, S2 HAV, S3 HBV and S4 HCV.

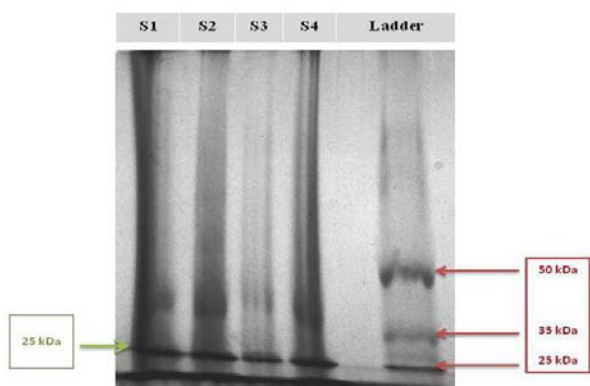


Image (3-1) : The electrophoresis of the G6PD enzyme purified from the red blood cells of the human being with a polyacrylamide gel in the absence of the teratogenic agents

The movement of proteins in a polyacrylamide gel is dependent on the amount of the molecular weight of the protein, and the amount of action of each protein is inversely proportional to its molecular weight, and on this basis, the proteins can separate into a polyacrylamide gel with the presence of the teratogenic agents, and in the absence of teratogenic agents, the movement of proteins depends on the protein charge, which sometimes produces erroneous results due to the proximity of the protein charge or similarity. The method of electrophoresing based on the acryl-amid and the PAGE-SDS to calculate the molecular weight based on the relationship between the logarithm of the molecular weights of the ladder solution containing the standard proteins and the relative movement of these proteins Mobility / Rm and as indicated in the table.

Table (3-5): The standard weights in the ladder solution used in the electrophoresis and the relative movement of each one.

Size	Iog	CM	Rm
10	1	0.5	0.07
15	1.18	1	0.14
25	1.4	2	0.29
35	1.54	2.5	0.36
50	1.7	3.3	0.47
75	1.9	4	0.57
100	2	4.5	0.64
150	2.18	5	0.71
225	2.35	5.3	0.76

Rm extracted from the distance travelled by the protein to the distance travelled by the blue bromophenol dye. Fig. (3-20) shows that the molecular weight of G6PD for all enzymatic extracts was 25 kD.

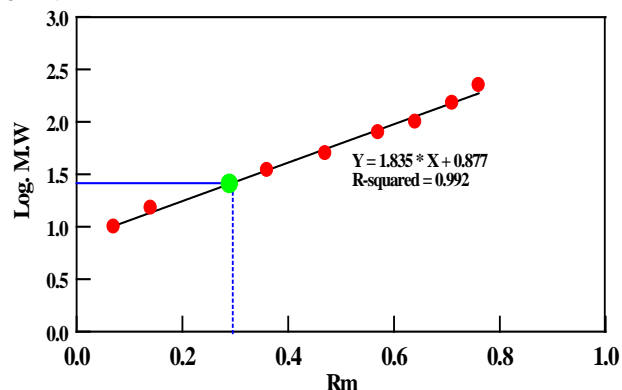


Figure (3-20) shows that the molecular weight of G6PD for all enzymatic extracts

The molecular weight of G6PD purified from human red blood cells was within the range mentioned above ⁽⁶³⁾, ranging from 22-58 KD. The difference in molecular weight estimation in the various studies is due to the length of the purification steps used in some studies, resulting in the enzyme breaking through these long phases and giving a molecular weight less than the real. These results differed with what was obtained in a study to purify the enzyme extracted from the adrenal gland of the cows. Using the ion exchange column DEAE-Cellulose, one protein peak obtained that fully matched the peak of enzymatic activity using the ion exchange technique, and a single protein packet was also obtained when using cellulose acetate migration and posting using the electrophoresis with an poly acryl amide gel with the presence of the teratogenic agents.

The G6PD enzyme was purified from coriander leaf ⁽⁶²⁾ using the ion-Exchange column DEAE-SEPHADEX-A50, and a single protein packet was obtained for the electrophoresis with a poly acrylamide gel with the presence of the teratogenic agents. It found that the G6PD enzyme purified from the small intestine of the rat gives a single package using a polyacrylamide without the use of the teratogenic agents and also when the agents used. The gel also provided a single peak. ⁽⁶⁴⁾

In the G6PD enzyme purified from human placenta by using ADP sepharose type columns, 2.5-it found that upon removal of polyacrylamide gel without the use of teratogenic agents, two protein packs appear fast and the other is slow to return G6PD, and when SDS used with the polyacrylamide gel, one packet was given, and the molecular weight of the enzyme calculated at 54 kD. ⁽⁶⁵⁾

It found that the G6PD enzyme purified from the liver of the dog had two packs when using a polyacrylamide gel without using the teratogenic agents, and these packages one fast and the other slow back to the G6PD, and when the SDS used, the gel gave one single package, and the molecular weight of the enzyme calculated at 52.5 kcal ⁽⁶⁶⁾. In the study of G6PD purified from sheep eye iris using a column of 2.5 ADP-Sepharose and utilising

a polyacrylamide in the presence of teratogenic agents, it found that the enzyme contained a single protein pack and its molecular weight compared to standard proteins was 54.95 kg.⁽⁵²⁾

CONCLUSION

Most of the biochemical variables studied were at an abnormal level in patients with HAV, and in the purification of the enzyme, the activity of the purified enzyme from the hepatitis C-infected sample was less effective than that of the other enzymatic extracts. The specific efficacy of most enzyme extracts was decreasing in the gel filtration step.

ACKNOWLEDGMENT

I am highly indebted to my supervisor Asst. Prof. Dr. Khalid F. AL-Rawi for his guidance and constant supervision as well as for providing necessary information regarding this research and also for his continuous support. I would like to express my special gratitude and thanks to Prof. Dr. Mohammed Qais AL-Ani for their support and sharing their knowledge and expertise in this study in addition to their honest and cooperative response to all question related to this study. My thanks and appreciation to my colleagues at Al Anbar University – College of science and all the people who helped me with their most abilities.

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