ESTIMATION OF SERUM COPPER LEVEL IN HAPATITIS-B PATIENTS BY USING SPECTROPHOTOMETER

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ABSTRACT: *Hepatitis B is a viral infection caused by hepatitis B virus and is a major health problem in all countries, especially in Pakistan. The purpose of this study was to determine the relationship of serum copper level with hepatitis B infection. For this purpose, indoor patients of Hepatitis B from DHQ, Hospital Gujranwala were selected, who don't know that they were infected by HBV. They were admitted in hospital due to other reasons such as accident, chest disease, etc. During their treatment, many medicines were used such as drips, injections, tablets and capsules. By the use of these medicines copper level is increased or decrease. To check the effect of variation of copper level on Hepatitis B infection, this research work was done. The serum copper level of hepatitis B patients and healthy persons was determined by using Spectrophotometer (digital photometer 5010). According to the results Copper level was decreased in some case and increased in some cases of hepatitis B patients. Minimum level of copper in hepatitis B patients was 63.0µg/dl while maximum level was 294.1µg/dl. However, overall results show that copper level is high in hepatitis B patients as compared to healthy persons. The copper level was highly changed in different hepatitis B patients may be due to the use of different medicines of hospital. However Further study is required for the confirmation of this research work.*

Key Words: Copper, Spectrophotometer, Hepatitis-B, Gujranwala, Estimation.

INTRODUCTION

Viral hepatitis is a major health problem in the world. It can be defined as the inflammation of the liver caused by hepatitis viruses [1]. The term hepatitis is derived from the Greek word 'hepar' means "Liver" and 'itits' mean "inflammation"[2]. It is a most serious liver disease may be affecting for a short time or may be lead to produce fibrosis on the liver and cause liver cirrhosis depend on the type of hepatitis [3].There are 5 main types of hepatitis viruses as (A, B, C, D and E). In which hepatitis A and E are caused by polluted water and resolve after a specific period of time. While hepatitis B, C and D are caused by blood to blood contact and some other factors. Hepatitis B and C are common and lead to chronic infection in millions of people in the world. These two types of hepatitis are the most common cause of liver cirrhosis and liver cancer [3].

Hepatitis B is a viral infection caused by hepatitis B virus, which envelops in DNA and affects the liver. Which may disturb the antioxidant system and play a role in the development of chronic liver diseases [3].

It is a major health problem in the world. It is estimated that worldwide about 2 billion people have evidence of past and present hepatitis B Virus infection [3]. It is very common worldwide; the high rate of hepatitis B is in Asia and Africa [4] . It is a serious disease can result in liver cancer, liver cirrhosis, failure of liver and death. Hepatitis B can be acute or chronic [5]. However. liver cirrhosis is the end stage of this disease [6].

Every person need to be regular monitoring the infection of hepatitis to establish that he is in the HBsAg- positive or HBsAg-Negative phase. HBV can be spread in healthcare workers by accidently needle stick injuries during the blood sampling of infected HBV patients. The vaccine to protect from hepatitis B infection is available, which give the immunity against HBV infection due to the presence of anti-HBs [3].

Many countries in the world start hepatitis B vaccine at birth or in childhood. The primary hepatitis B vaccination consists of three dose of vaccine having time period of 0, 1 and 6 month. It is 95% effective against the infection of hepatitis B[3].

During the initial infection of acute hepatitis B, many people do not have any symptoms and do not know that they are infected. However the symptoms of chronic hepatitis B are based on the physical conditions, the size of liver may be enlarged due to the formation of fibrosis on the surface of the liver. Extensive damage and scarring of liver lead to loss of weight, swelling of the legs and fluid in the abdomen [7]. However, symptoms for both acute and chronic hepatitis B are:-Yellow eyes and skin, Dark urine, Abdominal Pain, Nausea/Vomiting, Itchy skin, Weight loss [3].Hepatitis B virus (HBV) is the main cause of hepatitis B, which is spread when blood, semen or other body fluids of hepatitis B infected person enter the body of an uninfected person by:- Blood to Blood contact, Sharing contaminated medical equipment such as syringes, needles; Sharing personal items such as toothbrushes and razor [3].The risk of hepatitis B infection is also increased if the mother has acute hepatitis B during the pregnancy [3]. Hepatitis B is not spread by sneezing, hugging or coughing. However the virus can be found in saliva.In Pakistan 2.5 % peoples are infected and especially in Gujranwala 2.9 % people are infected with hepatitis B [7]. Many countries in the world use hepatitis B vaccine starting at the birth or in the childhood. Due to the use of vaccination the ratio of hepatitis B is decreased in most advanced countries [8].

Our body needs various trace elements like Co, Cu, Fe, Ni, Mn and Zn for the immune system of the body [9]. The importance of trace elements in human organism, especially in the blood cells, serum, liver and different parts of the body have been shown by many publications [10]. These elements are required in minute quantities in the form of enzymes and as a functional component of protein for the development and growth of the body [11]. Trace elements consist of about 0.01% of our body weight [12].

The relationship between Trace elements and liver diseases has not been understood clearly [13]. A number of evidences show that many trace elements are involved in many

biological processes such as (1) Involve in enzymatic reaction, act as an antioxidant, or other mechanism [5].

Normal range of Trace elements like Copper, Zinc, Iron, and selenium increase the protective effect against some diseases [5]. The imbalance in the concentration of trace elements such as Zinc, copper, magnesium and selenium may be responsible for many chronic liver diseases [15]. There are two reasons, by which we can say that trace elements are related to hepatitis; (1). Trace elements control many biological and biochemical activities of our body. (2). The concentration of trace elements in the blood is changed from different patients of Hepatitis [16].

As the activities of trace elements take place in the liver, so the concentration of trace elements may be related to liver diseases such as hepatitis [13]. Trace elements measurement in serum can be used as a good biomarker for the diagnosing different diseases [17]. Copper is the most important micronutrient for the growth of plants and animals. The daily requirement of copper is 0.5-1 mg for children and 1.2 mg for adults [18]. The major function of copper in the human body is to involve in oxidation-reduction reactions, that's why it is involved in many biological processes [19]. It plays an important role against hepatitis fibrosis in chronic diseases [13].

Copper can be found almost in every cell of the human body [20]. The composition and distribution of copper in different organs and tissues is not clear. The normal range of the copper depends on the gender and age such as:Men 70-140 µg/dL. Women 80-155 µg/dL, Children (6-12y) 80-190 µg/dL and Small babies 20-70 µg/dL [21].The concentration of copper is high in brain and liver [20]. A Very small amount of copper is required for our body [22]. Copper is necessary for the normal metabolism of iron and for the formation of red blood cells (Angelova et al, 2011) [20]. Water and oil are the main sources of copper. The concentration of copper in water and soil is different from place to place. However, dietary sources of copper are:- Potatoes, Nuts, Sunflower seeds, Mushrooms, Shellfish, Red meat [23].

There are many enzymes that require copper for their catalytic processes, known as copper enzymes[19]. The copper enzymes present in human bodyare:-Ceruloplasmin (Cp), Lysyl Oxidase enzyme, Superoxide dismutase, Cytochrome, Ascrobate oxidase and Tyrosinase etc [20]. Increasedcopper level in the blood can reach to different parts of the body and cause different health problems [9]. High concentration of copper is found in patients of liver cirrhosis, Wilson's disease, Tumor etc [20]. It may be associated with liver damage and kidney diseases [25].

The purpose of this research work was to determine the relationship of copper level and hepatitis B in patients who were admitted in DHQ Hospital Gujranwala due to different diseases. Hepatitis is a common disease in Gujranwala division and no institute or hospital is doing research work. Also literature does not show any evidence of use of spectrophotometer for estimation of Cu^{+2}level in hepatitis B patients by using Commercial copper kit. It was necessary to conduct a research work and find that how Hepatitis B is related to variation of Cu^{+2} concentration.

MATERIALS & METHODS

Apparatus Required: Tourniquet to tied arm, Disposable syringe, Red topped test tube, Water Bath, Centrifuge machine, Micro Pipette, Shaker Machine and Digital Photometer 5010.

Copper Kits: Commercial copper kit of Chema Diagnostic Company of Italy, contain three solutions, namely as Reagent A, Reagent B and Standard solution

Reagent A: - Acetate buffer of pH 4.90, Surfactants and preservatives.

Reagent B: -[3, 5- Di Br-PAESA]3, 5-Dibromo-2-Pyridylazo-N-Ethyl-N-Sulfopropylaniline.

Standard: - Standard solution of Copper 200 µg /dl.

Selection criteria: This study was conducted on 25 newly diagnosed indoor patients of Hepatitis B of DHQ Hospital, Gujranwala. They were admitted in hospital due to different reason other than Hepatitis B infection such as Accident, Chest disease. During treatment many medicines were used, some of these medicines may increase or decrease the level of copper in hepatitis B patients.For comparison of copper level, 15 healthy subjects having no HBV or HCV were also included. They were the employees of GINUM hospital, Gujranwala.

Ethical Consideration: A written informed consent form was taken from all indoor hepatitis B patients of DHQ, Hospital Gujranwala to collect the blood samples for this study. A complete history was taken from all the patients on the consent form, which include name, Age, Address, Hospital detail (Ward No & Registration ID).

Blood collection: The blood sample of 3ml was collected by using disposable syringes with the help of nursing staff of the DHQ Hospital, Gujranwala and then stored in red-topped tubes with proper labeling and then transferred to the laboratory for the separation of serum.

Separation of serum: After the collection of blood samples, these samples were incubated at 37^oC in water bath to clot the blood and then these clotted blood samples were centrifuged at 4000 rpm for 7 minutes to separate serum. These serum samples were stored in refrigerator at -20^oC in GINUM hospital until the performance of test.

Test procedure: The analysis of copper in the serum of Hepatitis B patients was done by using commercial copper kits and spectrophotometric technique in the GINUM hospital. In this procedure photometer 5010 is used, which have wavelength range of 340-800 nm. The Concentration of copper was detected using radiations of 580 nm.

The procedure performed for the determination of copper concentration included the steps:-Sample Preparation, Mixing of copper reagent and sample**,** Detectionand Result Calculation.

Sample Preparation: First of all, incubated all the serum samples in the water bath for 10 minutes and then take 1.5 ml of each serum sample in test tube with proper labeling.

Reagent and sample preparation:

Mix equal quantity of both Reagent A and B with the help of shaker for 10 minutes. Then take 1.5ml of mixed reagent in three test tubes to prepare blank, standard and sample solutions (Table 1).

Table 1. Scheme for the preparation of Blank, Standard and Sample solution

Solution	Blank	Standard	Sample
Reagent	1.5 ml	1.5 ml	1.5 ml
Water	100 µl	-------	--------
Standard	--------	100 µl	--------
Sample	--------	-------	100 µl

1. Take 100 µl of water in one test tube, add 1.5 ml of copper reagent and label as Blank.
2. Take 100 µl of standard solution of copper in saecond test tube, add 1.5 ml of copper reagent and label as standard.
3. Take 100 µl of serum sample in athird test tube, add 1.5 ml of copper reagent and label as sample solution.

Mix these solutions and then incubate at 37^0C for 5 minutes.

Detection of copper: The compound **[**3, 5- Di Br-PAESA] combines with copper to produce colored complex. The color of absorbance is directly proportional to concentration of copper in the sample. Absorbance of Copper complex was measured at the wavelength of 580nm by using photometer 5010.The absorbance of blank and standard solutions was adjusted in the photometer. Then the following factor was applied for the measurement of Concentration of copper from serum samples.

$$\text{Copper } (\mu g\ /dl) = \frac{\text{A(Sample)}- \text{ A (Blank)}}{\text{A (Standard)} - \text{ A (Blank)}} \times 200$$

Here 200 is the value of Standard solution of copper, which is provided with the commercial kit of copper. The advantage of the above factor is that we can get direct results of the quantity of copper in the serum samples.

RESULTS & DISCUSSIONS

Table 2 provide the information about the indoor patients of Hepatitis B, which was admit in the DHQ, hospital Gujranwala due to the different reasons such as accident, chest pain etc. This table 2 consist Name, Age, Address, DHQ Hospital detail about the patients history such as (Ward No, Registration No etc).

Table 2. Copper levels of Indoor hepatitis B patients.

Sr No	Age	Gender	Serum Copper (µg/dl)
1	13y	Male	105.7
2	14y	Male	141.4
3	16y	Male	127.2
4	17y	Male	167.0
5	22y	Male	117.4
6	22y	Male	119.5
7	26y	Male	142.0
8	28y	Male	95.4
9	30y	Male	129.8
10	30y	Female	122.4
11	32y	Male	143.4
12	35y	Male	294.1
13	36y	Male	122.5
14	45y	Male	112.2
15	45y	Female	126.2
16	50y	Male	134.7
17	50y	Male	199.0
18	50y	Male	162.7
19	52y	Male	73.8
20	55y	Female	170.7
21	55y	Female	197.5
22	60y	Male	136.2
23	60y	Female	88.4
24	60y	Male	63.0
25	65y	Female	128.5

Note: Minimum Age = 13y, Minimum Copper level = 63.0(µg/dl), Maximum Age = 65y, Maximum Copper level = 294.1(µg/dl) Mean Age =38.72y and Mean Copper level = 136.8 µg/dl.

Table 3. Copper levels of Male hepatitis B patients.

Sr No	Age	Gender	Serum Copper (µg/dl)
1	13y	Male	105.7
2	14y	Male	141.4
3	16y	Male	127.2
4	17y	Male	167.0
5	22y	Male	117.4
6	22y	Male	119.5
7	26y	Male	142.0
8	28y	Male	95.4
9	30y	Male	129.8
10	32y	Male	143.4
11	35y	Male	294.1
12	36y	Male	122.5
13	45y	Male	112.2
14	50y	Male	134.7
15	50y	Male	199.0
16	50y	Male	162.7
17	52y	Male	73.8
18	60y	Male	136.2
19	60y	Male	63.0

Note: Minimum Age, Minimum Copper level = 63.0(µg/dl),Maximum Age = 60y, Maximum Copper level = 294.1(µg/dl)Mean Age =34.63 and Mean Copper level = 136.16(µg/dl)

Table 4. Copper level of Female hepatitis B patients

Sr No	Age	Gender	Serum Copper (µg/dl)
1	30y	Female	122.4
2	45y	Female	126.2
3	55y	Female	170.7
4	55y	Female	197.5
5	60y	Female	88.4
6	65y	Female	128.5

Note: Minimum Age = 30y, Minimum Copper level=88.4 (µg/dl), Maximum Age = 65y, Maximum Copper level = 197.5 (µg/dl)Mean Age = 51.66, Mean Copper level = 138.95 (µg/dl)

Table 5. Copper level of healthy person

Sr No	Age	Gender	Serum Copper (µg/dl)
1	25y	Female	146.7
2	26y	Female	105.5
3	26y	Male	110.4
4	27y	Male	114.1
5	31y	Male	105.5
6	32y	Male	120.2
7	33y	Male	126.9
8	34y	Male	108.6
9	35y	Male	129.5
10	35y	Male	114.5
11	39y	Male	114.5
12	39y	Male	131.0
13	40y	Male	139.1

14	41y	Male	150.1
15	42y	Male	136.6

Note: Minimum Age=25y, Minimum Copper level =105.5 (µg/dl), Maximum Age =45y, Maximum copper level =150. (µg/dl)Mean Age = 33.66, Mean Copper level = 123.55 (µg/dl)

Table 6. Copper level of Male healthy person

Sr No	Age	Gender	Serum Copper (µg/dl)
1	26y	Male	110.4
2	27y	Male	114.1
3	31y	Male	105.5
4	32y	Male	120.2
5	33y	Male	126.9
6	34y	Male	108.6
7	35y	Male	129.5
8	35y	Male	114.5
9	39y	Male	114.5
10	39y	Male	131.0
11	40y	Male	139.1
12	41y	Male	150.1
13	42y	Male	136.6

Note: Minimum Age =26y, Minimum Copper level=105.5 (µg/dl), Maximum Age= 42y, Maximum copper level =150.1 (µg/dl)Mean Age = 34.92, Mean Copper level = 123.15 (µg/dl)

Table 7. Copper level of Female healthy person

Sr No	Age	Gender	Serum Copper (µg/dl)
1	25y	Female	146.7
2	26y	Female	105.5

Note: Minimum Age=25y, Minimum Copper level=105.5(µg/dl), Maximum Age = 26y, Maximum copper level =146.7 (µg/dl)Mean Age =25.5, Mean Copper level =126.1 (µg/dl).

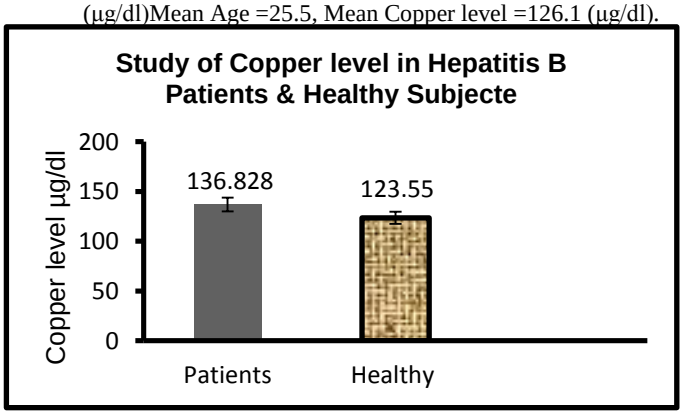

Figure 1. Graphical comparison of mean copper level of hepatitis B patients of Group-A with healthy subjects of Group-B:Group-A represents the mean copper level of hepatitis B patients. Group-B represents the mean copper level of healthy subjects.

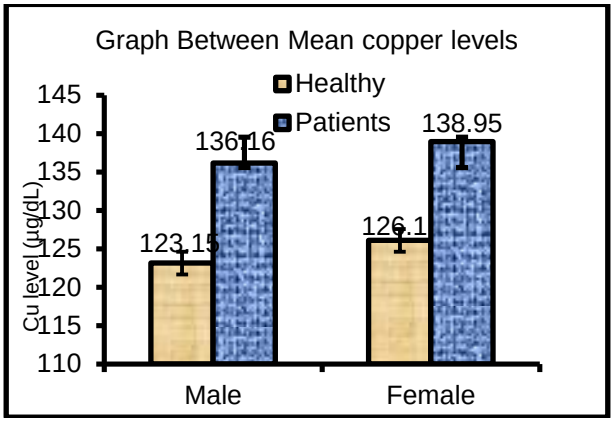

Figure 2. Comparison of average copper level in male healthy & male patients as well as female healthy & female patients.

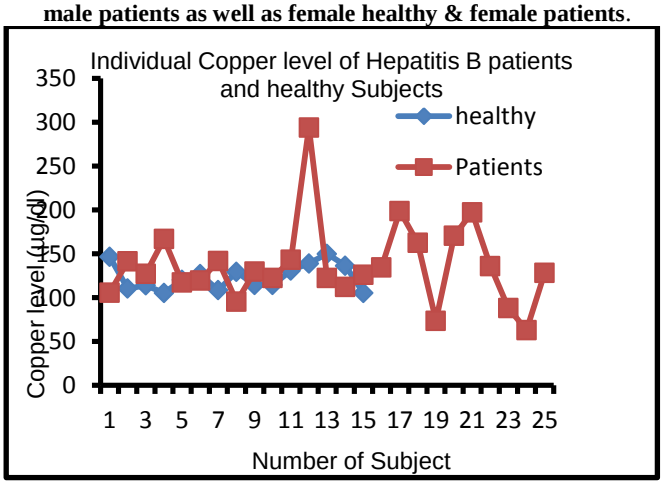

Figure 3. Comparison of individual copper level of patients of hepatitis B infection and healthy volunteers.

DISCUSSION

The present study was carried to determine the relation of serum copper level with hepatitis B infection. For this purpose, 25 indoor patients of hepatitis B were selected (19 males and 6 females) (Table 2), who were admitted in DHQ, hospital Gujranwala due to other reasons and they don't know that they had hepatitis B infection and they have not used medicine of hepatitis B, the main purpose was to check the effect of medicines other than hepatitis B medicine on copper level of hepatitis B patients.For comparison of the copper level of hepatitis B patients, 15 healthy subjects were also included in this study (Table 5), all the healthy subjects were workers of GINUM hospital, GRW. They all were vaccinated and had no hepatitis B infection.The serum copper level in the samples was determined by using spectrophotometer (photometer 5010). The results of copper level of hepatitis B patients and healthy subjects were shown in Table 2 and Table 5 Respectively.

As shown in Table 5 the copper level of healthy subjects who were workers of GINUM, GRW ranged between 105.5 to 150.1µg/dl individually and the mean copper level in healthy subjects was 123.55µg/dl. The table 2 shows that the copper level of 15 out of 25 hepatitis B patients was higher than mean copper level of healthy Subjects (123.55µg/dl). Our findings are similar to [11, 12, 26, 27-29], who reported in

their different research studies that serum copper level increased in the patients of hepatitis B.

This study also shown that 07 hepatitis B patients have individual copper level lower than mean value of healthy subjects (123.55µg/dl). The lower copper level may be due to the use of medicine or malnutrition of the protein or it may be due to the drinking of polluted water.

While the rest of three patients have serum copper level almost equal to the average copper level of healthy subjects. These findings are supported by the study of Martos et al., (1998)[30], Cesur et al., (2005)[31], Balamtekin et al., (2010)[32], who reported that there were no significant difference of copper level in healthy people & hepatitis B patients. However according to the graph overall result showed that mean copper level was increased in hepatitis B patients as compared with healthy subjects.

Manser et al (1989) and many other [33-38] researchers determined the level of trace elements by using atomic absorption spectrophotometer, which is very expensive as compared with spectrophotometer. So novelty of this research work is confirmed.

CONCLUSION

Copper level was increased in most cases of hepatitis B patients and was decreased in few cases may be due to the use of medicine or other factors i.e. polluted water, malnutrition or imbalance intake of copper diet. Copper level may be affected by hepatitis B infection. There is a biochemical relationship between Hepatitis B and serum copper level. Spectrophotometric method is cheaper, valid and time saving method use for the detection of trace elements, as compared to atomic absorption spectrophotometer. The objectives of this study were achieved successfully however further study is required for this research work.

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REFERENCES

[1] Reddy, P. R. N., Kishore, A., Franklin, A. and Reddy, P. E. Evaluation of trace elements in hepatitis B. *Journal of pharmaceutical and biomedical sciences*, **21**(21): 1-3(2012).

[2] Cebeci, S.A., Cesur, S., Kavas, G. O., Aksaray, S. and Tezeren, D. Serum copper and zinc concentration in patients with chronic hepatitis B. *Journal of Infection*, **51**(1):38-40(2005).

[3] Ilyas, M., Iftikhar, M. and Rasheed, U. Prevalence of hepatitis B and hepatitis C in populations of college students in Gujranwala. *Biologia (Pakistan)*, **57**(1&2): 89-95(2011).

[4] Pasha, Q., Malik, S. A., Shaheen, N. and Shah, M. H. Investigation of trace metals in the blood plasma and scalp hair of gastrointestinal cancer patients in comparison with controls. *Cin Chim Acta*, **411**(7-8): 531-539(2010).

[5] Lin, c. c., Huang, F. J., Tsai, Y. L. and Huang, L. Y. Selenium, iron, copper and zinc level and copper to zinc ratio in serum of patients at different stages of viral hepatitis disease. *Biological trace element research* **(109):** 15-22(2006).

[6] Hoffmann, g., Van Den Ingh, M, A., Bode, P. and Rothuizen, J. copper-associated chronic hepatitis in labrador retrievers, *j vet intern med*,**20:** 856-861(2006)..

[7] Abediankenari, S., Ghasemi, M., Nasehi, M. M., Abedi, S. and Hosseini, V. Determination of Trace Elements in Patients with Chronic Hepatitis B. *Acta medica Iranica*, **49(**10**):** 667-669(2011)..

[8] Elsayed, A, F., Ibrahim, B. O. and Albashir, A. S. Liver Abnormality in Carriers of Hepatitis B Virus (HBV). *J Pharma Biomed Sci***6**(6):347–349(2016).

[9] Nazir, F., Sarfraz A.R., Qadoos, A. and Riaz, R. Trace elements profile of hepatitis C patients in Faisalabad, Pakistan. *World applied science journal*, **28**(8):1093-1097(2013).

[10] Tautkus, s., Kazlauskas, R., Kareiva, A. and Irnius, A. Rapid quantitiative determination of metals in blood and liver by FAAS, *Chemija*,**16:** 29-33(2005).

[11] Elzeiny, A. M., Elzefzafy, M. W., Shahin, S. R., Atef, N. And Ahmed, G. M. Serum levels of selenium, zinc, copper and iron in patients with post viral hepatitis liver cirrhosis& hepatocellular carcinoma. *Asian Academy of Management Journal* 8(1):132-137 (2010).

[12] Al-Shakly, K. M. Determination the level of some trace elements in sera of Iraqi patients with viral hepatitis B infection. *Diyala journal for pure sciences*,**7(2):**84-193(2011).

[13] Mohammed, A. M., Abd-Elmaksood, D. M., Abd El maksood, N., El-Mezayen, H. and El-Garaam, H. Serum level of trace elements in Egyptian patients with chronic hepatitis C under interferon therapy. *Journal of genetic engineering and biotechnology*, **10:** 81-86(2012).

[14] Mehde, A, A., Mehdi, A, W., Jasim, M, A. Study several biochemical parameters into patient's with hepatitis B virus, *Global journal of medical research disease*, **13(**2**):**1-4(2013).

[15] Lichun, T., Jiongshan, Z. Min, D., Fenglin, W. and Hongzhi, Y. Correlation between Traditional Chinese Medicine symptom patterns and serum concentration of zinc, iron, copper and magnesium in patients with hepatitis B and associated liver cirrhosis. Journal of Traditional Chinese medicine, **35(**5**):** 546-550(2015).

[16] Elnimr, T., Morsy, R., Fert, E. A. and Ismail, A. Evaluation of some trace elements in biological samples of Egyptian viral hepatitis patients under nutrition therapy. *International journal of chemical, environmental & biological sciences*,**3**(4):322-328(2015)..

[17] Javed, S., Khattak, N. N., Khattak, A. S., Khokhar, Y., Aslam, M., Nangyal, H. and Khan, U. Comparison of Serum Trace Metals Concentration in Blood of Hepatitis B, C and Healthy Individuals and Their Correlation with Viral Load. *American-Eurasian J. Agric. & Environ. Sci.*,**15 (9):** 1895-1902(2015)..

[18] Saghir, M., Shaheen, N. and Shah, H, M. Comparative evaluation of trace metals in the blood of hepatitis C patients and healthy donors. *Biological Trace Element Research*, **143(2):** 751-763(2011).

[19] Krupanidhi, S., Sreekumar, A. & Sanjeevi, B. C. Copper & biological health. *Indian J Med Res*, **128,** 448-461(2008).

[20] Angelova, M., Asenova, S., Nedkova, V. & Kolarova, K. R. Copper in the human organism. *Trakia Journal of Sciences*, **9(1):**88-98(2011).

[21] Pramoolsinsap, C., Promvanit, N., Komindr, S., Lerdverasirikul, P. and Srianujata, S. Serum trace metals in chronic viral hepatitis and hepatocellular carcinoma in Thailand, *Journal of Gastroenterology*, **29(**5**):** 610-615(1994).

[22] Turkoglu, O. and Soylak, M. Spectrophotometric Determination of Copper in Natural Waters and Pharmaceutical Samples with Chloro(phenyl) glyoxime, *Journal of the Chinese chemical society*, **52:** 575-579(2005).

[23] Kalochi, N. F., Kazi, T.G., Afridi, H. I., Kazi, N., Kandhro, G.A., Shah, A. Q., Baig, J. A., Wadha, S. K., Khan, S. Shah, F., Jamil, M. K. and Arain, M. B. Distribution of copper iron and zinc in biological samples (scalp hair, serum, blood and urine) of Pakistani viral hepatitis (A-E) patients and control. *Biological Trace Element Research*, **143**(1):116-30(2011).

[24] Ahmed, J. M. and Zannat, T. A simple spectrophotometric method for the determination of copper in some real, environmental, biological food and soil samples using salicylaldehyde benzoyl hydrazone. *Pak. J. Anal. Environ. Chem*, **13(1):** 22-35(2012).

[25] Kavitha, C., Babu, S. M. and Saraswathi, KSpectrphotometric determination of copper in natural water and pharmaceutical samples with chloro(Phenyl) glyoxime. *journal of the chinese chemical society*, **52:** 575-579 (2013).

[26] Selvaraju, R., Rama, G. R., Narayanaswamy, R., Valliappan, R. and Baskaran, R. Trace element analysis in hepatitis b affected human blood serum by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Romanian J. Biophysics, **19**(1):35-42(2009).

[27] Afridi, I. H., Kazi, G. T., Kazi, G. N., Jamil, K. M., Sarfaraz, A. R., Arian, B. M., Kandhro, A. G., Shah, Q. A., Baig, A. J., Jalbani, N. and Ansari, R. Determination of Copper and Iron in the Biological Samples of Viral Hepatitis (A–E) Female Patients. *Biological Trace Elements Research*,**129**(1):78-87(2009).

[28] Arain, A. S., Kazi, G. T., Afridi, I. H., Talpur, N. F., Mughal, A. M., Shah, F. Arain, S. S. and Panhwar, H.A. Estimation of copper and iron burden in biological samples of various stages of hepatitis C and liver cirrhosis patients. *Biological trace element research*,**160**(2): 197-205(2014).

[29] Marghilani, A. A., Al-Hossainy, F.A. and Mansour, F. R. Support vector machine for simultaneous determination of ultra trace concentrations of copper and cadmium in serum of patients with chronic hepatitis by adsorptive stripping voltammetry. *Chemistry and Materials Research*,**3**(11): 56-72(2013).

[30] Martos, C., Alarcon, M., Lagos, F., Serrana, H., Valero, V. and Martinez, M. C. Serum zinc and copper concentration and Cu/Zn ratio in patients of hepatopathies or diabetes. *J Trace Elem Med Biol.* 12(1):44-9.(1998).

[31] Cesur, S., Cebeci, A., S. Kavas, O. G., Yilmaz, N. and Buyukkagnici, I. D. Serum copper and zinc concentrations in patients with chronic hepatitis C. *Journal of Infection*, **51**(1): 35-37(2005).

[32] Balamtekin, N., Kurekci, E. A., Atay, A., Kalam, S., Okutan, V., Gokcay, E., Aydin, A., Sener, K., Safali, M. and Ozcan. Plasma level of trace elements have an implication on interferon treatment of children with chronic hepatitis B infection. *Biological Trace Elements Research*, **135**(1): 153-161(2010).

[33] Manser, T. W. W. and Khan, A. M. trace element studies on Karachi populations, part 1: normal ranges for blood copper, zinc and magnesium for adults. JPMA, **39(**43**):** 43-49(1989).

[34] Singh, V., Bhattacharya, S. K., Sunder, S. and Kachhawaha, J. S. Serum and urinary copper in acute hepatic encephalopathy. *J Assoc Physicians India*, **38**(7): 467-469(1990).

[35] Hatano, R., Ebara, M., Fukuda, H., Yoshikawa, M., Suglura, N., Kondo, F., Yukawa, M. and Saisho, H. Accumulation of copper in the liver and hepatic injury in chronic hepatitis C. *Journal of Gastroenterology and Hepatology*. **15(7):** 786-791(2000).

[36] Fota-Markowska, H. Dynamics of serum copper level in patients in the acute phase of hepatitis B and in early convalescence. *Przeql Epidemiol*,**55(3):** 156-163(2001).

[37] Kalkan, A., Bulut, V., Avci, S., Celik, L. and Bingol, N. K. "Trace elements in viral hepatitis". *J Trace Elem Med Bio*, **16**(4): 227-230(2002).

[38] Poo, J. L., Rosas, R. R., Montemayor, A. C., Isoard, F. and Uribe M. "Diagnostic vaue of copper/zinc ration in hepatocellular carcinoma: a case control study". *Journal of Gastroenterology*, **38(1):** 45-51(2003).