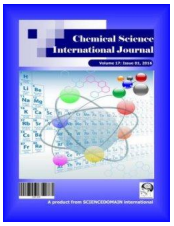

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Selenium Concentration of Iraq Patients Infected with Chronic Hepatitis B Using Different Analytical Techniques

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Authors' contributions

This work was carried out in collaboration between all authors. Author TAZ designed the study. Author WMS wrote the protocol and wrote the first draft of the manuscript. Author SMS reviewed the experimental design and all drafts of the manuscript. Authors HMA and AWAA managed the analyses of the study. Author HMA identified the plants. Authors WMS and AWAA performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The study aimed to use different analytical techniques for determination Selenium, related to patients with chronic hepatitis (B).
Study Design: Selenium was estimated by using AAS and ICP-MS and The analytical results undergoes comparison for priority of the methods. One hundred sixty patients samples and thirty healthy individuals were investigated in this study. The patients were divided into five groups.
Place and Duration of Study: The study was carried out in Fallujah teaching hospital for the period

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from 1 May to 3 November at viral hepatitis consultation clinic in the hospital.
Methodology: Selenium was estimated by using AAS and ICP-MS and The analytical results undergoes comparison for priority of the methods. One hundred sixty patients samples and thirty healthy individuals were investigated in this study. The patients were divided into five groups. Count/spleen diameter ratio was also calculated.
Results: In order to determine the appropriate method for measuring selenium, concentrations in the serum, scatter plotting method was employed. The measured readings of AAS and ICP-MS for Selenium, was compared. The results of the study suggest that hydride generation was the preferred method to estimate the concentration of selenium.
Conclusion: According to the results of application of scatter plotting statistical technique, it has been shown Selenium concentration can be determinate by AAS better than ICP-MS. The serum selenium concentrations were significantly lower in chronic hepatitis B patients than healthy individuals.

Keywords: Selenium; ICP-MS; hepatitis B; PCR.

1. INTRODUCTION

Chronic Hepatitis B virus (HBV) infection is a major liver disease that clinical outcome of infection is linked to immune response [1]. The disease may develop to liver carcinoma during infection. An estimated 350 million persons worldwide are chronically infected with HBV [2]. In the United States, there are an estimated 1.25 million hepatitis B carriers and approximately 75% of them live in Asia and Africa [3], defined as persons positive for hepatitis B surface antigen (HBs Ag) for more than 6 months [2]. Carriers of HBV are at increased risk of developing cirrhosis, hepatic decompensation, and hepatocellular carcinoma (HCC) [4,5]. Although most carriers will not develop hepatic complications from chronic hepatitis B, 15% to 40% will develop serious sequelae during their lifetime [5]. Alanine aminotransferase (ALT), also called Serum Glutamate Pyruvate Transaminase (SGPT) is an enzyme present in kidney, heart, muscle and greater concentration in liver compared with other tissues of the body [6]. ALT catalyzes the transfer of the α-amino acids of aspartate and alanine respectively to the α-keto group of ketoglutaric acid (alanine + α-ketoglutarate = pyruvate + glutamate) [7]. Aspartate aminotransferase (AST) also called Serum Glutamic Oxaloacetic Transaminase (SGOT). AST catalyses transamination reaction (alanine + α-ketoglutarate = oxaloacetate + glutamate) [6,8]. Up to date, many studies have shown that trace elements have an important role in metabolic activity and health condition [9]. It is demonstrated that trace elements that occur in the earth's crust at levels of 50-90 g/kg with the higher levels present in some sedimentary, volcanic and carbonate rocks. have a major role in protein synthesis, pregnancy abnormalities and immune function [10]. Selenium itself exists in a variety of chemical forms including selenite and selenate as well as elemental selenium and it is often found associated with sulphur-containing compounds. Very small quantities of selenium are required to maintain proper health in both animals and humans and this selenium must be obtained through dietary sources [11]. The necessity for selenium is most likely related to its presence in particular proteins termed selenoproteins. There are around 25 seleno-proteins inside human body and many of these are enzymes that act to protect the body against oxidative damage. Without selenium, the function of the selenium-requiring proteins can be compromised which results in the signs and symptoms of deficiency. Since the ageing process, as well as certain diseases, including cancer and cardiovascular disease, is associated with an increase in oxidative damage, maintaining adequate selenium intakes may provide some protection against these processes [12]. In humans, selenium deficiency can result in Keshan disease, a juvenile cardiomyopathy apparent in the Keshan region which can have particularly low intake levels (<15 μg/dL). There is also evidence for selenium deficiency being involved in impaired immune function, and increased incidence of cancer, cardiovascular and other degenerative diseases as well as overall mortality [13].

2. MATERIALS AND METHODS

2.1 Clinical Study

About one hundred sixty sera samples were collected from patients with chronic hepatitis B who were referred for advisory on hepatitis B at Fallujha teaching Hospital unit. All the patients included in this research were tested positive for HBS Ag test and their specimens were examined

by profile test which include (HBS Ab, HBe Ag, HBe Ab, HBc Ab, (IgM-IgG), HCV and HIV) and also viral load (HBV PCR) with unit (copies/ml) were carried to test the patients sera. The healthy control groups (n=30) were selected based on: non-alcohol drinkers, with no smoking habits, no history of viral hepatitis and absence of any acute or chronic pathology. Six to eight ml of blood was collected from the veins of both the patients and the control groups and kept in sterile tubes without adding any anticoagulant agent. Patients did not take any antiviral treatment prior to this study. Collected blood samples were placed in sterile place and allowed to clot. The blood samples were centrifuged and then the collected serum was stored in plastic vials at –20 C0 until further analysis.

-All of patients were HBs Ag positive and all of them HBc IgG Ab positive.

2.2 Procedure

All the solutions and standards were prepared in double distilled water. A standard solution (1000 µg/L) of selenium was obtained from Merck, Germany. The concentration of Selenium in serum was determined using Atomic Absorption Spectroscopy (hydride generation). Standards were prepared by appropriate dilution of the stock solution with (0.5% v/v) HNO3. HNO3 (65%), the sodium borbhydride pellets were dissolved in a 1% sodium hydroxide solution to give 3% NaBH4. Three milliliters of 3% super pure HCl was used for hydride generator with continuous flow generation system HS-55. A sample of the serum (1 ml) was transferred to a Teflon beaker for mineralization, then 3 ml of HNO3/HCLO4 (1:1 v/v) was added. The temperature of the sample was then brought gradually to boiling point on a hot plate, until fumes of HClO4 appeared. Samples were then heated according to the following temperature/time scheme: 175°C/60 min, 200°C/60 min and finally 250°C/60 min. The mixture was then heated according to the following (temperature/ time) scheme: 175°C for 60 min, 200°C for 60 min, and finally 250°C for 60 min. The mixture was then left to cool down to room temperature. Then 10 ml of (6N) HCl was added and the sample was heated again on hot plate to 170°C for 30 min to reduce Se (VI) to Se (IV) [14].

2.2.1 Sample analysis

The digested serum samples were analyzed for selenium using atomic absorption spectrophotometer equipped with hydride generation system, model Analytic Jena (novAA350). The analytical calibration method was accomplished with aqueous standards in 0.5% (v/v) HNO3. Fresh calibrations were made each time before analysis. The concentrations of Selenium also was determined using inductively Coupled plasma- Mass spectrometry to compare with AAS techniques.

2.3 Sample Preparation and Digestion

2.3.1 Materials

A 150 µL aliquot of serum samples were thawed at room temperature for 20 minutes and pipette into pre-cleaned polyethylene tubes. 150 µL of optima grade nitric acid and 100 µL of trace select grade hydrogen peroxide were then added to the samples. The tubes were tightly capped, centrifuged for 10 minutes at a speed of 4400 r/min in a centrifuge. Samples were then placed in a hot block digester and digested at 95°C for 90 minutes with the tube caps loosened. Following digestion the samples were diluted to 150 µL [15].

2.3.2 Procedure

Multi element working standards were prepared containing Se by diluting high purity 1000 mg L-1 stock solutions with deionized water and nitric acid. The standard solutions were kept at 4°C in dark room to reduce the risk of contamination. All works were carried out under clear room conditions. Ultra-pure de-ionized water was used for sample preparations. Standard solutions were prepared freshly from the stocks, with dilute nitric acid (3% v/v). In order to obtain appropriate. ICP-MS responses, the experiments were performed using different concentration levels.

2.3.3 Sample analysis

The digested serum samples were analyzed for selenium using Inductively Coupled plasma-Mass spectrometry (Agilent Technology, Japan). The analytical calibration method was accomplished with aqueous standards in 0.5% (v/v) HNO3. Fresh calibrations were made each time before analysis. Triplicates were carried out and the results were consistent.

2.4 Statistical Analysis

Our study as well as most other studies uses statistical analysis to determine the relationships between variables. The analysis is performed by

using the statistical package for the social sciences (SPSS, version 22) (SPSS Inc., Chicago, IL, USA) for windows. Continuous variables were expressed as mean ± standard deviation (SD).Pearson correlation test was used to correlate between different variables among the studied groups [16]. Pearson's correlation and analysis of variance (ANOVA) were conducted.

3. RESULTS AND DISCUSSION

3.1 Prevalence of Chronic Hepatitis B

Samples the detail of patients and healthy group are shown in Table 1.

The male patients were higher in number 84, while female patient were 76 patients from urban area were 60 patients (37.5%), while patients from rural area were 100 patients (62.5%), Table 2. This difference in statistically showed that increasing of the incidences of the disease in rural areas compared to the urban regions. This increasing of incidence can be attributed to lack of the health awareness and not to follow the instructions to avoid the spread of diseases. In addition, the people in rural areas did not immunize themselves by taking hepatitis B vaccine.

3.1.1 Serum liver markers

Viral load (HBV PCR) of the patients with chronic hepatitis B was measured from the present study revealed low serum albumin and higher serum S.ALT and S.AST in patients with chronic hepatitis B compared to those of healthy individuals. In addition, HBV-DNA was significantly higher in patients with chronic hepatitis B (Tables 3 and 4).

Table 1. Symbols and details for study groups

Symbols	Details	Numbers
1A	Patients HBeAg (+ve)normal liver function PCR= (>100000)	36
2A	Patients*, HBeAg (+ve)*abnormal liver function PCR= (>100000)	28
1B	Patients HBeAg (-ve) PCR= (>100000)	30
2B	Patients *HBeAg (-ve)* PCR=4000-100000	36
3B	Patients HBeAg (-ve) PCR=N0N	30
C	Healthy individuals	30

Table 2. Gender and residence distribution of groups for patients with chronic hepatitis B and healthy group

Parameters	1A	2A	1B	2B	3B	C	Total
Male	20	15	15	17	17	21	105
Female	16	13	15	19	13	9	85
Urban	9(25%)	8(28.5%)	9(30%)	18(50%)	16(53.3%)	16(53.3%)	76
Rural	27(75%)	20(71.4%)	21(70%)	18(50%)	14(46.6%)	14(46.6%)	114

Table 3. Serum levels T.S.B, ALT and AST of groups for HBV patients and healthy groups

Parameters	1A	2A	1B	2B	3B	C
T.S.B (mg/100 ml)	0.82±0.13	0.86±0.21	0.84±0.18	0.88±0.30	0.87±0.20	0.69± 0.06
ALT(IU/l)	20.44 ±6.70	38.38±17.50	31.85±15.16	23.77±11.33	32.30±12.70	23.66±5.40
AST(IU/l)	26.93±15.00	52.46±18.40	36.35±16.30	23.83±9.13	30.66±14.48	19.76±7.20
ALT\AST	0.90±0.40	0.82±0.49	0.95±0.43	1.21±0.70	1.24±0.61	1.37±0.64
Albumin (gm .dL^{-1})	3.98±0.60	4.17±0.43	4.00±0.37	4.17±0.69	4.05±0.59	4.38±0.33

Table 4. Viral load (HBV PCR) of patients with chronic hepatitis B

Parameters	1A	2A	1B	2B	3B
Level of HBV DNA (copies/ml)	1510055884	286109934.8	31818160*	33446.188*	NON

**Correlation is significant at the 0.05 level*

3.1.2 Pearson's correlation and ANOVA of liver function

The results of Pearson's correlation coefficients and their significant levels ($p<0.05$) are listed in Table 5. ALT/AST showed a positive and negative significant relationships with ALT and AST, respectively. ANOVA analysis (Appendix 1) showed significant differences (at $p < 0.05$) in all the liver functions indices between the groups 1A, 2A and 1B and the healthy individual as a control group. For the group 2B, there are significant differences in TSB and S.AST and insignificant differences in S.SAT, ALT/AST and Albumin liver function indices. ANOVA suggested significant differences in all liver functions except ALT/AST between the group 3B and the healthy individual. Significant differences were recorded in functions S.ALT and A.AST and insignificant differences in the other functions for group C.

Table 5. Pearson product moment correlations between biochemical parameters and HBV PCR in patient with chronic hepatitis B

	T.S.B	ALT	AST	ALT\AST	Albumin	HBVDNA
1A						
T.S.B	1					
ALT	.244	1				
AST	.039	.010	1			
ALT\AST	.077	*.620**	-.645*	1		
Albumin	-.412	-.226	-.001	-.239	1	
HBVDNA	.041	-.308	.338	-.313	.324	1
2A						
T.S.B	1					
ALT	.022	1				
AST	-.128	*.634*	1			
ALT\AST	.228	.395*	-.398*	1		
Albumin	.055	.017	-.016	.038	1	
HBVDNA	-.137	.187	.096	.063	.031	1
1B						
T.S.B	1					
ALT	.214	1				
AST	-.224	-.084	1			
ALT\AST	.135	.740*	-.575*	1		
Albumin	-.066	.087	.084	.032	1	
HBVDNA	-.125	-.191	.433	-0.300	.200	1
2B						
T.S.B	1					
ALT	-.029	1				
AST	-.133	-.068	1			
ALT\AST	.090	*.613**	*-.571**	1		
Albumin	-.222	-.157	.230	-.260	1	
HBVDNA	*.613**	.072	-.046	.214	-.229	1
3B						
T.S.B	1					
ALT	-.256	1				
AST	-.056	.176	1			
ALT\AST	-.215	*.538**	*-.594**	1		
Albumin	.151	-.030	-.125	.115	1	
HBVDNA						
C						
T.S.B	1					
ALT	-.097	1				
AST	.172	-.102	1			
ALT\AST	-.306	*.488**	*-.828**	1		
Albumin	-.130	-.003	-.080	.017	1	
HBVDNA						

Table 6. Selenium concentration for patients with chronic hepatitis B and healthy groups

Parameters	1A	2A	1B	2B	3B	C
Selenium ($\mu g\ L^{-1}$)	53.12±7.00	51.10±4.96	51.89±4.92	56.65±7.33	64.39±7.39	67.68±7.60

3.1.3 Selenium concentration

In this study, Selenium serum concentration was significantly lower in chronic hepatitis B patients in comparison to healthy individual, Table 6. The obtained results using atomic absorption spectrophotometer equipped with the hydride generation system. It is adopted in this study because the selenium concentrations in serum samples from one hundred sixty patients samples and thirty healthy individuals show that the content of selenium is relatively low, ranging from 29.1 to 80.9 µg L-1 from using HGAAS. While the obtained results using ICP-MS were less accurate in order to compare with the results of other studies. The results were also less accurate comparing with the results obtained from hydride generation technique. The results of ICP-MS were used only for statistical comparison.

3.1.4 Pearson's correlation and ANOVA of selenium

Pearson's correlation coefficients of Se and HBVDNA in HBV e Ag (+ve), HBV e Ag (-ve) and healthy individuals are shown in Table 7. A positive significant correlation (at $p < 0.05$) was observed between HBVDNA in group 1B and group 1A. Statistically, we found that there is no distinct comparison between the correlation relationships of Se level measured by AAS and that measured by ICP-MS as an analytical methods. The comparison showed only that the majority of correlation relationships for Se measured by ICP-MS were positive and for Se measured by AAS were negative. ANOVA results of Se levels between all groups patients and healthy individual were listed in Appendix 1. Significant differences were recorded for groups 1A, 1B and 2B and insignificant for groups 2A and 3B at $p < 0.05$. The comparison of Se level measured by GFAAS and ICP-MS was performed using ANOVA. Insignificant differences (at $p < 0.01$) were noted suggesting use any method without priority to measure the Se level in serum.

Comparison of the readings of Se level measured by AAS and ICP-MS was performed using scatter plotting, Fig. 1. The linear equation between y(AAS) and x (ICP-MS) was listed in the Figure. The slope of the line equals to -0.1492. Statistically, increasing of the slope value suggests significant differences in readings of Se level measured by AAS and ICP-MS. According to this result, we recommend use the common method (AAS) to measure Se level.

A significant decrease in serum levels of albumin in patients with chronic hepatitis B compared to healthy groups may suggest decreased hepatic production due to decreased liver function following hepatocellular disease. However, when it is certain that the cause of their alteration is liver disease, serum albumin levels is useful tests for monitoring liver synthetic activity [17]. Since AST activity is found in many tissues, it is not considered liver specific and increases in this enzyme may be associated with liver or muscle damage [18]. ALT, a cytosolic enzyme, is found in its highest concentrations in the liver and is more specific to the liver [19]. The most common causes of elevated AST levels are chronic hepatitis B autoimmune hepatitis, non-alcoholic steatohepatitis, hemochromatosis, Wilson's disease, celiac sprue, muscle damage and myocardial infarction [20]. Serum level of HBV DNA may be used as a major risk predictor for hepatocellular carcinoma, independent of HBeAg serostatus, serum ALT level, and the presence of liver cirrhosis. Monitoring the change in serum HBV DNA level is recommended for the management of chronic hepatitis B patients. Randomized controlled trials comparing different therapeutic strategies in patients with elevated serum HBV DNA level but a normal ALT level may further contribute to the development of appropriate treatment guidelines in these patients. These patients, especially those seronegative for HBeAg, account for an increasing majority of chronically infected individuals and are at an increased risk of future hepatocellular carcinoma [21]. In the present study, decrease in serum Selenium might indicate the development and progression of HBV, Fig. 2. It also links to the disease progress of some viral agents in relation to the biosynthesis of selenoproteins [22]. The selenium requirement for prevention of chronic disease has not yet been definitively determined [23]. On the other hand Khan et al. [14] also identified selenium deficiency in patients with viral hepatitis.

Table 7. Pearson' correlation of Se and HBVDNA for all groups HBV patients

Variables	Groups	Se	Se	Se	Se	HBVDNA	HBVDNA	HBVDNA	HBVDNA
Se	1A	1							
Se	2A	.183	1						
Se	1B	-.035	-.221						
				1					
Se	2B	.103	-.032	-.053	1				
HBVDNA	1A	.240	-.044	.039	.009				
						1			
HBVDNA	2A	-.189	-.326	-.102	.141	.253			
							1		
HBVDNA	1B	.088	.363	.059	-.030	*.569**	-.105	1	
HBVDNA	2B	-.096	.102	.134	.109	.010	.192	.428	
									1

**Correlation is significant at the 0.05 level*

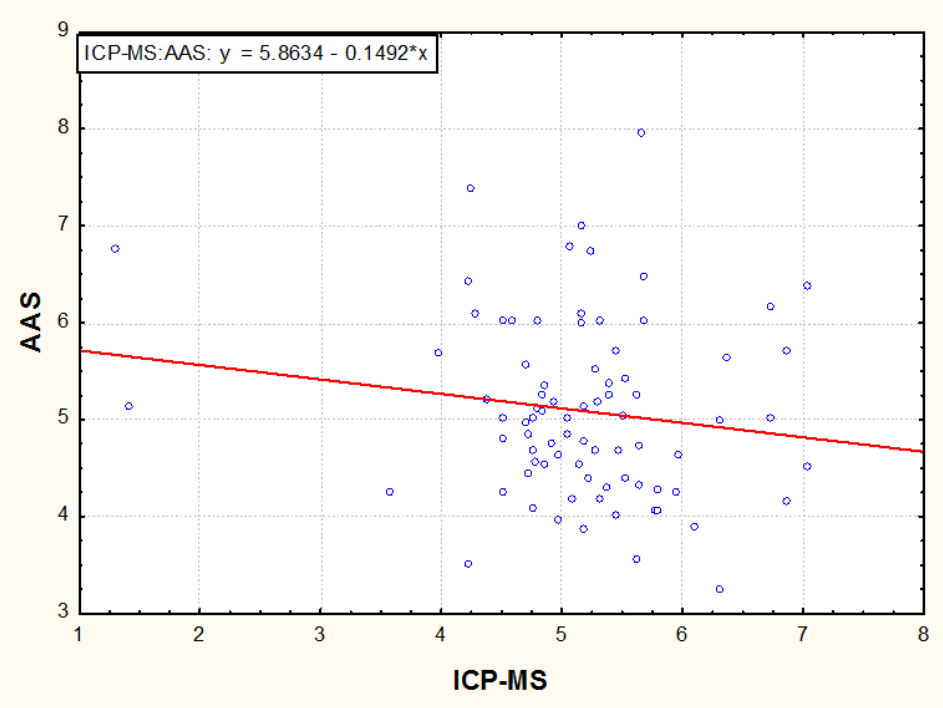

Fig. 1. Scatter plot between the selenium level measured by AAS and those measured by ICP-MS

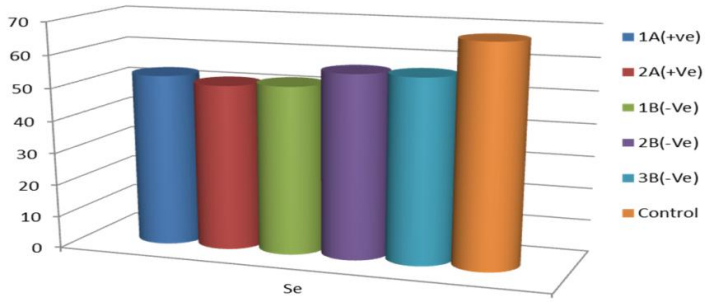

Fig. 2. Serum levels selenium of different groups for HBV patient and healthy individuals

4. CONCLUSION

According to the results of application of scatter plotting statistical technique, it has been shown Selenium concentration can be determinate by AAS better than ICP-MS. ICP-MS. The serum selenium concentrations were significantly lower in chronic hepatitis B patients than healthy individuals.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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