DOI: https://doi.org/10.54393/pbmj.v8i8.1279

PAKISTAN BIOMEDICAL JOURNAL

https://www.pakistanbmj.com/journal/index.php/pbmj/index

ISSN (E): 2709-2798, (P): 2709-278X

Volume 8, Issue 08 (August 2025)

Original Article

OPEN ACCESS

Comparative Analysis of Serum Trace and Heavy Metals in Hepatitis B, C, and D Patients from Shaheed Benazirabad, Pakistan

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ARTICLE INFO

Keywords:

Viral Hepatitis, Hepatitis B, Hepatitis C, Hepatitis D, Trace Metals, Heavy Metals, Serum Analysis

How to Cite:

Dahri, Z. A., Shah, A., Khuhawar, T. M. J., Shaheen, S., & Buriro, F. (2025). Comparative Analysis of Serum Trace and Heavy Metals in Hepatitis B, C, and D Patients from Shaheed Benazirabad, Pakistan: Analysis of Serum Trace and Heavy Metals in Hepatitis B, C, and D Patients. Pakistan BioMedical Journal, 8(8), 09–15. https://doi.org/10.54393/pbmj.v8i8.1279

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Received Date: 26th July, 2025

Revised Date: 8th August, 2025

Acceptance Date: 13th August, 2025

Published Date: 31st August, 2025

ABSTRACT

Trace and heavy metals play essential roles in liver metabolism and immune regulation, but their imbalance may exacerbate complications in viral hepatitis. **Objectives:** To compare serum levels of key trace elements (zinc [Zn], iron [Fe], copper [Cu]) and heavy metals (lead [Pb], cadmium [Cd], chromium [Cr], aluminum [Al], arsenic [As], manganese [Mn], nickel [Ni], cobalt [Co]) among patients with Hepatitis B (HBV), Hepatitis C (HCV), and Hepatitis D (HDV). **Methods:** A cross-sectional study was conducted on 130 patients (aged 15–70 years) in Shaheed Benazirabad, Pakistan (2022–2024). Serum metals were quantified using Flame and Graphite Furnace Atomic Absorption Spectrometry (FAAS/GFAAS). Statistical analyses included ANOVA/t-test for normally distributed data and Kruskal–Wallis/Mann–Whitney U for non-normal data. For metals with concentrations reported below the method's limit of detection (LOD), a value of half the LOD (LOD/2) was assigned for statistical analysis. This common strategy minimizes bias and allows for the inclusion of all data points in the analysis, preventing the loss of information that would occur from exclusion. **Results:** Cr was significantly elevated in HCV compared to HBV (p=0.0035). Manganese (Mn) was significantly reduced in HDV compared to HBV (p=0.011). Other metals (Zn, Fe, Cu, Pb, As, Al, Cd, Ni, Co) showed no statistically significant differences, although trends of accumulation were observed. **Conclusions:** Elevated chromium in HCV and reduced manganese in HDV suggest that hepatitis type influences metal homeostasis, potentially contributing to disease progression.

INTRODUCTION

Hepatitis is a transmissible inflammation of the liver, most often caused by viruses, which alters the functioning of the liver and causes accumulation of toxins. Symptoms are fever, nausea, joint pain, dark urine and abdominal pain [1, 2]. Essential micronutrients play a critical role in the liver metabolic activities including enzymatic activity, protein synthesis, immune system, and antioxidant protection [3, 4]. Reactive oxygen species (ROS) exacerbate liver damage, contributing to hepatocellular carcinoma, particularly in viral hepatitis [5]. Hepatitis A (HAV) does not progress to a chronic stage, whereas Hepatitis B (HBV) can become chronic in about 10% of cases, often driven by immune-mediated mechanisms [6, 7]. Hepatitis C (HCV) tends to persist due to inadequate antiviral immune responses. HDV and Hepatitis E (HEV) involve varying immune-pathogenic mechanisms, with HAV and HEV typically not leading to chronic disease [8]. Cytokines such as interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6), released during stress or infection, influence trace element levels as part of the immune response [9, 10]. Among these, zinc (Zn) is essential for immune integrity and liver function, impacting the activity of over 300 enzymes [11]. Zn deficiency contributes to complications in chronic liver disease, including hepatic

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encephalopathy. Studies have reported reduced plasma Zn levels in patients with HBV-related cirrhosis. Iron (Fe) and copper (Cu) levels are also altered during infection. Cu accumulation, particularly in chronic HCV, can induce oxidative damage and liver injury [12, 13]. Similarly, hepatic iron overload is associated with fibrosis, cirrhosis, and hepatocellular carcinoma, although direct causality remains uncertain [14, 15]. Notably, iron reduction has been shown to improve response to antiviral therapy [16]. Trace elements are crucial for protein synthesis, immune function, and pregnancy health [17]. Accurate measurement of these elements requires sensitive methods such as atomic absorption spectrometry (AAS) [18, 19], often aided by microwave-assisted digestion [20], which reduces acid consumption and vapor production [21, 22].

This study aims to explore liver disease complications in relation to demographic factors and to analyze serum levels of essential and heavy metals in affected patients.

METHODS

This analytical cross-sectional study included 130 viral hepatitis patients (aged 15–70 years, both sexes) recruited from Peoples Medical College Hospital (PMCH), Nawabshah, Rural Health Center (RHC) Shahpur Chakar, and nearby villages such as Qazi Muhammad Jumandahri and Ahmed Abad, covering adjoining areas of Shaheed Benazirabad (SBA). The Ethical Committee of the Institute of Biochemistry approved the study (Ref. IOB/294m/2022). All the participants signed informed consent and the study was performed according to the principles of the Declaration of Helsinki. The study period was from August 2022 to July 2024. The sample size of 130 patients was based on feasibility and availability of hepatitis B, C, and D cases within the study period at the selected hospitals. Although no formal power analysis was performed before data collection, this number exceeds the minimum recommended sample ($n \approx 90$) calculated post hoc using G*Power software (effect size $f = 0.25$, $\alpha = 0.05$, power = 0.80, three groups, one-way ANOVA). Thus, the study was sufficiently powered to detect medium differences in serum trace and heavy metal concentrations across the hepatitis groups. Patients were recruited through a consecutive sampling technique, enrolling all eligible hepatitis B, C, and D cases presenting at the study sites during the research period. Inclusion criteria were age 15–70 years, confirmed viral hepatitis diagnosis, and informed consent. Patients with co-infections, chronic kidney disease, or on metal supplements were excluded. From each participant, 7 mL of venous blood was drawn using sterile disposable syringes. Out of this, 3 mL was placed in EDTA tubes and stored at 4°C for lead (Pb) and cadmium (Cd) analysis. The remaining sample was transferred to gel tubes, allowed to clot, centrifuged at 3000 rpm for 10 minutes, and the serum was stored at –20°C in Eppendorf tubes for up to two months for the analysis of Zn, Cu, and Fe. Zinc, copper, and lead concentrations were measured using Flame Atomic Absorption Spectrometry (FAAS); cadmium was analyzed using Graphite Furnace Atomic Absorption Spectrometry (GFAAS); and iron levels were determined using a CECIL CE 1011 spectrophotometer with a human manual kit [23, 24]. Descriptive statistics were used to summarize demographic and biochemical data. Data distributions were assessed using the Shapiro–Wilk test [25, 26]. Normally distributed variables were summarized as mean ± SD, while skewed variables were expressed as median with interquartile range (IQR). Based on data distribution, independent t-tests and one-way ANOVA were applied to normally distributed variables [27], while Mann–Whitney U and Kruskal–Wallis tests were used for non-normally distributed data [28]. Appropriate statistical methods were applied to evaluate associations between trace metal levels and hepatitis across demographic groups. Boxplots were used for data visualization.

RESULTS

This study analyzed trace and heavy metal concentrations in HBV, HCV, and HDV patients, using the Shapiro–Wilk test to classify distributions with descriptive Statistics. Non-normally distributed metals—Al, Cd, Fe, Mn, Ni, Zn exhibited marked skewness and variability. Aluminium was elevated across all types, especially in HCV, indicating possible bioaccumulation. Cadmium had negative means, suggesting levels near detection limits, slightly higher in HDV. Iron showed extreme variability, particularly in HCV, pointing to potential overload. Manganese declined from HBV to HDV with decreasing variability, indicating tighter regulation. Nickel increased modestly from HBV to HDV, with detection issues implied by negative minima. Zinc remained stable, slightly higher in HCV. Normally distributed metals, As, Co, Cr, Cu, and Pb, showed consistent trends. Arsenic rose with disease progression. Cobalt remained negligible, likely due to low bioavailability. Chromium was highest among these metals, elevated and variable in HCV and HDV, suggesting altered metabolism. Copper remained stable. Lead showed high variability and right-skew in HCV and HDV, hinting at environmental or occupational exposure. Overall, chromium and lead showed the highest variability and concentration, while arsenic accumulated progressively, and cobalt appeared insignificant (Table 1).

DOI: https://doi.org/10.54393/pbmj.v8i8.1279

Table 1: Descriptive Statistics and Shapiro-Wilk Normality Test for Metal Variables

Non-Normal Variables: Variables that Follow A Non-Normal Distribution									
Variables	**Al-B**	**Al-C**	**Al-D**	**cd-B**	**cd-C**	**cd-D**	**fe-B**	**fe-C**	**fe-D**
Mean	2.26	2.54	2.16	-0.19	-0.19	-0.10	26.87	29.17	17.24
Median	1.54	1.89	1.50	-0.05	-0.03	-0.03	8.23	5.49	7.00
Std Dev	1.78	2.06	1.84	0.25	0.33	0.14	35.16	47.60	21.28
Min	0.91	0.82	0.82	-1.02	-1.44	-0.47	0.95	0.87	0.87
Max	10.10	10.10	10.10	-0.01	0.01	0.01	148.82	209.80	79.97
Shapiro Stat	0.64	0.73	0.62	0.75	0.64	0.75	0.75	0.63	0.74
Shapiro p-value	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Variables	**Mn-B**	**Mn-C**	**Mn-D**	**Ni-B**	**Ni-C**	**Ni-D**	**Zn-B**	**Zn-C**	**Zn-D**
Mean	0.05	0.04	0.04	0.03	0.04	0.05	0.91	1.01	0.91
Median	0.04	0.03	0.03	0.04	0.05	0.05	0.91	0.95	0.93
Std Dev	0.03	0.03	0.01	0.03	0.05	0.03	0.44	0.60	0.42
Min	0.02	0.02	0.02	-0.07	-0.14	0.00	0.33	0.26	0.26
Max	0.15	0.17	0.07	0.09	0.11	0.18	2.49	2.66	2.35
Shapiro Stat	0.81	0.65	0.87	0.93	0.83	0.87	0.83	0.85	0.91
Shapiro p-value	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.01
Normal Variables: Variables That Follow A Normal Distribution									
Variable	**As-B**	**As-C**	**As-D**	**Co-B**	**Co-C**	**Co-D**	**Cr-B**	**Cr-C**	**Cr-D**
Mean	0.03	0.04	0.04	-0.03	-0.04	-0.03	0.10	0.12	0.12
Median	0.03	0.04	0.04	-0.03	-0.03	-0.03	0.09	0.12	0.11
Std Dev	0.02	0.02	0.02	0.01	0.02	0.01	0.02	0.03	0.04
Min	-0.01	-0.01	0.02	-0.06	-0.06	-0.06	0.03	0.07	0.01
Max	0.07	0.07	0.07	-0.01	0.00	0.00	0.16	0.17	0.19
Shapiro Stat	0.98	0.99	0.97	0.98	0.97	0.97	0.94	0.94	0.95
Shapiro p-value	0.68	0.94	0.44	0.80	0.53	0.54	0.11	0.08	0.19
Variables	**Cu-B**	**Cu-C**	**Cu-D**	**Pb-B**	**Pb-C**	**Pb-D**	**-**	**-**	**-**
Mean	0.07	0.07	0.08	0.11	0.11	0.11	-	-	-
Median	0.07	0.07	0.08	0.13	0.13	0.12	-	-	-
Std Dev	0.04	0.05	0.04	0.11	0.11	0.10	-	-	-
Min	0.01	0.00	0.00	-0.07	-0.06	-0.06	-	-	-
Max	0.18	0.19	0.19	0.34	0.36	0.36	-	-	-
Shapiro Stat	0.94	0.97	0.98	0.96	0.95	0.97	-	-	-
Shapiro p-value	0.09	0.41	0.91	0.24	0.15	0.42	-	-	-

Boxplot visualizations were used to assess differences in the concentrations of As, Co, Cr, Cu, and Pb among patients with Hepatitis B, C, and D. The boxplot for Chromium (Cr) showed a clearly elevated median and wider spread in Hepatitis C patients compared to groups B and D, indicating a notable difference in distribution. This visual trend was supported by the ANOVA result (p = 0.0092), confirming a statistically significant variation. In contrast, the boxplots for Arsenic, Cobalt, Copper, and Lead displayed overlapping medians, similar interquartile ranges, and consistent overall shapes across all three groups. These visual patterns suggest no meaningful differences in the distributions of these four metals. Thus, boxplot analysis highlights Chromium as the only metal with a distinct concentration pattern, particularly elevated in Hepatitis C patients, while the other metals remained visually consistent regardless of hepatitis type (Figure 1).

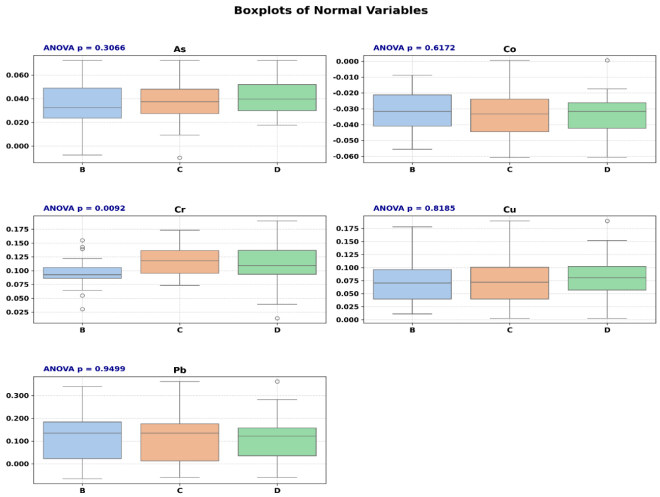

Figure 1: Boxplot of Normally Distributed Metal Variables

Boxplot visualizations were used to assess differences in the concentrations of Al, Cd, Fe, Mn, Ni, and Zn among groups B, C, and D. The boxplot for Manganese (Mn) revealed a noticeably higher median and broader spread in Group B compared to Groups C and D, suggesting a distinct variation in its distribution. This visual pattern was supported by the Kruskal-Wallis test (p = 0.0352), indicating a statistically significant difference. In contrast, the boxplots for Aluminum (Al), Cadmium (Cd), Iron (Fe), Nickel (Ni), and Zinc (Zn) showed overlapping medians, comparable interquartile ranges, and similar distribution shapes across all three groups. These consistent visual patterns imply no substantial differences in the distributions of these five metals. Therefore, the boxplot analysis highlights Manganese as the only element with a distinct concentration pattern, particularly elevated in Group B, while the others remained visually uniform across the groups (Figure 2).

DOI: https://doi.org/10.54393/pbmj.v8i8.1279

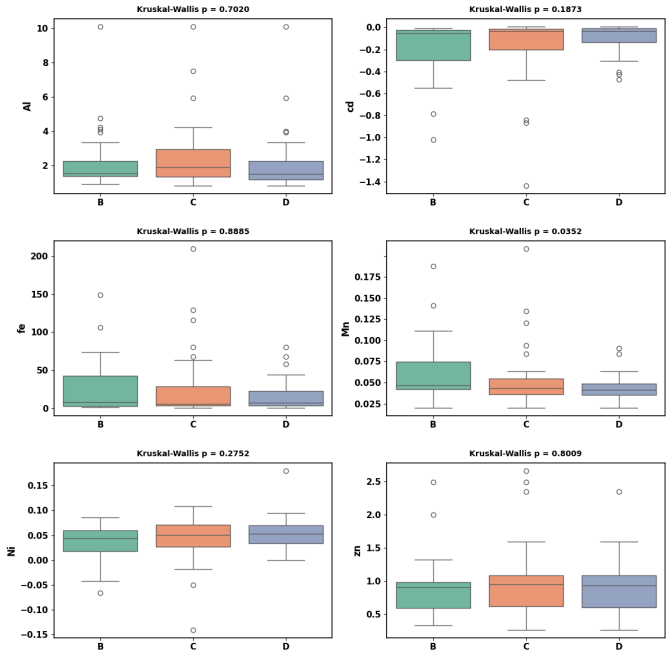

Figure 2: Boxplot of Non-Normally Distributed Metal Variables

To investigate potential differences in heavy metal concentrations among patients with Hepatitis B (HBV), Hepatitis C (HCV), and Hepatitis D (HDV), statistical analyses including One-Way ANOVA and independent sample t-tests were conducted for each metal. The analysis revealed that chromium (Cr) was the only metal showing a statistically significant difference among the three groups. The ANOVA result for chromium was F = 4.9386 with a p-value of 0.0092, indicating a significant variation. Specifically, the pairwise comparison between HBV and HCV showed a significant difference (t = -3.4058, p=0.0035), suggesting elevated chromium concentrations in HCV patients. No significant differences were observed between HBV and HDV or between HCV and HDV. In contrast, arsenic (As), cobalt (Co), copper (Cu), and lead (Pb) showed no statistically significant differences across the hepatitis types. Both ANOVA and pairwise t-tests confirmed uniform concentrations of these metals among all patient groups, indicating that their levels are not influenced by hepatitis type (Table 2).

Table 2: Hypothesis Testing of Normally Distributed Metal Variables Using Parametric Tests

Test	One-Way ANOVA	T-test			One-Way ANOVA	T-test		
		B vs C	B vs D	C vs D		B vs C	B vs D	C vs D
As					**Co**			
Statistic	1.198	-0.506	-1.522	-1.069	0.4851	0.9429	0.7783	-0.2032
p-value	0.307	1	0.4	0.869	0.6172	1	1	1
Cr					**Pb**			
Statistic	4.9386	-3.4058	-2.3491	0.4457	0.0515	-0.2238	0.0745	0.3165
p-value	0.0092	0.0035	0.0664	1	0.9499	1	1	1
Cu					-			
Statistic	0.2007	-0.0296	-0.5737	-0.536				
p-value	0.8185	1	1	1				

To investigate potential differences in heavy metal concentrations among patients with Hepatitis B (HBV), Hepatitis C (HCV), and Hepatitis D (HDV), non-parametric statistical tests were used due to non-normal data distribution. The Kruskal-Wallis test assessed overall group differences, followed by Mann-Whitney U tests for pairwise comparisons. Among the metals analyzed, only manganese (Mn) showed a statistically significant difference across the hepatitis types. The Kruskal-Wallis test yielded a statistic of 6.6921 with a p-value of 0.0352, indicating significant variation. Pairwise comparisons revealed a significant difference between HBV and HDV (U=661.5, p=0.011), while differences between HBV and HCV and between HCV and HDV were not significant. In contrast, aluminium (Al), cadmium (Cd), iron (Fe), nickel (Ni), and zinc (Zn) showed no statistically significant differences across the groups. Both Kruskal-Wallis and Mann-Whitney U tests confirmed that concentrations of these metals remained consistent among all hepatitis types, suggesting they are not influenced by hepatitis infection status (Table 3).

Table 3: Hypothesis Testing of Non-Normally Distributed Metal Variables Using Non-Parametric Tests

Test	Kruskal-Wallis	Mann-Whitney U			Kruskal-Wallis	Mann-Whitney U		
		B vs C	B vs D	C vs D		B vs C	B vs D	C vs D
Ai					**Cd**			
Statistic	0.7075	460.5	520	538.5	3.3496	397.5	351.5	439.5
p-value	0.702	0.7836	0.5829	0.4181	0.1873	0.2454	0.0704	0.5684
Fe					**Mn**			
Statistic	0.2365	487.5	516	502.5	6.6921	605	661.5	526.5
p-value	0.8885	0.9271	0.6221	0.7621	0.0352	0.0808	0.011	0.5216
Ni					**Zn**			
Statistic	2.5808	400	370	453.5	0.4439	435.5	456	507.5
p-value	0.2752	0.26	0.1214	0.709	0.8009	0.5309	0.7354	0.709

DISCUSSION

This study revealed disease-specific alterations in trace and heavy metal concentrations among patients with hepatitis B, C, and D, highlighting the importance of metal metabolism in viral hepatopathology. The significantly elevated chromium (Cr) levels observed in HCV patients compared to HBV are of particular interest. Chromium has been implicated in oxidative stress-mediated hepatocellular damage and fibrosis [29]. The association between Cr dysregulation and chronic HCV infection may reflect virus-induced disruption of redox balance and warrants consideration as a potential biomarker of disease severity. Manganese (Mn) levels showed a progressive decline from HBV to HDV, which may indicate differences in

DOI: https://doi.org/10.54393/pbmj.v8i8.1279

hepatic processing or biliary excretion. Previous studies have emphasized the dual role of Mn in health and disease, with excess linked to neurotoxicity and deficiency contributing to impaired enzymatic activity [30]. This trend suggests that Mn metabolism could be differentially affected by the viral etiology of hepatitis, although mechanistic clarification requires further research. Although aluminium (Al), cadmium (Cd), and nickel (Ni) levels were elevated but non-significant, their presence underscores the possible contribution of environmental exposures to liver injury in chronic viral hepatitis. Similar findings in experimental hepatology indicate that such metals exacerbate oxidative stress and mitochondrial dysfunction [29]. These results suggest that subclinical metal accumulation may contribute cumulatively to hepatocellular injury without clear disease-specific patterns. Iron (Fe) variability, especially in HCV patients, is notable. While our findings were not statistically significant, iron overload is a recognized cofactor for hepatic fibrosis and carcinogenesis in chronic liver disease [31]. The absence of a strong association in this study may reflect the need for larger cohorts or stratification by fibrosis stage. Similarly, copper (Cu) and lead (Pb) showed wide inter-individual variability, likely influenced by heterogeneous environmental or occupational exposures rather than direct viral effects [32]. Interestingly, zinc (Zn) levels remained stable across all groups despite its essential role in hepato-protection and immune modulation. Prior studies have reported zinc depletion in advanced liver disease, particularly cirrhosis [30]. Our results may suggest either compensatory mechanisms at earlier disease stages or uniform depletion across hepatitis types that obscures group-specific differences. Taken together, these findings support the hypothesis that viral hepatitis not only causes direct hepatocellular damage but also perturbs systemic metal homeostasis. This dysregulation may contribute to oxidative injury, altered immune responses, and progression to fibrosis or cirrhosis. The novelty of this study lies in comparing multiple viral hepatitis types, revealing both shared and unique patterns of metal imbalance. Clinically, trace metal profiling could aid in risk stratification and guide supportive interventions such as nutritional correction or chelation in selected cases. However, this study has limitations, including a modest sample size, a lack of dietary and occupational exposure data, and a cross-sectional design, which precludes causal inference.

CONCLUSIONS

This study demonstrates that specific trace and heavy metals, particularly chromium and manganese, exhibit significant variations among patients with different types of viral hepatitis. Chromium levels were notably elevated in hepatitis C, while manganese showed a progressive decline from hepatitis B to hepatitis D. Although other metals did not display strong statistical associations, their potential contribution to liver dysfunction cannot be excluded and warrants continued monitoring. Overall, these findings provide deeper biochemical insights into viral hepatitis and underscore the importance of incorporating trace and heavy metal profiling into future diagnostic, prognostic, and therapeutic strategies.

Authors Contribution

Conceptualization: ZAD
Methodology: ZAD, AS
Formal analysis: ZAD, AS, TMJK, SS, FB
Writing review and editing: ZAD, SS

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Source of Funding

The author received no financial support for the research, authorship and/or publication of this article.

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