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TOXICITY STUDY IN CYPRINUS CARPIO FOR COMPOSITION OF HEAVY METALS USING FTIR AND AMINO ACID ANALYSIS

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Abstract
Mercury (Hg) and lead (Pb) causes aquatic pollution and affects cell growth and pigment content of marine microalga. In this study mercury (Hg) and lead (Pb) effects the biochemical constituents of kidney, liver and brain of Cyprinus carpio by using Fourier-transform infrared (FT-IR) techniques was examined. Significant variations in absorbance intensities between the exposed liver tissues and the control liver tissues were found by FT-IR, indicating changes to important metabolic components. There was a significant increase in fifteen amino acids levels in liver of Cyprinus carpio when compared with the control, mercury and Lead treated liver. According to an examination of amino acids, Pb has a stronger affinity for tissues than Hg. Between 1620 and 1640 cm-1, absorption is brought on by the beta sheet structure.

Keywords: Cyprinus carpio, Mercury, Lead, Fourier Transform Infrared Mass Spectroscopic (FTIR-MS), Amino Acid.

Introduction
Mercury (Hg) was once known as hydrargyrum and is now more generally known as quicksilver. Mercury poisoning can be brought on by swallowing any form of mercury, inhaling vapours, or being exposed to water-soluble forms of mercury (such mercuric chloride or methylmercury). Mercury and the majority of its compounds must be handled carefully since they are highly poisonous. When spills of mercury occur (for example, from some thermometers or fluorescent light bulbs), special cleaning techniques are used to prevent exposure and control the spill. Organic

forms of mercury, such methylmercury and dimethylmercury, are the most hazardous. Both acute and chronic poisoning from mercury are possible. Natural concentrations of mercury, frequently in the form of the extremely lethal organic mercury complex methylmercury, are common in the bodies of fish and shellfish. By microbial mediation to the water column and sediments, Hg is changed into methylmercury in the environment. Fish swallow methylmercury, one of the most poisonous forms of mercury, which bioaccumulates in the food chain (Burjer et al,2005). Fish and seafood intake is the main way that humans are exposed to mercury.(Hightower et al,2003) The World Health Organization (WHO) limits the amount of mercury in fish that can be consumed by humans to 0.5 mg/kg.(World Health Organization,1996).

Lead (Pb) is a neurotoxin that accumulates in soft tissues and bones; it damages the nervous system and interferes with the function of biological enzymes, causing neurological disorders ranging from behavioral problems to brain damage, and also affects general health, cardiovascular, and renal systems. Aquatic organisms can bioaccumulate Pb through their feed and drinking water. Pb builds up in a number of fish organs, including the digestive system, gills, liver, spleen, and kidneys (Jezierska et al, 2006) When lead passes the blood-brain barrier, it can have a number of negative impacts on fish's bodies, well-being, and lifespan (Hodson et al).

Protein, peptide, and amino acid-containing substances can have their amino acid content determined via amino acid analysis. The majority of the time, proteins are long, linear polymers of amino acids that are joined together by peptide bonds. These peptide bonds must be hydrolyzed as the initial step in amino acid analysis. After that, the released amino acids are separated, found, and measured. HCl acid hydrolysis was used to develop the technique for the first time by Moore, Stein, and colleagues in the 1950s, and despite a lot of work by many people, the fundamental approach hasn't altered much over the years. The overview and strategic planning for amino acid analysis are covered in this subject, along with a variety of approaches and problems.

The biophysical technique known as Fourrier-transform infrared (FTIR) spectroscopy is used in biochemical analysis to analyse the structures of proteins, lipids, carbohydrates, and nucleic acids as well as to provide data from all tissues' constituents. It is a crucial method for examining molecular alterations in biological samples. Due to its simplicity, FTIR is a vibrational spectroscopic technique that is frequently utilized (Peak et al,2005) Additionally, this technique is quick, sensitive, and simple to use, and it provides a precise measurement without the need for external calibration (Depguch, 2017). This method allows for quick data collection and allows for the study of materials in any condition. The absorption peaks of an infrared spectrum, which correspond to the frequencies of vibrations between the bonds of the atoms that make up a sample, are like the fingerprints of the sample. Another clear indicator of the quantity of macromolecules present in the spectrum is the magnitude of the peak (Maquelin et al, 2003). FTIR spectroscopy offers qualitative biochemical data for the evaluation of structural and functional changes of macromolecules in biological samples (Naumann et al, 1991). Utilizing infrared (IR) absorption spectra, FTIR spectroscopy is a productive and trustworthy method for determining the

biochemical signature of intricate biological systems from small samples (Cakmak et al,2006) The current study employed FTIR to assess changes in the brain, kidney and liver of Cyprinus carpio.

Materials and Methods

Amino Acid Analysis

Principles of Amino Acid Analysis of Mercury and Lead: Amino acid analysis was carried out by using Beckman Model (118 BL and 119 CL method) (1990). The loading of the sample (100 ml) is done at the sample injector. The buffer pump and the Ninhydrin pump were put on. The function of the pump was to pump the eluant buffer through the column at a constant rate. The retention time of each amino acid was constant. As the eluant passes through the column at a constant set of parameters, like temperature of the column, buffer flow rate, concentration and pH of the buffer, it eluated the amino acid. The effluent which carried the amino acid was met with a stream of Ninhydrine reagent, which was deliverd by another Ninhydrine pump. The mixture of the effluent and Ninhydrine reagent were made to pass through a reaction coil kept immersed in boiling water bath. The color development took place depending on the concentration of the particular amino acid. The intensity of the colored complex was read by means of cuvette in a Builtin calorimeter. All the amino acids gave a purple color in the reaction with Ninhydrine reagent which was read at 570nm excepting Proline and Hydroxyproline which give yellow color and which was read at 440 nm. The amino acids were recorded in the form of peaks and integrated with reference to a standard run of amino acids. The area of the curve of the sample was compared with that of the standard and concentration of the amino acid in the sample was calculated.

Area of the amino acid in the sample X Concentration (nm) of the standard = nm of the amino acid in the sample

$$\frac{\text{Area of the amino acid in the standard}}{}$$

This was expressed in µM/L of the sample.

Items	Buffer A 0.2N, pH 2.83	Buffer B 0.2N, pH 3.70	Buffer C 1.0 N, pH 3.75
Citric acid	42.09 g	42.09g	56.08 g
LiOH monohydrate	33.62g	33.62g	167.76 g
Concentrated HCL	56.0 ml	49.0 ml	288.0 ml
Tiodiglycol	40.0 ml	40.0 ml	--
Ockanoic acid	0.4 ml	0.4 ml	0.4 ml
Isopropanol	--	--	240.0 ml
Final volume	4 L	4 L	4240 ml

Sample preparations for Amino acid analysis of Mercury and Lead: To 0.1 ml of the cell lysate, from control, mercury and cadmium treated kidney, liver and brain added 50mg Sulphosalicylic acid (SSA). Mixed and centrifuged for 10 minutes at 4000 rpm. Decanted and diluted the supernatant 1:3 with diluting buffer. Lithium citrate buffers were used for the elution of amino acids.

Ninhydrine Reagent

Methylcellosolve (peroxide free) - 2.85L

Sodium acetate buffer (4 Normal) - 950 ml

Ninhydrine - 76.0 g

Stannouschloride - 0.50 g

The ninhydrine reagent was kept in an amber bottle under nitrogen pressure.

FTIR MS - Binding Site Determination

The FTIR-MS analysis could bring out the binding sites of the various amino acids present in the stress protein which is triggered by the corresponding gene. The samples were dehydrated with graded alcohol series first and then fixed in glutaraldehyde fixative. Later prepared the potassium bromide (KBr) pellets of the dried sample and introduced to the FTIR MS (Nicolet 700 FTIR Mass Spectrophotometer) monitor and obtained the graphical representations. Both these experiments proved that the HSP 70 gene is activated by bioaccumulation and is having amino acid combinations and concentration.

Results

Physicochemical analysis of freshwater: The physical and chemical properties of the freshwater in which C. carpio was found and analyzed for the presence of dissolved salts and toxic metals. The dissolved oxygen content was found to be 6.2 0.4 mg/l, with a neutral pH of 7.3 0.01. The total hardness of the water was determined to be 345 99 mg/l, whereas the free CO2 concentration was calculated to be 2.1-0.12 mg/l. In the tested water, there were no residues of mercury or cadmium, though there were traces of calcium (81 88 mg/l) and magnesium (34 mg/l). In addition, the water sample contained high levels of sulphates and chlorides (Table 1). This test showed that C. carpio had not been exposed to mercury before the trial began.

Table 1. The physicochemical characteristics of water were analyzed by using standard methods (APHA, 1995 and 2005).

Parameters	**Values**
Dissolved Oxygen	6.2 ± 0.4 mg/l
pH	7.3 ± 0.01 m
Temperature	28 ± 2°C
Total hardness	345 ± 99 mg/l
Free CO_2	2.1 ± 0.12 mg/l

Ca	81 ± 88 mg/l
Mg	34 ± 0.0 mg/l
Hg	Nil
Sulphates	112 ± 0.9 mg/l
Chlorides	234 ± 22 mg/l
Pb	Nil
Specific conductance	2340 (Micro siemens/cm) at 2°C

Amino Acid Analysis of Mercury and Lead

Table 2 and Figures 1A, Figure 1B, Figure 1C, Amino Acid Analysis Showing the Amino Acid concentration in the Control, Hg and Pb treated Liver of Cyprinus carpio

Figure 1 A – Amino acid profile present in the Control Liver of *Cyprinus carpio* on 28th day of exposure.

Figure 1 B – Amino acid profile present in the Mercury treated Liver of *Cyprinus carpio* on 28th day of exposure.

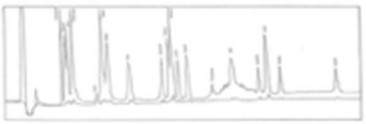
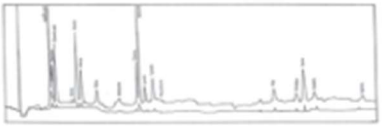


Figure 1 C – Amino acid profile present in the Lead treated Liver of *Cyprinus carpio* on 28th day of exposure.



Amino Acid	Amino acid concentration (mg/l)		
	Control Liver	Mercury treated Liver	Lead treated Liver
Aspartic acid	57.94	201.22	72.04
Threonine	46.54	108.06	47.87
Serine	41.29	74.97	49.10
Glutamic acid	39.43	161.34	126.18
Proline	83.49	25.96	17.01
Glycine	43.91	155.99	81.99
Alanine	67.89	105.99	78.90
Valine	36.17	79.58	60.98
Methionine	31.43	50.97	33.28
Cysteine	42.83	262.60	69.86
Isoleucine	56.92	169.00	172.0
Leucine	24.33	137.85	61.35
Tyrosine	26.93	154.08	49.79
Phenylalanine	–	35.06	03.61
Lysine	35.43	207.99	128.01
Histidine	18.99	46.15	09.19
Arginine	03.49	69.08	11.07

Table 3and Figures 1D, Figure 1E, Figure 1F Amino Acid Analysis Showing the Amino Acid concentration in the Control, Hg and Pb treated Brain of Cyprinus carpio.

Figure 1 D – Amino acid profile present in the Control Brain of *Cyprinus carpio* on 28th day of exposure.

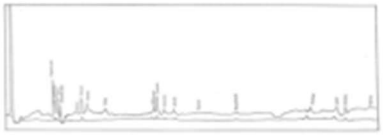


Figure 1 E – Amino acid profile present in the Mercury treated Brain of *Cyprinus carpio* on 28th day of exposure.

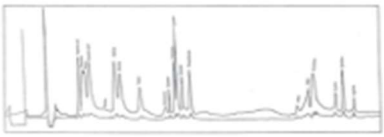


Figure 1 F – Amino acid profile present in the Lead treated Brain of *Cyprinus carpio* on 28th day of exposure.

Amino Acid	Amino acid concentration (mg/l)		
	Control Brain	Mercury treated Brain	Lead treated Brain
Aspartic acid	59.80	207.98	89.07
Threonine	22.91	107.92	39.29
Serine	51.06	85.56	77.60
Glutamic acid	65.01	-	-
Proline	24.98	39.15	97.83
Glycine	47.23	404.53	118.32
Alanine	28.82	122.39	89.89
Valine	48.90	184.61	26.88
Methionine	20.01	48.14	28.97
Cystine	11.02	90.99	34.94
Isoleucine	13.39	185.10	114.83
Leucine	55.45	127.18	125.01
Tyrosine	10.18	97.15	57.04
Phenylalanine	20.19	61.71	68.16
Lysine	48.99	170.77	69.89
Histidine	65.21	137.21	89.68

Table 4 and Figure1G, Figure 1H, Figure 1I Amino Acid Analysis Showing the Amino Acid concentration in the Control, Hg and Pb treated Kidney of Cyprinus carpio

Figure 1 G – Amino acid profile present in the Control Kidney of *Cyprinus carpio* on 28th day of exposure

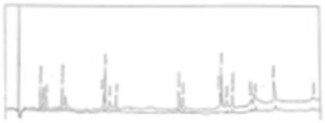


Figure 1 H – Amino acid profile present in the Mercury treated Kidney of *Cyprinus carpio* on 28th day of exposure.

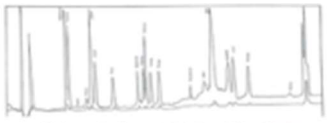


Figure 1 I – Amino acid profile present in the lead treated Kidney of *Cyprinus carpio* on 28th day of exposure.

Amino Acid	Amino acid concentration (mg/l)		
	Control Kidney	Mercury treated Kidney	Lead treated Kidney
Aspartic acid	54.82	284.99	80.90
Threonine	34.95	191.60	99.26
Serine	44.53	188.41	52.80
Glutamic acid	11.16	200.89	114.98
Proline	18.62	63.99	69.14
Glycine	49.89	197.47	66.69
Alanine	25.88	123.82	54.06
Valine	47.96	116.01	44.62
Methionine	69.43	28.12	98.31
Cystine	48.28	291.42	18.27
Isoleucine	35.63	96.82	34.87
Leucine	39.83	207.04	77.22
Tyrosine	82.31	132.08	23.42
Phenylalanine	83.29	171.39	30.22
Lysine	26.88	82.83	69.84
Histidine	69.98	74.68	34.87
Arginine	61.79	39.16	36.99

FTIR analysis of mercury and lead

Figure 1 FTIR-MS Values for Liver

Functional Groups	CL	HgL	PbL
$-NH_2$	3300 cm^{-1} to 3440 cm^{-1}	3300 cm^{-1} to 3340 cm^{-1}	3340 cm^{-1} to 3420 cm^{-1}
-CO	1630 cm^{-1}	1620 cm^{-1}	1640 cm^{-1}
$-CH_2$	1380 cm^{-1}	1360 cm^{-1}	1320 cm^{-1}

Figure 2 FTIR-MS Values for Brain

Functional Groups	CBr	HgBr	PbBr
$-NH_2$	3440 cm^{-1} to 3840 cm^{-1}	3300 cm^{-1} to 3900 cm^{-1}	3350 cm^{-1} to 3680 cm^{-1}
-CO	2900 cm^{-1} to 1680 cm^{-1}	2900 cm^{-1} to 1630 cm^{-1}	2910 cm^{-1} to 1630 cm^{-1}
$-CH_2$	1380 cm^{-1}	1330 cm^{-1}	1350 cm^{-1}

Figure 3 FTIR-MS Values for Kidney

Functional Groups	CK	HgK	PbK
$-NH_2$	3210 cm^{-1} to 3600 cm^{-1}	3300 cm^{-1} to 3360 cm^{-1}	3360 cm^{-1} to 3596 cm^{-1}
-CO	1620 cm^{-1}	2860 cm^{-1} to 1620 cm^{-1}	2860 cm^{-1} to 1620 cm^{-1}
$-CH_2$	1330 cm^{-1}	1330 cm^{-1}	1330 cm^{-1}

Discussion

The liver of Cyprinus carpio had an increase in fifteen of the seventeen amino acids compared to the control, mercury, and lead-treated livers. The control liver had a higher concentration of alanine, which is thought to be a major form of inter-tissue transport for amino acid carbon. Alanine increases in muscle, plasma, and the liver. (Mommsen et al., 1980 and Moses et al., 2018) The amino acids released by cortisol-mediated proteolysis can also be transaminated with pyruvate to create alanine (Henderson and Garland, 1980). In many transporter proteins, SH groups on cysteine residues account for functional alterations that represent regulatory mechanisms since they are highly sensitive (Pan et al., 1995; Grunewald et al., 1998; Seal and Amara, 1998 ; Zarbiv et al., 1998). The metals Hg, Pb, Cu, Cd, and Zn have the highest binding affinities for cysteine in stress proteins. Stress proteins have 60–61 amino acids, of which 20 are cysteines, as one of their distinguishing features. The cysteines are ordered in the following ways: Cys-X-X-Cys, Cys-X, Cys, or Cys-Cys. The absence of histidine and aromatic amino acids in the stress protein. Two metal-binding domains of the protein, each able to bind three to four metal ions, make up the structure (Kagi and Schaffer, 1988). Out of the seventeen amino acids found in the brain of Cyprinus carpio, the current study also revealed a general increase in trend for twelve of them. In the current investigation, glutamic acid levels in the control brain were noticeably greater. The concentration of glutamic acid was significantly reduced in the mercury and lead-treated brain.Twelve amino acids had an increase in trend in their respective quantities in the kidney case. The amino peak values in the spectra of CL, PbL, and HgL showed a significant difference, but all other peaks remained constant, demonstrating that the metals are bonded to the amino sites. The N-electron atom's is taken up by Hg and Pb, who then form a covalent link with it. As a result, the tissues' amino groups serve as the metals' binding sites. Because to the H-bonding in the amino groups, the peaks of the -NH2 group are wider than the carbonyl peaks. On exposure, the amino acid's -NH2 begins to coordinate with Hg or Pb, and complexes involving metal-nitrogen bonds start to form. This shift in the amino acid's -NH2 may be seen in the IR spectra. The peaks of the

tissues' amino group that were recorded before and after interacting with the metals changed, according to the IR spectra that were taken. Pb is more tightly bonded to the tissues than Hg. The beta sheet structure is responsible for absorption between 1620 and 1640 cm-1. (Byler and Susi, 1986) The FTIR absorption produced by the alpha-helical configuration falls between 1650 and 1658 cm-1 (Byler and Susi, 1986; Surewicz and Mantsch, 1988; Haris and Chapman, 1992). The ATPase-active domain and the peptide-binding domain make up the structure of HSP70 (Moise et al., 2019) The presence of regions with extremely low structural stability and regions with high stability define the binding sites' dual nature. The poor stability sections are frequently loops that, upon binding, become stable and cover a large percentage of low molecular weight ligands. A key function in the communication of information to the catalytic site of allosteric enzymes. Small ligands are advantageous for improving binding affinity. SEM and EDAX were used to determine surface bioaccumulation, histopathological alterations, and total energy loss. Studies on the relationship between the total surface bioaccumulation and the tissue damage were conducted, and a favourable relationship was found. The molecular bioaccumulation in ppb levels in the liver, kidney, and brain of Cyprinus carpio could be found using ICP-MS tests, and it was found to be positively connected with the accumulation of heavy metal. Studies using the FTIR-MS technique were carried out to identify the various functional groups and the places where amino acids bind. Automated Ion Exchange Chromatography, Atomic Absorption Spectroscopy, and Amino Acid Analyzer were used to quantitatively determine the total free amino acids.

Conclusion

This study reveals significant biochemical outcomes of Hg and Pb in fish liver, kidney and brain and reveals that complicated alterations due to the exposure to heavy metal ions in cytochrome P450-dependent metabolism in fish. Comparing the livers of Cyprinus carpio to control, mercury- and lead-treated livers, a rise in fifteen of the seventeen amino acids was observed. Alanine, which is regarded to be a significant means of inter-tissue transfer for amino acid carbon, was found in higher concentrations in the control liver. The current study also indicated a general increase in trend for twelve of the seventeen amino acids detected in the brain of Cyprinus carpio. In the current study, glutamic acid concentrations in the control brain were significantly higher. The brain of those exposed to mercury and lead had considerably less glutamic acid. In the kidney case, the trend for each of the twelve amino acids' individual levels was upward. ICP-MS tests were used to identify the molecular bioaccumulation in ppb levels in the liver, kidney, and brain of Cyprinus carpio, and it was discovered to be positively associated with the accumulation of heavy metal. The IR spectra were collected before and after the tissues interacted with the metals, and the peaks of the tissues' amino group were recorded before and after. Compared to Hg, Pb has a stronger connection to the tissues. Between 1620 and 1640 cm-1, absorption is caused by the beta sheet structure.

Declaration of competing interest

The authors alone are responsible for the content and writing of this article. We declare that there are no competing interests.

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