FTIR STUDY OF ARSENIC INDUCED BIOCHEMICAL CHANGES ON THE LIVER TISSUES OF FRESH WATER FINGERLINGS *LABEO ROHITA*

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Abstract. Arsenic is an ubiquitous element present in various compounds throughout the earth's crust and it is identified as a human carcinogen. In this study, we focus on the arsenic induced biochemical changes in the liver tissues of freshwater fish *Labeo rohita*, using Fourier transform infrared (FTIR) spectroscopy. The spectrum of liver tissue is quite complex and contains several bands arising from the contribution of different functional groups belonging to proteins, lipids and others. The FTIR spectra reveal significant differences in absorbance intensities and areas between control and arsenic intoxicated liver tissues; this variation shows the alteration in biochemical contents like proteins and lipids in the liver tissues due to arsenic intoxication. The detailed spectral analyses were performed in two distinct wave number regions, namely 3600–2800 cm^{-1}, and 1800–1000 cm^{-1} and curve fitting analyses were performed in the amide I region for the detailed analysis of protein secondary structures. In the present study, we observed a decrease in α–helical structure and an increase in β-sheet structure due to arsenic intoxication.

Key words: Arsenic, FTIR, *Labeo rohita*, liver, biochemical contents, protein secondary structure.

INTRODUCTION

Arsenic (As) is a member of group V of the periodic table of elements along with nitrogen, phosphorus, antimony, and bismuth. It is classified as a metalloid, having intermediate chemical properties between typical metals and non-metals. Thus, arsenic is capable of forming alloys with metals, but it also readily forms covalent bonds with carbon, hydrogen, and oxygen. The chemistry of arsenic is rather complex, and the compounds it forms are numerous. This is largely because arsenic possesses several different valence or oxidation states, which result in the markedly different biologic behavior of its compound. The arsenic compounds are used in pigments and dyes, as a preservative of animal hides, in glass manufacture, agricultural pesticides, and various pharmaceutical substances [5].

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The aquatic environment is particularly sensitive to the toxic contaminants, because a considerable amount of the chemicals used in industry, urbanization, and agriculture enters the marine and other aquatic environments [4]. The discharge of potentially toxic trace metals into the marine and freshwater environments has become a global problem. Continuous exposure of freshwater organisms to low concentrations of heavy metals may result in bioaccumulation, causing changes in the activities of several liver enzymes [19]. Fish, as living bioindicator species, play an increasingly important role in the monitoring of water pollution, because they respond with great sensitivity to changes in the aquatic environment [1, 14].

Fourier transform infrared (FTIR) spectroscopy has been used extensively to probe structural changes in proteins and lipids [2, 6]. In the present paper, we focus our attention on the arsenic induced biochemical changes in the liver tissues of freshwater fish *Labeo rohita* using Fourier transform infrared spectroscopy.

MATERIALS AND METHODS

TEST SPECIES

The freshwater fingerlings, *Labeo rohita* (length 6±1 cm and weight 8±1 g) were procured from the Government fish farm at Lalpet, Tamil Nadu, India. The collected fish were acclimated to laboratory condition for 15 days [15]. For the entire duration of the experiment, the fish were fed with commercial fish feed (Himalaya company, India).

TEST CHEMICALS

The AnalaR grade Arsenic trioxide (As_2O_3) was obtained from Sigma Aldrich Company, Bangalore, India, and used without further purification for the experiment.

LETHALITY STUDIES

The LC_{50} value for arsenic was determined by using Litchfield and Wilcoxon [12] method and was found to be 124.5 ppm. Arsenic stock solution was prepared by dissolving 1.3202 g of As_2O_3 in one liter of dilute acidic water. The acclimated fish were stocked in a 30-litre glass trough of dimension 60×30×30 cm equipped with continuous air supply. The physico-chemical parameters such as pH, total alkalinity, total hardness, calcium and magnesium were measured according to APHA [3] and maintained throughout the experiment (7.2, 120 mg/l, 200 mg/l, 50

mg/l and 18 mg/l). The water was changed along with waste feed and fecal materials every day at 7 a.m. by slowly siphoning the water from each container. Daily the containers were refilled and redosed with metal toxicant.

EXPERIMENTAL STUDY

Fourteen-day exposure to 1/3rd LC_{50} of arsenic was conducted and the liver tissues were collected from both control and arsenic intoxicated fishes. The experimental design and calculations for the acute toxicity were based on the procedure given by Finney [11] and Sparks [20]. The acclimated test fish were divided into two groups, each containing twenty-five fish. Group I was used as control and reared in dechlorinated tap water. The test fish belonging to group II were exposed to a higher sub-lethal concentration (41.5 ppm) of arsenic for 14 days (sub-acute exposure). After this period, the fish were sacrificed, and liver tissues were removed and stored at –80 ºC until sample preparation for FTIR spectroscopic studies [2].

SAMPLE PREPARATION

The liver tissues were kept in lyophilizer to remove water. The samples were then ground in an agate mortar and pestle in order to obtain liver powder. The liver powder was mixed with completely dried potassium bromide (at a ratio of 1/100), and then the mixture was subjected to a pressure of 5×10^6 Pa in an evacuated die to produce a KBr pellet for use in a FTIR spectrometer.

SPECTROSCOPIC ANALYSIS

FTIR spectra were recorded with a Perkin Elmer–Spectrum RxI Spectrometer equipped with a mullard I–alanine doped triglycine sulfate (DTGS) detector installed at Centralised Instrumentation and Services Laboratory (CISL), Annamalai University. The spectrometer was continuously purged with dry nitrogen to eliminate atmospheric water vapour and carbon dioxide (CO_2). Pellets were scanned at room temperature (25 ± 1 ºC) in the 4000–400 cm^{-1} spectral range. To improve the signal to noise ratio for each spectrum, 100 interferograms with a spectral resolution of ±4 cm^{-1} were averaged. Background spectra, which were collected under identical conditions, were subtracted from the sample spectra automatically. The frequencies for all sharp bands were accurate to 0.001cm^{-1}. Each sample was scanned under the same conditions with three different pellets. These replicates were averaged and then used. Absorption intensity of the peaks was calculated with base-line method. The variations in the frequencies and band areas were determined accurately from the original baseline-corrected spectra

belonging to the corresponding control and treated groups. Special care was taken to prepare the pellets at the same thickness by taking the same amount of sample and applying the same pressure. Therefore, in the present study it is possible to directly relate the intensities of the absorption bands to the concentration of the corresponding functional groups [6]. The spectra were analyzed using ORIGIN 8.0 software.

RESULTS

The present study was carried out to analyze the toxic effects of arsenic in the liver tissues of freshwater fingerlings *Labeo rohita* by using FTIR spectroscopy. The intensity and/or more accurately the area of the absorption bands in FTIR spectrum are directly related to the concentration of the molecules [6]. Fig. 1 shows the average FTIR spectra of control and arsenic intoxicated liver tissues of *Labeo rohita* in the 4000–400 cm^{-1} range. The spectra were normalized with respect to the amide A (3293 cm^{-1}) band. As seen from Fig. 1, the spectrum is quite complex and contains several bands arising from the contribution of different functional groups belonging to proteins, lipids and others. Therefore, the detailed spectral analyses were performed in two distinct wave number ranges, namely 3600–2800 cm^{-1} and 1800–800 cm^{-1} [2, 6, 10]. For the accurate measurements of the spectral parameters, all the spectra were normalized with respect to specific selected bands and considered separately. The main absorption bands and their assignments were defined in Table 1. The band area values of the selected bands were presented in Table 2.

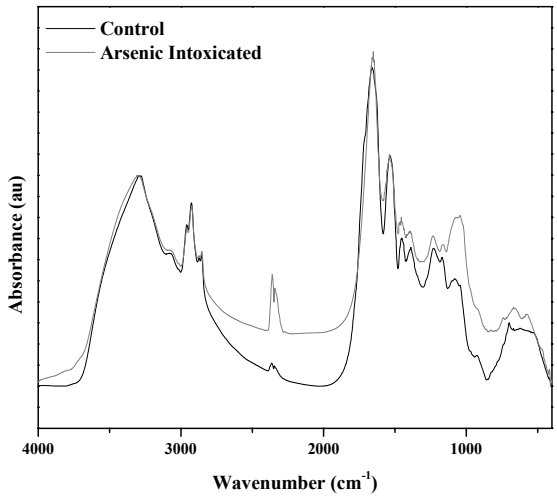

Fig. 1. The average FTIR spectra of control and arsenic intoxicated liver tissues of Labeo rohita in the region 4000–400 cm^{-1}. The spectra were normalized with respect to the amide A band.

Table 1

General band assignments of the FTIR spectra of control and arsenic intoxicated liver tissues of freshwater fingerlings *Labeo rohita* based on literature [6, 7]

S. No.	Wave number (cm^{-1})		Definition of the spectral assignments
	Control	Arsenic intoxicated	
1.	3293	3292	Amide A: mainly N–H stretching of proteins
2.	3081	3077	Amide B: N–H stretching of proteins
3.	2958	2958	CH_3 asymmetric stretch: mainly lipids
4.	2926	2926	CH_2 asymmetric stretch: mainly lipids
5.	2872	2873	CH_3 symmetric stretch: mainly proteins
6.	2854	2854	CH_2 symmetric stretch: mainly lipids
7.	1659	1655	Amide I: mainly C═O stretching of proteins
8.	1534	1534	Amide II: N–H bending and C–N stretching of proteins
9.	1451	1458	CH_2 bending: mainly lipids
10.	1388	1399	COO^- symmetric stretch: fatty acids and amino acids
11.	1230	1234	PO_2^- asymmetric stretch: mainly nucleic acids
12.	1170	1157	CO–O–C asymmetric stretching: glycogen and nucleic acids
13.	1081	1079	PO_2^- symmetric stretch: mainly nucleic acids
14.	1044	1043	C–O stretching: polysaccharides

Table 2

The band area values of selected bands for control and arsenic intoxicated liver tissues of *Labeo rohita*

Wave number (cm^{-1})	Control	Arsenic intoxicated
3293	128.87	126.01↓
3081	1.36	1.26↓
2926	6.35	5.42↓
2854	0.997	0.867↓
1659	74.32	47.31↓
1534	16.88	9.46↓
1451	2.41	1.15↓
1388	2.43	1.10↓
1230	4.60	2.65↓

As could be seen from Fig. 1, differences were seen in this region where the spectrum is populated by absorptions arising from the amide A, amide B, C–H stretching vibrations of $–CH_2$ (methylene) and $–CH_3$ (methyl) groups. The bands at ~3293 cm^{-1} and ~3081 cm^{-1} are generally assigned to amide A and B bands stemming from N–H stretching modes of proteins. The spectra show shifts in the wave numbers for both amide A and B bands. Further, the area of amide A and B

band decreases from control to arsenic intoxicated as 128.87 to 126.01 and 1.36 to 1.26 respectively. Lipids play a key role in the membrane fluidity. By affecting the conformation of membrane proteins, they govern exposure and diffusion of membrane components. The changes in lipid fluidity can be detected by analyzing the methylene stretching bands of the lipid hydrocarbon chains. In the present study, the band area of the CH_2 asymmetric stretching vibration (2926 cm^{-1}) decreases from 6.35 to 5.42 due to arsenic intoxication. This decrease indicates a change in the composition of the acyl chains [6]. In addition, the area of the symmetric CH_2 stretching (2854 cm^{-1}) band was found to have decreased in the arsenic intoxicated tissues from 0.997 to 0.867. This result suggests the decreased proportion of the CH_2 groups in the arsenic intoxicated liver tissues of *Labeo rohita*.

The bands between 1700 cm^{-1} and 1600 cm^{-1} in the spectra of liver tissues mainly stem from the amide I vibrational modes of tissue proteins and exhibit a high sensitivity to conformational changes in the secondary structure. The strong band observed at 1659 cm^{-1} can be assigned to α–helical structure. This amide I band is due to in-plane stretching of the C=O bond, weakly coupled with C–N stretching and in-plane N-H bending. Each type of secondary structure, i.e., α-helix, β-sheet, β-turn and unordered gives rise to different C=O stretching frequencies, and results in characteristic band positions. Hence, band positions are used to determine the secondary structural types present in each protein [9]. Further analysis has been carried out for the proteins present in the tissue samples by resolving the amide I band using a curve fitting method. By taking the second derivative of the arithmetical function, the number of peaks and their relative positions were determined. The underlying bands of amide I band as deduced by curve fitting analysis for control and arsenic intoxicated groups are given in Figs. 2 and 3 respectively. The absorption band areas determined by the curve fitting method are presented in Table 3. As seen from Table 3, the peak around 1693 cm^{-1} arises from β-sheet; the peak around 1682 cm^{-1} arises from β-turns; the peak at ~1659 cm^{-1} is due to α-helix structures; the peak around ~1648 cm^{-1} is assigned to random coil; the peak around 1624 cm^{-1} arises from β-sheet structures [9, 10, 21, 22]. It is also observed that the α-helix, β-turns and random coil structures decrease and β-sheet structure increases due to arsenic intoxication. The decrease in α-helix structure of the liver tissues might be responsible for the increase in β-sheet structure, which is consistent with the mechanism of β-sheet formation [7, 8, 9]. And the amide II vibrational mode of tissue proteins arises in the region 1600–1500 cm^{-1}. The amide II band mainly stems from the N–H bending and C–N stretching. As seen from Fig. 1, the absorbance intensities of the amide bands decrease due to arsenic intoxication when compared to control tissues. Also, the area of the amide I and II bands decreases respectively from 74.32 to 47.31 and from 16.88 to 9.46 in arsenic intoxicated tissues. The decrease in the band areas and absorbance intensity of both amides I and II bands indicate the destructive

effect of arsenic, since it is suggested [13] that free radical damage could cause a reduction in protein synthesis. Further, such an oxidative action of arsenic is supported by the observation that arsenic treatment decreases the protein content in the brain tissues of rat [16]. These changes reflect the loss of protein levels in the arsenic intoxicated liver tissues. This loss of protein provides verification of increased protein oxidation in the liver tissues with arsenic intoxication. Loss of function of protein may result from a change in critical side chain or from a break in the hydrogen or disulfide bonds, which maintain the secondary and tertiary structures. This break can lead to a partial unfolding of the tightly coiled peptide chain. It results in a disorganization of the internal structure [22]. Samuel *et al*. [16] have also reported decreased sulfhydryl proteins in the rat brain regions due to arsenic treatment. Decreased levels of protein thiols observed in the present study in the arsenic intoxicated liver tissues of fish are suggestive of an excess free radical production and binding of arsenic with various sulfhydryls that exist in the tissue cells. Webb [23] has reported that arsenic compounds interact with thiol groups strongly and specifically. Interactions between trivalent arsenic and thiol containing residues in proteins and peptides have generally been regarded as the basis for the effects of this metalloid on the structure and function of these molecules [17]. Loss of thiol groups is considered to be one of the immediate responses to an elevation in the level of oxidation stress [18].

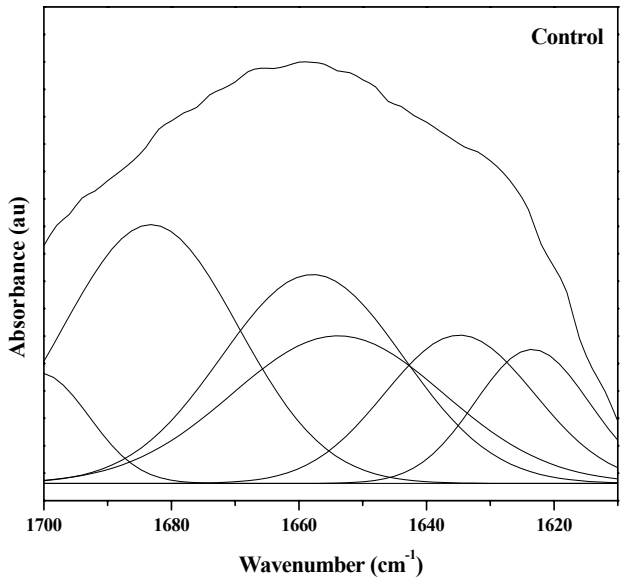

Fig. 2. The underlying bands of amide I bands in the 1700–1600 cm^{-1} region, as deduced by curve-fitting analysis for average spectra of the control liver tissues of *Labeo rohita*.

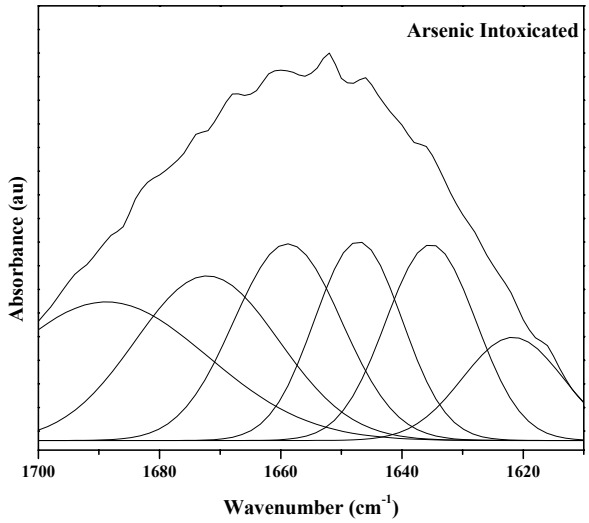

Fig. 3. The underlying bands of amide I bands in the 1700–1600 cm^{-1} region, as deduced by curve-fitting analysis for average spectra of the arsenic intoxicated liver tissues of *Labeo rohita*.

Table 3

A summary of the results of curve fitting analysis expressed as a function of percentage areas of main protein secondary structures and their band assignments for control and arsenic intoxicated liver tissues of *Labeo rohita*

Control			**Arsenic intoxicated**		
Wavenumber (cm^{-1})	**Areas (%)**	**Assign.**	**Wavenumber (cm^{-1})**	**Areas (%)**	**Assign.**
1693	6.725	β-sheets	1694	23.718	β-sheets
1682	27.751	β-turns	1683	19.959	β-turns
1659	23.315	α-helix	1655	17.904	α-helix
1648	19.204	Random coil	1646	14.703	Random coil
1624	13.627	β-sheets	1622	14.970	β-sheets
1618	9.378	Others	1616	8.747	Others

The band observed at ~1451 cm^{-1} is due to bending vibration of the CH_2 in the lipids and proteins. As seen from Table 2, a decrease in the area from 2.41 to 1.15 was observed at 1451 cm^{-1}. The band centered at ~1388 cm^{-1} can be attributed to COO^- symmetric stretching vibration of amino acid side chains and fatty acids. A decrease in the area of 2.43 to 1.14 is also observed in this band. The bands at 1230 cm^{-1} and 1081 cm^{-1} are respectively due to asymmetric and symmetric

stretching modes of phosphodiester groups in nucleic acids [6]. The decrease in the area of these bands implies a decrease in the relative content of the nucleic acids in the arsenic intoxicated tissues.

CONCLUSIONS

The decreased band areas of symmetric and asymmetric CH_2 stretching modes observed in the arsenic intoxicated tissues suggests the decreased composition of the acyl chains in the arsenic intoxicated liver tissues of *Labeo rohita*. The decrease in the band areas and intensities of amide bands in the arsenic intoxicated tissues indicate a decrease in the protein quantity of the system and the destructive effect of the arsenic in the liver tissues. The decrease in the band areas of symmetric and asymmetric stretching modes of phosphodiester groups suggest a decrease in the relative content of the nucleic acids in the arsenic intoxicated liver tissues. The decrease in α-helix structure and an increase in β-sheet structure of the liver tissues are consistent with the mechanism of β-sheet formation.

In conclusion, the present study shows that the liver tissues are vulnerable to arsenic intoxication. The result further suggests that arsenic intoxication induces significant alteration on the major biochemical constituents such as lipids, proteins and nucleic acids, which can be easily evidenced by FTIR spectroscopy.

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