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Impact of Diclofenac Drug on the biochemical composition of the fresh water fish, *Cirrhinus mrigala*

S. Binukumari[1], V. Priyadarsini[2] and J. Vasanthi[3*]

[1,2]PG and Research Department of Zoology, Kongunadu Arts and Science College, Coimbatore, Tamil Nadu, India
[3]PG and Research Department of Zoology, Nirmala College for women, Coimbatore, Tamil Nadu, India
*Corresponding author

Abstract

Fishes belonging to the species *Cirrhinus mrigala* were exposed to 2.8 per cent concentration of Diclofenac drug for 24, 48, 72, 96 hours and 10 days, 20 days and 30 days respectively. Bioassay studies were conducted to calculate 50 per cent mortality of *Cirrhinus mrigala* in 96 hour by exposing to different concentrations of Diclofenac drug. Biochemical characteristics like total protein, carbohydrate and lipid were observed in blood and tissues like gill, liver and kidney. The parameters was found to be decreased in blood and the tissues when compared to control.

Keywords: Drug, *Cirrhinus mrigala*, bioassay, biochemical.

Introduction

Water pollution brings a reduction in diversity of aquatic life and eventually destroys the balance of life and influences the stream and river. Pollution of water is responsible for a very large number of mortalities and in capacitiations throughout the world. Polluted state of the water resources has led to the steady decline in fisheries and also affected the irrigated land (Shashikant, 1986). Pollution and its effect constitute one of man's greatest crimes against himself. The extent despoilment in rendering waters unfit for human consumption and unsuitable for aquatic life and other important uses is particularly alarming near large centers of population. The damaging effects of this scourge also appear in remote places in the vicinity of coal mines, tanneries and paper mills (Lagler, 1972).
The new group of pollutants of all aquatic ecosystems is the pharmaceutical drugs. Pharmaceuticals are synthetic chemicals belonging to a wide group of different chemical families and may also react differently in the environment. Most pharmaceuticals are deposited in the environment through human consumption and excretion and are often flitered ineffectively by waste water treatment plants which are not designed to manage them. Pharmaceuticals may also be deposited in the environment through improper disposal, runoff from sludge fertilizer and reclaimed waste water irrigation and leaky sewage.
Pharmaceuticals reach the environment mainly in three ways:

- They are excreted from humans and animals, intact or metabolized mainly into the urine, passing on to the environment directly or via sewage plants.

- Unused reach the environment either via household water or via urban solid garbage handling.
- Manufacturing plants producing the active substances might unintentionally release pharmaceuticals into the environment.

Some pharmaceuticals are degraded to various extents in sewage treatment plants, but others leave the plant in active forms. Active residues of pharmaceuticals have been detected in surface water and they may persist in the environment for long periods of time. Large amounts of antibiotics and other pharmaceuticals have been found downstream from sewage plants for pharmaceutical industries. EPPPs from sewage sludge used as fertilizer are absorbed by soya and antibiotics have been found in the leaves.
Unmetabolized pharmaceuticals are often the most non- biodegradable substances in the environment (Stuer – Lauridsen *et al*., 2000). Their intrinsic medicinal properties give them the tendency to bioaccumulate in other organisms besides humans and thereby potentially provoke effects on the biota of aquatic and terrestrial ecosystems (Halling – Sorensen, 1998). Many pharmaceuticals are often persistent and lipophilic- able to pass through cell membranes, which allow them to carry out specific biological functions. Many pharmaceuticals are relatively stable to avoid being biologically inactivated before carrying out their intended pharmaceutical effects in the body.

Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) taken or applied to reduce inflammation and as an analgesic reducing pain in certain conditions. It is supplied as or contained in medications under a variety of trade names. A bioassay involves the use of live animal or plant (in vivo) or tissue or cell (in vitro) to determine the biological activity of a substance such as a hormone or drug. Bioassays are typically conducted to measure the effects of a substance on a living organism and are essential in the development of new drugs and in monitoring environmental pollutants.

Carbohydrates, proteins and lipids play a major role as energy precursors for fishes under stress conditions (Idler and Clemens, 1959). Biochemical parameters were often used when clinical diagnosis of fish physiology was applied to determine the effects of external stressors and toxic substances (Osman *et al*., 2001). In general biochemical profiles in fish and other aquatic organisms under stress serve as important bioindicators in the monitoring of aquatic environment.

Fish blood is widely used in toxicological research and environmental monitoring as a promising indicator of physiological and pathological changes of the whole body (Velisek *et al*., 2010). Blood plasma reflects the physiological state of an animal because they are the products of intermediate metabolism (Artacho *et al*., 2007; Firat and Kargin, 2010) and the parameters of blood plasma are important in diagnosing the structural and functional status of fish (Adhikari *et al*., 2004).

Materials and Methods

Cirrhinus mrigala is the freshwater carp found in Northern India, Punjab, West Bengal and Orissa. It has a wider mouth and thinner lips. Body silvery, dark grey alomg with the back, sometimes with coppery tinge. Adult attains a maximum length of 90 – 100 cm and a weight of 1.4 to 2.8 kg. The growth of about 20 cm is recorded in 8 months. This growth data is based on an experiment conducted on 6000 fingerlings.

Analytical test for water chemistry

The tap water free from contaminants was used as dilution water for the present study. The physico – chemical analysis of water used in the experiments was carried out using the methods of APHA, (2005).
Physico – chemical parameters of the tap water used for the present study are as follows: Temperature 27.2 ± 0.9 (0C), pH 7.1 ± 0.1, Dissolved oxygen 5.4 ± 0.4 (mg/l), Calcium 120 ± 1.1 (mg/l), Magnesium 50 ± 0.2 (mg/l).

Collection and maintenance of fish

The fingerlings of the freshwater fish, *Cirrhinus mrigala* ranging in weight from 4 kg to 8 kg and measuring (4 cm to 6 cm in length) were procured from Aliyar. The procured bulk samples of *Cirrhinus mrigala* were transported to the laboratory in well aerated polythene bag and acclimatized to the laboratory conditions under natural photo period for one week in large plastic containers at (26 ± 5 0C). The tank was previously washed with potassium permanganate to prevent any fungal infection. The fishes were maintained in dechlorinated tap water of the quality used in the test and water was renewed every day to provide freshwater rich in oxygen.

Continuous artificial aeration was maintained throughout the acclimation and exposure periods. During the periods of acclimation they were fed everyday with oil cake mixed with rice flour.

Unhealthy fish and those with infections were removed. Feeding was stopped two days prior to the experiment to maintain same state of metabolic requirements. Fish belonging to both sexes were selected for the present investigation. All the precautions laid down on recommendations of the toxicity tests to aquatic organisms are followed Anon, (1975).

Toxicant

Analytical grade Diclofenac 2 [(2,6 – Dichlorophenyl) amino] benzene acetic acid sodium salt [DCF, 99.9% pure]; CAS No.15307-79-6 were purchased from Sigma-Aldrich chemic GmbH, Germany, Dimethyl sulphoxide (DMSO) (CAS No 67-68-5) was purchased from Fischer Scientific India Pvt. Ltd, India and 0.2 ml/l used to prepare the stock solution at different concentrations (1, 10 and 100 µg/L due to their low water solubility. IUPAC Name is 2 [(2, 6 – Dichlorophenyl) amino] benzene acetic acid sodium salt. Molecular formula is $C_{14}H_{10}C_{12}NNaO_2$ and molecular weight is 381.13.

Evaluation of median lethal concentration

The concentrations of the pollutant at which 50 percent of the test animals die during a specific test period of time is referred to as median lethal concentration (LC_{50}) or median tolerant limit. In aquatic toxicology the traditional LC50 test is often used to measure the potential risk of a chemicals (Jack de Bruijin *et al*., 1991).

Batches of 10 healthy fishes were exposed to different concentrations of drug, Diclofenac to calculate the LC_{50} value. One more set of fishes are maintained as control in tap water. To find the wide range of concentration 100-600 ml were chosen and the number of dead or affected fishes was counted at regular intervals upto 48 hrs. The level of the dissolved oxygen, pH, alkalinity and hardness were monitored and maintained constant.

Appropriate narrow range of concentration was used to find the median lethal concentration, using a minimum of 6 fishes for each concentration and the mortality was recorded for every 24 hrs upto 96 hrs. It was found as for 96 hrs, using probit analysis method (Finney, 1971). From the stock solution various sublethal concentrations were prepared for bioassay studies.

Three groups of fishes were exposed to 1/10 of the drug 'Diclofenac' for 24, 48, 72, 96 hrs, 10 days, 20 days and 30 days respectively. Another group was maintained as control. All the groups received the same type of food and other conditions were maintained similarly. At the end of each exposure period, fishes were sacrificed and tissues such as gill, liver and kidney were dissected and removed. The tissues (10 mg) were homogenized in 80% methanol, centrifuged at 3500 rpm for 15 minutes and the clear supernatant was used for the analysis of different parameters.

Estimation of total protein

Total protein concentration was estimated by the method of Lowry *et al*. (1951), based on the following principle.

In alkaline medium protein in the sample form a complex with copper ions. The amino acids containing aromatic groups, tyrosin and tryptophane, present in copper protein complex react with Folin Cliocalteu phenol reagent to give blue colour due to the reduction of phosphomolybdate. The intensity of the colour developed is proportional to the concentration of protein present in the sample. The value is expressed as mg/g of tissues.

Estimation of carbohydrate

Quantitative estimation of carbohydrate in the tissues was done following the method by Hedg's and Hofreiter, (1962).

Carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone, a green coloured product with an absorption maximum at 630 nm.

Estimation of lipid

Estimation of lipid was estimated by the method of Richmond, (1973) based on the following principle.
Cholesterol esterase hydrolyses cholesterol esters into free cholesterol and fatty acids. In the second reaction cholesterol oxidase converts cholesterol to cholest-4-en-3- one and hydrogen peroxide. In the presence of peroxidise, hydrogen peroxide oxidatively coupies with 4-aminoantipyrine and phenol to produce red quinoeimine dye which has absorbance maximum at 510 nm (500-530). The intensity of red colour is proportional to the amount of total cholesterol in the specimen.

Results and Discussion

Total protein content

The amount of protein estimated in different tissues and blood of the fish, *Cirrhinus mrigala* subjected to short term and long term exposures are presented in Table 1.

The amount of protein in gill tissue was 2.66, 2.50, 2.47 and 2.29 mg/g in the fishes exposed to short term duration of Diclofenac drug after 24, 48, 72 and 96 hours respectively. The fishes which were exposed to long term duration of Diclofenac drug for 10, 20 and 30 days were found to contain 2.03, 1.99 and 1.68 mg/g while the control fish contained 3.00 mg/g of protein.

2.98, 2.55, 2.24 and 2.11 of protein were present in the liver tissue respectively after 24, 48, 72 and 96 hours of short term exposure of the fishes. The protein content in the fishes that were subjected to long term exposure was 2.01, 1.85 and 1.25 mg/g respectively. The mean control value was 3.26.

Kidney recorded 2.85, 2.25, 2.01 and 1.74 mg/g of protein in fishes exposed to short term duration of Diclofenac drug for 24, 48, 72 and 96 hours respectively. 1.45, 1.21 and 1.01 mg/g were recorded in the kidney of fishes exposed to long term duration of Diclofenac drug for 10, 20 and 30 days. The control value was 3.02.

Blood recorded 4.12, 3.50, 2.01 and 1.95 mg/g of protein in fishes exposed to short term duration of Diclofenac drug for 24, 48, 72 and 96 hours respectively. 1.00, 0.89 and 0.50 mg/g were recorded in the blood of fishes exposed to long term duration of Diclofenac drug for 10, 20 and 30 days. The mean control value was 4.21 mg/g.

Total Carbohydrate content

The amount of carbohydrate in the tissues estimated after subjecting the fishes to short term and long term exposure periods on the Diclofenac drug presented in Table 2.

The gill of fishes exposed to 2.8% Diclofenac drug for 24, 48, 72 and 96 hours was found to contain 15.12, 11.82, 9.63 and 7.80 mg/g of carbohydrate respectively. The fishes maintained as control were found to contain a mean of 16.50 mg/g in their gill tissue.

Liver tissue was found to contain 9.29, 7.01, 6.24 and 5.02 mg/g of carbohydrate respectively in 24, 48, 72 and 96 hours exposures in 2.8 per cent concentration of Diclofenac drug. Under treatment of Diclofenac drug for 10, 20 and 30 days exposures, the values were 4.09, 3.20 and 3.05 mg/g respectively. The mean carbohydrate content in the liver of the control was 11.35.

29.30, 25.81, 18.47 and 15.44 mg/g of carbohydrate were found in the kidney tissue of 24, 48, 72 and 96 hours treated fishes respectively. Values of carbohydrate estimated in the fishes exposed to long term periods 10, 20 and 30 days in 2.8 per cent Diclofenac drug were 11.31, 9.01 and 4.27 mg/g in their kidney tissue. The mean control value was 35.26.

Blood tissue was found to contain 35.10, 28.70, 26.20 and 25.01 mg/g of carbohydrate respectively in 24, 48, 72 and 96 hours exposures in 2.8 per cent concentration of Diclofenac drug. Under treatment of diclofenac drug for 10, 20 and 30 days exposures, the values were 20.40, 18.20 and 15.71 mg/g respectively. The mean carbohydrate content in the liver of the control was 40.04 mg/g.

The Lipid content

The amount of lipid in the tissues estimated after exposing the fishes to short term and long term periods of the Diclofenac drug are presented in Table 3.

The lipid content in the gill tissue of fishes exposed to short term periods in term of 24, 48, 72 and 96 hours were 11.01, 10.00, 9.06 and 8.12 mg/g respectively. The fishes exposed to long term periods of 10, 20 and 30 days in 2.8 per cent Diclofenac drug contained 7.10, 6.25 and 5.50 mg/g of lipid in their gill respectively against an average of 14.92 mg/g in the control.

Liver tissue was found to contain 16.49, 11.20, 9.88 and 8.02 mg/g of lipid in short term exposure periods of 24, 48, 72 and 96 hours. The fishes subjected to long term periods were found to contain 5.71, 4.64 and 3.90 mg/g of lipid. The mean control value was 22.01 mg/g.

Kidney recorded 35.96 mg/g in the control fishes. The fishes exposed for short term periods were found to contain 29.73, 23.34, 16.50 and 9.94 mg/g of lipid.

Table 1: Changes in the Protein content in the Tissues and Blood of *Cirrhinus mrigala* on short and long term exposure

Sample mg/gwet tissue	Exposure periods							
	Control	24 hrs	48 hrs	72 hrs	96 hrs	10 days	20 days	30 days
Gill		2.66 ± 0.28	2.50 ± 0.46	2.47 ± 0.53	2.29 ± 0.36	2.03 ± 0.30	1.99 ± 0.21	1.68 ± 0.30
't' value	3.00 ± 0.38	0.62^{ns}	0.90^{ns}	0.77^{ns}	1.74^{ns}	2.68^{*}	4.66^{**}	4.60^{**}
% change		-11.33	-16.00	-17.66	-23.66	-32.33	-33.66	-44.00
Liver		2.98 ± 0.30	2.55 ± 0.30	2.24 ± 0.36	2.11 ± 0.30	2.01 ± 0.28	1.85 ± 0.30	1.25 ± 0.29
't' value	3.26 ± 0.33	1.34^{ns}	2.33^{*}	3.02^{*}	3.98^{*}	4.31^{**}	5.32^{**}	8.22^{**}
% change		-8.58	-21.77	-31.28	-35.27	-38.34	-43.25	-61.65
Kidney		2.85 ± 0.34	2.25 ± 0.34	2.01 ± 0.25	1.74 ± 0.29	1.45 ± 0.27	1.21 ± 0.22	1.01 ± 0.24
't' value	3.02 ± 0.41	4.15^{**}	15.77^{**}	23.63^{**}	29.12^{**}	34.21^{**}	40.81^{**}	43.86^{**}
% change		-5.62	-25.49	-33.44	-42.38	-51.98	-59.93	-66.55
Blood		4.12 ± 1.26	3.50 ± 1.09	2.01 ± 0.92	1.95 ± 0.89	1.00 ± 0.73	0.89 ± 0.55	0.50 ± 0.56
't' value	4.21 ± 1.00	0.39^{ns}	0.83^{ns}	2.04^{*}	3.08^{**}	3.96^{**}	4.70^{**}	4.98^{**}
% change		-2.13	-16.86	-52.25	-53.68	-76.24	-78.86	-88.12

Values are mean ± SD, n=5, Figures in Parenthesis are percentage decrease over control.
* - Significant at 5% (t<0.05); ** - Significant at 1% (t<0.01); NS – Non Significant

Table 2: Changes in the Carbohydrate content in the Tissues and Blood of *Cirrhinus mrigala* on short and long term exposure

Sample mg/gwet tissue	Exposure periods							
	Control	24 hrs	48 hrs	72 hrs	96 hrs	10 days	20 days	30 days
Gill		15.12 ± 0.41	11.82 ± 0.43	9.63 ± 0.37	7.80 ± 0.34	6.55 ± 0.31	5.32 ± 0.35	4.01 ± 0.37
't' value	16.50 ± 0.37	2.91^{*}	14.13^{**}	22.04^{**}	29.45^{**}	34.19^{**}	36.35^{**}	39.22^{**}
% change		-8.36	-28.36	-41.63	-52.72	-60.30	-67.75	-75.69
Liver		9.29 ± 0.30	7.01 ± 0.42	6.24 ± 0.35	5.02 ± 0.41	4.09 ± 0.35	3.20 ± 0.37	3.05 ± 0.38
't' value	11.35 ± 0.29	7.65^{**}	13.17^{**}	18.32^{**}	20.53^{**}	26.63^{**}	28.77^{**}	28.78^{**}
% change		-18.15	-38.23	-45.02	-55.77	-63.96	-71.80	-73.12
Kidney		29.30 ± 0.32	25.81 ± 0.38	18.47 ± 0.41	15.44 ± 0.33	11.31 ± 0.42	9.01 ± 0.42	4.27 ± 0.37
't' value	35.26 ± 0.34	21.88^{**}	32.30^{**}	54.19^{**}	71.41^{**}	75.87^{**}	81.74^{**}	106.19^{**}
% change		-16.90	-26.80	-47.61	-56.21	-67.92	-74.44	-87.89
Blood		35.10 ± 1.54	28.70 ± 0.74	26.20 ± 0.43	25.01 ± 0.70	20.40 ± 0.73	18.20 ± 0.48	15.71 ± 0.48
't' value	40.04 ± 2.12	2.70^{**}	7.61^{**}	9.39^{**}	10.12^{**}	8.22^{**}	15.31^{**}	17.68^{**}
% change		-12.33	-28.32	-34.56	-37.53	-49.05	-54.54	-60.76

Values are mean ± SD, n=5, Figures in Parenthesis are percentage decrease over control.
* - Significant at 5% (t<0.05); ** - Significant at 1% (t<0.01); NS – Non Significant

Table 3: Changes in the Lipid content in the Tissues and Blood of *Cirrhinus mrigala* on short and long term exposure

Sample mg/gwet tissue	Exposure periods							
	Control	**24 hrs**	**48 hrs**	**72 hrs**	**96 hrs**	**10 days**	**20 days**	**30 days**
Gill	14.92 ± 0.43	11.01 ± 0.41	10.00 ± 0.41	9.06 ± 0.32	8.12 ± 0.35	7.10 ± 0.39	6.25 ± 0.29	5.50 ± 0.36
't' value		9.66**	12.71**	17.24**	19.48**	21.49**	26.36**	27.77**
% change		-26.20	-32.97	-39.27	-45.57	-52.41	-58.10	-63.13
Liver	22.01 ± 0.38	16.49 ± 0.36	11.20 ± 0.36	9.88 ± 0.34	8.02 ± 0.39	5.71 ± 0.36	4.64 ± 0.37	3.90 ± 0.36
't' value		18.81**	35.85**	42.75**	43.94**	55.01**	57.88**	61.44**
% change		-25.08	-49.11	-55.11	-63.56	-74.05	-78.91	-82.28
Kidney	35.96 ± 0.34	29.73 ± 0.34	23.34 ± 0.36	16.50 ± 0.31	9.94 ± 0.30	7.21 ± 0.35	6.52 ± 0.30	4.08 ± 0.25
't' value		6.02**	11.11**	16.99**	22.91**	24.73**	25.72**	27.67**
% change		-17.32	-35.09	-54.11	-72.35	-79.95	-81.86	-88.65
Blood	61.70 ± 0.67	51.48 ± 0.71	44.70 ± 0.62	39.00 ± 0.68	30.70 ± 0.69	28.00 ± 0.64	25.14 ± 0.64	20.00 ± 0.58
't' value		17.85**	30.80**	39.99**	54.10**	61.66**	67.27**	80.45**
% change		-16.56	-27.55	-36.79	-50.24	-54.61	-59.25	-67.58

Values are mean ± SD, n=5, Figures in Parenthesis are percentage decrease over control.
* - Significant at 5% (t<0.05); ** - Significant at 1% (t<0.01); NS – Non Significant

However those exposed to longer durations contained 7.21, 6.52 and 4.08 mg/g.

Blood recorded 61.70 mg/g in the control fishes. The fishes exposed for short term periods were found to contain 51.48, 44.70, 39.00 and 30.70 mg/g of lipid. However those exposed to longer durations contained 28.00, 25.14 and 20.00 mg/g.

The biochemical profiles of blood can provide important information about the internal environment of the organism. The impacts of pharmaceuticals on the biochemical parameters of fish can help to understand the mechanism and mode of action of drugs (Li *et al*., 2011).

Vutukuru, (2005) reported that there is an appreciable decline in different biochemical constituents in various tissues in fresh water fish, *Labeo rohita* under chromium stress. Kannan *et al*. (2010) reported the decreased protein content on gill, brain and muscle of *Mystus vittatus* when exposed to mercuric chloride. The decreased trend of protein content in various tissues of *C. mrigala* in the present study may be due to metabolic utilization of keto acids in the synthesis of glucose or for the osmotic and ionic regulation.
Carbohydrate is an essential component of living cells and sources of energy for animals. The results of the present findings showed a significant decrease in carbohydrate content in all the tissues studied. Shazia Quadir *et al*. (2014) observed a significant decrease in glucose content by the exposure of Imidacloprid. Tissue specific depletion of carbohydrates as observed in the present study may be due to its rapid utilization to meet the energy demands under the impact of drug.

Lipid is an important normal body constituent used in the structure of cell membranes, synthesis of bile acid and synthesis of steroid hormones. Remia *et al*. (2008) reported that the decline of lipid may be due to utilization of fatty deposits instead of glucose for energy purpose of the fish, *Tilapia mossambica* on exposed to Monocrotophos. Mohsen Abdel – Tawwab *et al*. (2013) reported that the significant decrease in lipid content.

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