Journal of Hazardous Materials 195 (2011) 188–194

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Ecotoxicological impacts of clofibric acid and diclofenac in common carp (*Cyprinus carpio*) fingerlings: Hematological, biochemical, ionoregulatory and enzymological responses

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ARTICLE INFO

Article history:
Received 18 March 2011
Received in revised form 6 August 2011
Accepted 8 August 2011
Available online 16 August 2011

Keywords:
Clofibric acid
Diclofenac
Cyprinus carpio
Acute toxicity
Hematology
Biochemical parameters

ABSTRACT

Investigation on the toxic effects of pharmaceutical drugs namely clofibric acid (CA) and diclofenac (DCF) were studied in a common carp *Cyprinus carpio* at different concentrations such as 1, 10 and 100 $\mu g\,L^{-1}$ for a short-term period of 96 h under static bioassay method. At all concentrations, red blood cell (RBC), plasma sodium (Na^+), potassium (K^+), and glutamate oxaloacetate transaminase (GOT) levels were decreased in fish treated with CA and DCF. Contrastingly, white blood cell (WBC), plasma glucose, protein, lactate dehydrogenase (LDH) and gill Na^+/K^+-ATPase level were increased. However, a mixed trend was observed in hemoglobin (Hb), hematocrit (Hct), plasma chloride (Cl^-), mean cellular volume (MCV), mean cellular hemoglobin (MCH), mean cellular hemoglobin concentration (MCHC) and glutamate pyruvate transaminase (GPT) levels. There was a significant ($P<0.01$ and $P<0.05$) change in all parameters measured in fish exposed to different concentrations of CA and DCF. In summary, the alterations in hematological, biochemical, ionoregulatory and enzymological parameters can be used as biomarkers in monitoring the toxicity of CA and DCF in aquatic environment. However, more detailed studies on using of specific biomarkers to monitor the human pharmaceuticals are needed.

1. Introduction

Pharmaceuticals drugs are produced and prescribed to cure the diseases, and to improve human health [1,2]. These drugs enter into aquatic environment through domestic waste waters, disposal from medical centres, excretion via water and sewage treatment systems [3–5]. The occurrence and detection of various pharmaceutical drugs in the environment, particularly in surface water, ground water, drinking water and influents and effluents from the wastewater treatment plant has been reported [6–9]. Rosal et al. [4] reported that the presence of pharmaceutical drugs even at low concentration level (ranging from $\mu g\,L^{-1}$ to $ng\,L^{-1}$) may lead to public health problems. Clofibric acid (CA) is an active derivative substance of clofibrate, designed to improve lipid metabolism in human [10]. Occurrence of CA in the environment has been reported in many countries. For example, 1 $ng\,L^{-1}$ in the North Sea [11]; 0.55l $\mu g\,L^{-1}$ in surface waters of Swiss lakes [12]; 1.6 $\mu g\,L^{-1}$ in effluents from sewage treatment plants, Germany [13]; 0.8–2 $\mu g\,L^{-1}$ in USA [14] and 5 $ng\,L^{-1}$ in Greece [15].

Diclofenac (DCF), one of the most important non-steroidal anti-inflammatory drugs (NSAID) is used in conditions like inflammation, arthritis, menstrual, dysmenorrheal and rheumatic disease and also acts as a cyclooxygenase inhibitor [16]. DCF is detected extensively in different water bodies throughout the world because of its higher amount of usage and production. There are many reports available on the occurrence of DCF such as 1030 $ng\,L^{-1}$ in surface waters [17]; 2 $ng\,L^{-1}$ in drinking water well (Mediterranean region) [18]; 0.38 $\mu g\,L^{-1}$ in groundwater (Berlin) [19] and 195 $ng\,L^{-1}$ in Mersey Estuary (UK) [20] and 6.2 $ng\,L^{-1}$ in Estuary of the River Elbe (North Sea) [11]. The continuous discharge and occurrence of pharmaceutical drugs in the aquatic ecosystem has become a major problem due to either high persistence or biological activity [2]. Many studies have been conducted based on acute toxicity test using laboratory organisms belonging to different tropic levels. Embry et al. [21] reported that aquatic toxicity data on acute responses to anthropogenic chemicals by fish plays a very important role. Powers [22] suggested that fish models are increasingly used in the early phases of pharmaceutical development and its toxicity evaluation. However, most investigations have been limited to lethal effects during acute exposures [23].

Assessment of polluted water bodies and aquatic animal health biomarkers are widely used as early diagnostic tools [24]. Hematological, biochemical, ionoregulatory and enzymological parameters have been routinely used as valuable biomarkers to assess the toxicity of environmental contaminants in aquatic ecosystem [25]. The hematological variables such as hemoglobin (Hb), hematocrit

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doi:10.1016/j.jhazmat.2011.08.029

(Hct), red blood cell (RBC) count, white blood cell (WBC) count, and hematological indices such as mean cellular volume (MCV), mean cellular hemoglobin (MCH) and mean cellular hemoglobin concentration (MCHC), and biochemical parameters like plasma glucose and protein are widely used to assess the toxic stress induced by environmental contaminants. Ion levels in plasma as measured by osmolality or specific ion concentrations of sodium (Na^+), potassium (K^+), and chloride (Cl^-) have potential as sensitive biomarkers of environmental chemical exposure. However these ions are very sensitive to environment stressors [26,27]. Enzyme activities have also been used as sensitive indicator of stress in fish exposed to diverse group of water pollutants and also to predict the possible level of threat to life [28]. Among the enzymes, transaminases like glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) play a vital role in protein and carbohydrate metabolism and act as an indicator for tissue damage and cell rupture [29]. Lactate dehydrogenase (LDH) also used as indicative criteria of exposure due to chemical stress and anaerobic capacity of tissue [30]. In addition to these, gill Na^+/K^+-ATPase is involved in osmoregulation of fish and widely used as a sensitive indicator of environmental contaminants.

Studies on the potential adverse ecological impacts of pharmaceutical drugs and its residues on the physiology of aquatic organisms are scarce. CA is persistent for a long time (e.g., approximately 21 years) in the environment [7,31] and is considered as a potential endocrine disruptor, because it interferes with the synthesis of cholesterol [32]. On the other hand, lethality and teratogenicity were observed in DCF exposed zebra fish embryos (*Danio rerio*) after 96 h exposure to $480 \pm 50\,\mu g\,L^{-1}$ (LC_{50}/96 h) and $90 \pm 20\,\mu g\,L^{-1}$ (EC_{50}/96 h), respectively [33]. Further, cytological alterations in liver, kidney and gills even at $1\,\mu g\,L^{-1}$ in rainbow trout have also been observed [34]. More specifically, DCF is responsible for higher population decline among vultures in Indian subcontinent, Pakistan, and Nepal [35]. However, the knowledge on the toxicity and effects of pharmaceutical drugs on the aquatic organisms are meager, particularly on freshwater fish. Consequently, we attempted to study the impacts of CA and DCF at different concentrations such as 1, 10 and $100\,\mu g\,L^{-1}$ on hematological, biochemical, ionoregulatory, and enzymological parameters in a common carp, *C. carpio*. The carp is widely cultivated in major water bodies of India.

2. Materials and methods

2.1. Chemicals

Clofibric acid (α-(*p*-Chlorophenoxy) isobutyric acid, CAS No. 882-09-7) and diclofenac (2-[(2, 6-Dichlorophenyl) amino] benzene acetic acid sodium salt, CAS No. 15307-79-6) were purchased from Sigma–Aldrich Chemie GmbH, Germany. Dimethyl sulphoxide (CAS No. 67-68-5) was purchased from Fischer Scientific India Pvt. Ltd, India and $0.2\,mL\,L^{-1}$ used to prepare the stock solution at different concentrations (1, 10, and $100\,\mu g\,L^{-1}$) due to their low water solubility.

2.2. Experimental fish and water

Fingerlings of *C. carpio* were obtained from Tamil Nadu Fisheries Development Corporation Limited, Aliyar Fish Farm, Tamil Nadu, India in the weight range of 8.0 ± 0.4 g and body length of 7.0 ± 0.5 cm (mean ± SD). They were safely brought to the laboratory and acclimatized for 20 days in a large cement tank (containing 1000 L of water) prior to the experiment. During the acclimatization period, fish were fed *ad libitum* with rice bran and groundnut oil cake in the form of dough one time a day. Water was renewed (one third of the water) daily and feeding was withheld 24 h before the commencement of the experiment. The tap water free from chlorine was used and the water had the following physicochemical characteristics [36]; temperature ($27.0 \pm 1.2\,^\circ C$), pH (7.2), dissolved oxygen ($6.2\,mg\,L^{-1}$), total hardness ($89\,mg\,L^{-1}$, as $CaCO_3$) and salinity ($0.4 \pm 0.02‰$). Before the experiment, fish were randomly divided into two groups which were housed in 200 L aquaria with tap water and continuously aerated. Photoperiod of the study was a 12:12 light-dark cycle.

2.3. Experimental design and acute toxicity test

A 96 h acute test was conducted in order to determine the short-term impacts of CA and DCF. The nominal concentrations of CA and DCF including 1, 10 and $100\,\mu g\,L^{-1}$ were added in each glass aquaria ($120\,cm \times 80\,cm \times 40\,cm$) containing 60 L of water. Three replicates were maintained for each concentration groups and 30 fish of equal size and weight were introduced. The test water was renewed at the end of 24 h and freshly prepared solution was added to maintain the concentration of CA and DCF at a constant level. A concurrent control of 30 fish in three different glass aquaria was maintained under identical conditions. The mortality/survival of fish was recorded in every 24 h. The dead fish were removed from the aquaria immediately. Feeding was withheld during the bioassay experiment. At the end of 96 h period fish from the control and drug treated groups were taken for further analysis.

2.4. Blood sample collection

Blood samples were collected by heart puncture using plastic disposable syringes fitted with 26 gauge needles. The syringe and needle were prechilled and coated with heparin (Beparine R heparin sodium, IP 1000 IU mL^{-1} derived from beef intestinal mucosa containing 0.15% w/v chlorocresol IP preservative), an anticoagulant manufactured by Biological E Limited, Hyderabad, India. The collected blood was transferred into small vials, which is previously rinsed with heparin. Whole blood was used for the estimation of hemoglobin, RBC and WBC counts. The remainder of the blood sample was centrifuged at 9392 g, at 4 °C for 20 min to separate the plasma, which was used for the estimation of biochemical parameters (glucose and protein), electrolytes (Na^+, K^+ and Cl^-) and enzymes (GOT, GPT and LDH).

2.5. Hematological studies

RBC and WBC were counted by haemocytometer method [37]. Hb content of the blood was estimated by the method of cyanmethemoglobin [38]. Hct was estimated by the microhematocrit method [39]. Erythrocyte indices of fish *viz*., MCV, MCH and MCHC were also calculated according to standard formulas.

$$\text{MCV(cubic micra)} = \frac{\text{HCT(\%)}}{\text{RBC (millions} \times \text{cu} \times 10^6)} \times 100 \quad (1)$$

$$\text{MCH(picograms)} = \frac{\text{Hb(g/dl)}}{\text{RBC (millions} \times \text{cu} \times 10^6)} \times 100 \quad (2)$$

$$\text{MCHC(g/dl)} = \frac{\text{Hb(g/dl)}}{\text{HCT(\%)}} \times 100 \quad (3)$$

2.6. Biochemical studies

2.6.1. Estimation of plasma glucose and protein

Plasma glucose was estimated following the method of Cooper and Mc Daniel [40]. To 5.0 mL of *O*-toluidine colour reagent, 0.1 mL of plasma was added and the content was mixed well and placed in

Table 1a
Hematological profiles (Hb, Hct, RBC, and WBC) in a freshwater fish *C. carpio* after 96 h exposure to different concentrations of CA and DCF.

Drug	Concentrations ($\mu g\,L^{-1}$)	Hb (g/dL)	Hct (%)	RBC (million/cu mm)	WBC (1000/cu mm)
CA	Control	4.627[b]	13.72[b]	0.332[a]	16.780[d]
	1	2.468[d]	7.20[d]	0.250[c]	24.663[c]
	10	3.667[c]	10.56[c]	0.244[c]	30.065[b]
	100	5.485[a]	16.02[a]	0.280[b]	47.101[a]
DCF	Control	4.716[a]	13.840[a]	0.377[a]	18.460[d]
	1	4.364[a]	12.664[a]	0.338[b]	25.874[c]
	10	3.316[b]	9.982[b]	0.284[c]	47.515[a]
	100	2.793[b]	7.380[c]	0.243[d]	43.205[b]
F-statistics#					
	Concentration (C, $F_{3,32}$)	15.09**	13.18**	43.76**	784.31**
	Drug (D, $F_{1,32}$)	3.09 ns	3.56 ns	27.93**	84.43**
	$C \times D$ ($F_{3,32}$)	38.06**	36.45**	15.95**	106.73**

ns, Not significant, **, Significant at $P < 0.01$.
Means in a column followed by common superscript for the drug are not significantly different ($P < 0.05$) according to DMRT.

boiling water for 10 min. The content was then cooled under running tap water for 5 min and the optical density (OD) of the sample was measured at 630 nm within 30 min in a UV spectrophotometer. Plasma protein was estimated following the method of Lowry et al. [41]. To 0.90 mL of distilled water, 0.10 mL of plasma was added and treated with 5.0 mL of solution C [50 mL of solution A (2.00 gm of sodium carbonate was dissolved in 100.00 mL of 0.1 N NaOH), was dissolved with 1 mL of solution B (500.00 mg of copper sulphate was dissolved in 100.00 mL of 1% sodium potassium tartarate solution)]. The prepared content was allowed to stand at room temperature for 10 min, and then 0.5 mL of Folin-phenol was added. After 15 min, the colour intensity was read at 720 nm in a UV spectrophotometer.

2.7. Analysis of plasma electrolytes

Plasma sodium (Na^+) and potassium (K^+) were estimated by the method of Trinder [42] and Sunderman [43] and chloride (Cl^-) was estimated by the modified method of Young et al. [44].

2.8. Enzymological analysis

2.8.1. Determination of GOT, GPT and LDH activity

Plasma GOT and GPT activities were estimated by 2,4-DNPH method described by Reitmen and Franckel [45], and LDH activity was measured following the methodology described by Tietz [46].

2.8.2. Estimation of gill Na^+/K^+-ATPase activity

The gills were isolated from the control and drug treated fish and 100 mg of each tissue was weighed and homogenized with Teflon homogenizer along with 1 mL of 0.1 M Tris–HCl buffer adjusted to pH 7.4). The homogenates were centrifuged at 93.9 g for 15 min at 4 °C and the clear supernatant was used for the estimation of Na^+/K^+-ATPase activity following the method of Shiosaka et al. [47].

2.9. Statistical analysis

All values were expressed as means and analyzed by analysis of variance (ANOVA), followed by a DMRT (Duncan Multiple Range Test) test to determine the significant differences ($P < 0.01$ and $P < 0.05$) among the concentrations, between the drugs, and the difference between the concentrations and drugs on each parameters.

3. Results

3.1. Hematological indices

The hematological parameters *viz*., Hb, Hct, RBC, WBC, MCV, MCH and MCHC in *C. carpio* exposed to CA and DCF for 96 h exposure showed alterations when compared to control groups (Tables 1a and 1b). Hb and Hct contents were decreased in both CA and DCF treatments (except in 100 $\mu g\,L^{-1}$ of CA treatment). RBC count was decreased in both treatments. However, WBC count was increased at all concentrations of CA and DCF treatments. Among the hematological indices, MCV and MCH values were increased at

Table 1b
Hematological indices (MCV, MCH, and MCHC) in a freshwater fish *C. carpio* after 96 h exposure to different concentrations of CA and DCF.

Drug	Concentrations ($\mu g\,L^{-1}$)	MCV (cu m)	MCH (pg)	MCHC (g/dl)
CA	Control	413.770[b]	139.369[b]	33.713[a]
	1	288.920[c]	99.074[c]	34.290[a]
	10	435.112[b]	151.073[b]	34.742[a]
	100	573.389[a]	196.327[a]	34.198[a]
DCF	Control	367.330[a]	125.144[a]	293.400[a]
	1	374.924[a]	129.166[a]	290.090[a]
	10	352.888[a]	117.032[a]	280.400[a]
	100	304.846[a]	115.396[a]	292.094[a]
F-statistics#	Concentration (C, $F_{3,32}$)	6.11**	9.52**	<1 ns
	Drug (D, $F_{1,32}$)	18.96**	20.04**	34105.59**
	$C \times D$ ($F_{3,32}$)	16.74**	17.33**	<1 ns

ns, Not significant, **, Significant at $P < 0.01$.
Means in a column followed by common superscript for the drug are not significantly different ($P < 0.05$) according to DMRT.

Table 2
Biochemical changes (plasma glucose and plasma protein) in a freshwater fish *C. carpio* after 96 h exposure to different concentrations of CA and DCF.

Drug	Concentrations ($\mu g\,L^{-1}$)	Glucose ($mg\,100\,mL^{-1}$)	Protein ($\mu g\,mL^{-1}$)
CA	Control	74.028^{c}	1.784^{b}
	1	111.866^{b}	2.426^{a}
	10	134.411^{a}	2.502^{a}
	100	132.401^{a}	2.472^{a}
DCF	Control	88.82^{c}	1.851^{c}
	1	106.08^{b}	2.619^{b}
	10	122.77^{a}	2.769^{a}
	100	132.30^{a}	2.606^{b}
F-statistics#	Concentration (C, $F_{3,32}$)	44.35**	229.16**
	Drug (D, $F_{1,32}$)	<1 ns	44.06**
	$C \times D$ ($F_{3,32}$)	2.63 ns	2.92*

ns, Not significant, *, Significant at $P < 0.05$; **, Significant at $P < 0.01$.
Means in a column followed by common superscript for the drug are not significantly different ($P < 0.05$) according to DMRT.

10 and 100 $\mu g\,L^{-1}$ of CA treatment, whereas these parameters were found to be decreased at 1 $\mu g\,L^{-1}$. In contrast, MCV and MCH values were increased at 1 $\mu g\,L^{-1}$ of DCF treatments whereas the values were found to be decreased at 10 and 100 $\mu g\,L^{-1}$ treatments. But the MCHC values in both drugs treated groups were found to be similar to control groups. A significant ($P < 0.01$) change was found in all hematological parameters among concentrations (C), between drugs (D) and also between the concentrations and drugs ($C \times D$) (Tables 1a and 1b).

3.2. Biochemical parameters

Plasma glucose level was elevated at all concentrations of CA and DCF exposed fish when compared with controls (Table 2). A significant ($P < 0.01$) difference was observed among the concentrations of CA and DCF. No significant difference in plasma glucose level was observed between the drugs and between the concentrations and drugs. Furthermore, plasma protein level was also increased at all concentrations of CA and DCF exposed fish comparatively to control groups (Table 2). A significant ($P < 0.01$) relationship was found among the concentrations of both drugs and also between the drugs. The interaction between the concentrations and drugs on plasma protein level showed a significant value at $P < 0.05$.

3.3. Plasma electrolytes

During 96 h exposure period, plasma Na^+ level was decreased at all concentrations of CA and DCF (Table 3). However, a maximum decreased level was observed in 100 $\mu g\,L^{-1}$ of CA and DCF concentrations when compared to other concentrations. A significant ($P < 0.01$) difference in plasma Na^+ level was observed among the concentrations of both CA and DCF. The difference between the drugs and also between the concentrations and drugs were noted at significant ($P < 0.05$) level on plasma Na^+ levels. Similarly, plasma K^+ level was also decreased in all CA and DCF concentrations (Table 3). Besides, a maximum decrease in K^+ level was noted in 100 $\mu g\,L^{-1}$ concentrations of both CA and DCF when compared to other concentrations. There was no significant difference in plasma K^+ level among the concentrations. However, a significant ($P < 0.01$) difference was noted between the drugs and also between the concentrations and drugs ($P < 0.05$). Plasma Cl^- level was found to be increased at all concentrations of CA whereas in DCF concentrations plasma Cl^- level was decreased when compared to control groups (Table 3). There was no significant difference among the concentrations of CA and DCF. A significant ($P < 0.01$) difference was found between the drugs and also between the concentrations and drugs.

3.4. Enzyme assay

The GOT activity was decreased at all concentrations (1, 10, and 100 $\mu g\,L^{-1}$) of CA and DCF treated fish. A maximum decrease was noted in CA concentrations when compared to DCF (except 1 $\mu g\,L^{-1}$) concentrations (Table 4). A significant ($P < 0.01$) difference was observed among the concentrations and also between concentrations and drugs. Between the drugs a significant difference in GOT activity was found at $P < 0.05$ level. GPT activity was increased at all concentrations of both CA and DCF (except 1 $\mu g\,L^{-1}$ concentration of CA) (Table 4). A maximum increase in GPT activity was noted in 100 and 10 $\mu g\,L^{-1}$ concentrations of CA and DCF, respectively. LDH activity was also increased at all concentrations of CA and DCF treatments (Table 4). However, a maximum increase was noted in CA treatment when compared to DCF treatment throughout the study period (96 h). A significant ($P < 0.01$) difference was observed in GPT and LDH activities among the concentrations, between the

Table 3
Ionoregulatory (Na^+, K^+ and Cl^-) responses in a freshwater fish *C. carpio* after 96 h exposure to different concentrations of CA and DCF.

Drug	Concentrations ($\mu g\,L^{-1}$)	Na^+ ($mmol\,L^{-1}$)	K^+ ($mmol\,L^{-1}$)	Cl^- ($mmol\,L^{-1}$)
CA	Control	142.57^{a}	9.345^{a}	125.76^{c}
	1	118.29^{b}	8.832^{a}	175.51^{a}
	10	110.05^{b}	9.280^{a}	144.75^{bc}
	100	116.34^{b}	7.935^{a}	164.09^{ab}
DCF	Control	136.52^{a}	8.985^{a}	132.41^{a}
	1	128.13^{ab}	7.797^{b}	109.08^{ab}
	10	124.64^{b}	7.887^{ab}	112.16^{ab}
	100	119.05^{b}	6.756^{ab}	89.89^{b}
F-statistics#	Concentration (C, $F_{3,32}$)	20.67**	2.05 ns	1.27 ns
	Drug (D, $F_{1,32}$)	5.07*	9.35**	43.51**
	$C \times D$ ($F_{3,32}$)	4.01*	3.90*	8.55**

ns, Not significant; *, Significant at $P < 0.05$; **, Significant at $P < 0.01$.
Means in a column followed by common superscript for the drug are not significantly different ($P < 0.05$) according to DMRT.

Table 4
Enzymological (GOT, GPT, LDH and Na^+/K^+-ATPase) responses in a freshwater fish *C. carpio* after 96 h exposure to different concentrations of CA and DCF.

Drug	Concentrations ($\mu g\,L^{-1}$)	GOT ($IU\,L^{-1}$)	GPT ($IU\,L^{-1}$)	LDH ($IU\,L^{-1}$)	Na^+/K^+-ATPase ($\mu g/h/g$)
CA	Control	40.468[a]	52.593[c]	1775.8[c]	18.88[b]
	1	14.052[b]	46.035[d]	1855.3[c]	24.32[a]
	10	14.608[b]	69.219[b]	2737.9[b]	25.92[a]
	100	14.007[b]	74.912[a]	2980.3[a]	25.44[a]
DCF	Control	32.604[a]	54.623[c]	1314.7[c]	19.04[b]
	1	32.463[a]	67.553[b]	1686.9[b]	22.08[a]
	10	11.950[b]	73.436[a]	1709.6[b]	22.96[a]
	100	29.230[a]	71.466[ab]	1985.1[a]	24.11[a]
F-statistics#	Concentration (C, $F_{3,32}$)	18.22**	79.56**	102.72**	15.33**
	Drug (D, $F_{1,32}$)	6.57*	29.63**	233.79**	5.43*
	$C \times D$ ($F_{3,32}$)	8.30**	23.31**	21.60**	<1 ns

ns, Not significant, *, Significant at $P < 0.05$; **, Significant at $P < 0.01$.
Means in a column followed by common superscript for the drug are not significantly different ($P < 0.05$) according to DMRT.

drugs, and also between the concentrations and drugs. The Na^+/K^+-ATPase activity in gill was increased in 1, 10, and 100 $\mu g\,L^{-1}$ of CA and DCF after 96 h exposure, compared with the respective controls (Table 4). Statistically, a significant ($P < 0.01$) difference was observed among the concentrations of both CA and DCF. Further, the differences induced between CA and DCF shows a significant value at $P < 0.05$. The interaction between concentrations and drugs on gill Na^+/K^+-ATPase activity was not significant.

4. Discussion

The occurrence of pharmaceutical chemicals and its residues in aquatic environments pose a major problem in most of the countries. Consequently, environmental risk assessment of these emerging pollutants is needed to evaluate their impacts on non target organisms. In ecotoxicological studies, static bioassay tests have been widely used for evaluating the impacts of toxic chemicals on aquatic organisms [48]. The scientific reports of APHA [49] accepted bioassay studies as standard methods for assessing the toxicity of any new chemical compounds that enter into the aquatic ecosystems. In bioassay method, acute toxicity test is commonly used to evaluate the potential threat of several aquatic pollutants.

4.1. Hematological indices

The decrease in Hct, Hb and RBC count levels (except in CA at higher concentration of 100 $\mu g\,L^{-1}$) may be indicators of anemia [50] and reduction in RBC count caused either by the inhibition of erythropoiesis or by the destruction of red cells by the drugs CA and DCF. Similar decrease in RBC count, Hb and Hct values were also reported in carps exposed to toxicants [27,51]. Further, the elevated level of Hb content in the CA exposure at 100 $\mu g\,L^{-1}$ might have resulted from replacement of oxidized denatured Hb and to supply more oxygen to tissues. In this study, the increase in Hct level at 100 $\mu g\,L^{-1}$ of CA treated fish indicates impaired respiratory capacity of the fish due to damage in the gill caused by the drug. Most of the pharmaceutical compounds in the aquatic environment enter into fish body through gill and food. Swelling of RBCs due to stress may also contribute an increase in Hct level [29].

The alterations in MCV, MCH and MCHC levels with the lower and higher concentrations of CA and DCF might be due to stress response to the drugs. The increase in MCV may also result from the increase of immature RBC [52]. Primarily, WBCs are involved in the regulation of immunological function in many organisms and the changes in WBC number to pollutants reflect the decrease in the non specific immunity of the fish [28]. In the present study also the increase in WBC count in both CA and DCF treatments indicate a generalized immune response to drug toxicity or the immune system may be stimulated by the drugs to protective against toxicity. An increase in MCV and MCH levels indicates the swelling of RBCs due to drug toxicity, whereas the decrease in these hematological parameters might have resulted from impaired oxygen uptake due to gill damage caused by the drugs. No significant change in MCHC value was observed in both the treatments at all concentrations. The changes in hematological parameters of rainbow trout (*Oncorhynchus mykiss*) exposed to a lipophilic drug, verapamil indicates a compensatory responses to maintain the gas transfer [53]. A similar mechanism may be operated in the present study on hematological parameters of CA and DCF treated fish.

4.2. Biochemical profiles

In general environmental contaminants in aquatic media induce significant changes in biochemical parameters of aquatic organisms. We found a significant increase in plasma glucose and protein level in fish exposed to various concentrations of CA and DCF. Basically, environmental stress alters carbohydrate metabolism in fish. An elevation of blood glucose level in the present study may indicate gluconeogenesis to compensate the increased metabolic demands imposed by the drugs [25]. Increased plasma protein level in both the treatments indicates an adaptation of the fish to the drug toxicity.

4.3. Ionoregulatory response

Gills of freshwater fish play an important role in the transport of ions like Na^+, K^+ and Cl^- to maintain acid base balance, osmotic pressure of the body and regulation of water influx and ion efflux [54]. In fish, gills due to their intimate contact with water likely to be the important target organ for aquatic pollutants. In this study, the reduced level of plasma Na^+ and Cl^- ions in CA and DCF treated fish might have resulted from histological alterations of gills or disturbances in the membrane permeability due to drug toxicity. Osmoregulatory failure may be also a reason for decreased levels of major plasma ions [55]. The decrease in plasma K^+ level indicates the inhibition of the Na^+/K^+-ATPase due to drug toxicity (in this study the inhibition of Na^+/K^+-ATPase was noted). The decrease in plasma Cl^- level in DCF treated fish indicates an apparent decrease of blood chloride concentrations in fish due to reduced activity of carbonic anhydrase or interference of cortisol [56]. The elevation in plasma Cl^- level in CA treatment may be due to transportation of chloride ions from other tissues into blood due to imbalances in the osmoregulation process [57]. Fletcher [58] suggested that loss

of water from the circulation may also leads to significant rise in plasma electrolytes in *Pseudopleuronectes americanus* during stress.

4.4. Enzymological activity

The enzymes GOT, GPT and LDH can be used for detection of tissue damage and bioindicators in animals subjected to acute and chronic exposure of xenobiotics [59]. The decrease in plasma GOT activity in both CA and DCF treatments indicates the accumulation and toxicity of these drugs in liver which might have caused the necrosis and death of liver cells. A similar observation was also made in *C. punctatus* exposed to As_2O_3 [60]. The enzyme GPT is mainly present in the liver and any damage to liver leads to release of these enzymes into blood stream. Moreover, the damage and severity of the organ (liver) is mainly depends on the exposure period and the type of toxicant [61]. In our study, the elevations of GPT activity indicate that the organism tries to mitigate the drug induced stress. The increase of GOT and GPT activity in *O. mykiss* exposed to verapamil indicates amplified transamination processes [53].

LDH involved in carbohydrate metabolism, can be used as a good indicator to chemical exposure and stress in fish. Jose et al. [62] suggested that the increase in LDH activity in cultures exposed to carbamazepine may be due to stabilization of cytoplasmic membrane. Elevation of LDH activity in mosquito fish, *Gambusia holbrooki* after acute exposure to CA may be due to stress response caused by CA [10]. Similarly, in carbamazepine treated rainbow trout *O. mykiss*, plasma LDH activity was increased during the chronic exposure [63]. The observed elevation of LDH activity in CA and DCF treated fish indicate structural damage of the cell membranes or hepatic or heart tissues. In addition, changes in protein and carbohydrate metabolism may cause a change in LDH activity [64].

Gill Na^+/K^+-ATPase is intimately involved in electrolyte balance and the determination of ATPase activity would prove to be an important index for tolerable levels of a large group of environmental contaminants [65,66]. However, the impacts of pharmaceutical drugs on gill ATPase activity in freshwater fish have not been given enough attention. The significant increase in gill Na^+/K^+-ATPase activity at all the concentrations of CA and DCF indicating the direct action of these drugs on ATPase function. Further, the increase in gill Na^+/K^+-ATPase activity may be a compensation for a dysfunctional regulation of ionic levels or a process to restore electrolyte levels [67,68]. On the whole, in our study a significant alteration in ionic levels were noted in both CA and DCF treatments and therefore we warrant a further in depth study.

5. Conclusion

Collectively this study concluded that different concentrations (1, 10, and 100 $\mu g\,L^{-1}$) of CA and DCF have a profound influence on the hematological, biochemical, ionoregulatory and enzymological profiles of freshwater fish *C. carpio*. These parameters could be effectively used as potential biomarkers of pharmaceutical toxicity to freshwater fish in the field of environmental biomonitoring. Furthermore, chronic effects of CA, DCF and their metabolites on these parameters along with other parameters such as hormonal and histopathological studies need to be investigated in the future studies.

Acknowledgments

The author (Manoharan Saravanan) would like to thank and acknowledge the Council of Scientific and Industrial Research (CSIR), New Delhi, Government of India for providing financial support in the form Senior Research Fellowship for this study (Fellowship award letter no: 09/472(0141)/2009-EMR-I, dated: 01.05.2009).

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