Ecotoxicology and Environmental Safety 74 (2011) 1216–1225

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Chronic exposure to diclofenac on two freshwater cladocerans and Japanese medaka

Jinyoung Lee [a], Kyunghee Ji [a], Young Lim Kho [b], Pilje Kim [c], Kyungho Choi [a,*]

[a] School of Public Health, Seoul National University, Seoul 151-742, Republic of Korea
[b] School of Human & Environmental Sciences, Eulji University, Seongnam, Gyeonggi 461-713, Republic of Korea
[c] National Institute of Environmental Research, Incheon 404-708, Republic of Korea

ARTICLE INFO

Article history:
Received 12 January 2011
Received in revised form
15 March 2011
Accepted 19 March 2011
Available online 13 April 2011

Keywords:
Diclofenac
Long-term exposure
Reproduction
Japanese medaka
Surface water

ABSTRACT

Consequences of long-term exposure to diclofenac up to 3 months were evaluated using freshwater crustaceans (*Daphnia magna* and *Moina macrocopa*) and a fish (*Oryzias latipes*). Marked decrease of reproduction was observed at 25 mg/L for *D. magna*, and at 50 mg/L for *M. macrocopa*. Three-month exposure of fish to 0.001–10 mg/L of diclofenac resulted in significant decreasing trend in hatching success and delay in hatch. The hatching of the eggs produced from the fish exposed to 10 mg/L was completely interfered, while fertility of the parent generation was not affected. Gonadosomatic index (GSI) of female fish was also affected at 10 mg/L. Predicted no effect concentration of diclofenac was estimated at 0.1 mg/L, which is a few orders of magnitude greater than those observed in ambient water. Therefore direct impact of diclofenac exposure is not expected. However its bioaccumulation potential through food web should warrant further evaluation.

1. Introduction

Diclofenac, a non-steroidal anti-inflammatory drug (NSAID), has been widely applied for veterinary and human use. This compound has been frequently detected in sewage treatment plants (STP) and surface waters worldwide. For example, median concentrations of 0.02 and 0.42 μg/L of diclofenac were detected, respectively, in streams and STP effluent of the United Kingdom (Ashton et al., 2004). In Germany, the median concentration of detection was 0.15 μg/L in rivers and streams, and 0.81 μg/L in STP effluent (Ternes, 1998). In Korea, diclofenac was detected on an average up to 0.016 μg/L in major rivers (Choi et al., 2009). Recently lethal toxicities of diclofenac on vulture species that has led to near extinction in south Asia and southern Africa were reported (Naidoo et al., 2009; Oaks et al., 2004), and its potential consequences in ecosystems received worldwide interest. Toxicities of diclofenac on aquatic species have been reported for various aquatic species including freshwater algae, water fleas, and fish, but most of the studies were limited to consequences of short-term exposures (Cleuvers, 2004; Ferrari et al., 2003). Due to rapid photolysis of diclofenac under sunlight (Packer et al., 2003), diclofenac remains for a relatively short time in water. However, continuous use of diclofenac might lead to consistent influx of this compound into water, and hence long-tem exposure is possible among freshwater organisms. However, there is limited information on the effects of long-term exposure to diclofenac among aquatic organisms.

Potential adverse effects of diclofenac on reproduction have been suggested in a couple of short-term studies (Ferrari et al., 2003; Hong et al., 2007). Over-expression of vitellogenin (VTG) was observed in *O. latipes* after 4 days (d) exposure to diclofenac at 1 μg/L, suggesting its endocrine disrupting potential (Hong et al., 2007). However, reproduction related effects of long-term exposure to diclofenac have rarely been reported to date.

Reproduction-related damages have been reported for ibuprofen, another most frequently medicated NSAID. In Flippin et al. (2007) ibuprofen induced changes in spawning pattern in breeding pairs of *O. latipes* after 6 weeks of exposure. Han et al. (2010) also showed a marked change of estradiol (E2) concentration in the human adrenocarcinoma (H295R) cell line after 48 hours (h) exposure to ibuprofen, and reproduction related damages in Japanese medaka after 120 d exposure. On the other hand, Ji et al. (2010) could not find hormonal changes in H295R cell line after diclofenac exposure up to 20 mg/L, suggesting a different mode of toxicity compared to ibuprofen.

As regulations on pharmaceutical residues in the environment are being considered by many countries (Choi et al., 2009), there is a growing need for toxicity data from long-term exposure. In this study, aquatic toxicological effects of long-term exposure to diclofenac were evaluated using several model test organisms, e.g., freshwater crustaceans (*Daphnia magna* and *Moina macrocopa*) and

* Corresponding author. Fax.: +82 2 765 9105.
E-mail addresses: kyungho@snu.ac.kr, kchoi1995@paran.com (K. Choi).

0147-6513/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2011.03.014

a fish (*Oryzias latipes*), and predicted no effect concentration (PNEC) for diclofenac was derived. *M. macrocopa* is an autochthonous species in Korea, which has recently been employed for toxicity assessment (Han et al., 2010; Ji et al., 2008).

Test organisms were chosen because of their importance in freshwater ecosystems. Macroinvertebrates have a pivotal role in the maintenance of food web. Especially daphnids are an important source of food of small fish (Anderson, 1944). Accordingly, extinction or even decrease in daphnids population might affect the complex food web of aquatic ecosystem. In addition Japanese medaka (*O. latipes*) is a useful test organism for developmental and reproductive effects of chemicals (Marsh et al., 2010; Metcalfe et al., 1999). Up to 3 months of exposure period was employed and potential effect on the second generation was also evaluated in fish. The results of this study will provide information for derivation of PNEC of this compound, which may be used in ecological risk assessment or in developing environmental quality criteria for this compound.

2. Materials and methods

2.1. Test chemical and chemical analysis

Diclofenac sodium salt (CAS RN: 15307-79-6) was obtained from Sigma Aldrich (St. Louis, MO, USA). The actual concentrations of diclofenac in exposure medium were measured 48 h before and after exposure, using high performance liquid chromatography (Series 1100, Agilent Technologies, Palo Alto, CA, USA) with triple quadruple mass spectrometry (MS/MS). Diclofenac was separated on a 2.0×150 mm Luna C18 column (Phenomenex, Torrance, CA, USA). Injection volume was 5 μL and the flow rate was 200 μL/min. Analytes were separated in isocratic mode with mobile phases of 90% of A (5 mM ammonium acetate in water) and 10% of B (methanol) (v/v).

Identification and quantification of analytes were accomplished by use of an API 4000 triple MS/MS system (Applied Biosystems, Foster City, CA, USA), operated in the electrospray ionization (ESI) negative mode with multiple reaction monitoring (MRM). The ESI conditions for the analysis of diclofenac were optimized to the following conditions: ion source voltage—4.5 kV and ESI temperature 400 °C. The mass analyzer was operated in the MRM mode: m/z 294→214.

2.2. Test organisms and maintenance

Test organisms were cultured in Environmental Toxicology Laboratory of Seoul National University (Seoul, Korea) following the method described in Ji et al. (2008). Water quality parameters (hardness, alkalinity, pH, temperature, conductivity, and dissolved oxygen) were routinely measured (American Public Health Association, 1992, Supplemental Materials Tables S1 and S4).

2.3. Acute and chronic exposures of D. magna and M. macrocopa to diclofenac

The 48 h acute toxicity tests with *D. magna* and *M. macrocopa* were carried out following U.S. EPA (2002). The range of test concentrations was determined by preliminary range finding toxicity tests. Test concentrations were prepared by dissolving diclofenac sodium salt in culture media with 2-fold serial dilution (control, 12.5, 25, 50, 100, and 200 mg/L for *D. magna*, and control, 25, 50, 100, 200, and 400 mg/L for *M. macrocopa*). After 24 and 48 h of exposure to diclofenac, the numbers of survivals were counted.

For chronic exposure, test solutions were prepared in 3-fold serial dilution (control, 0.93, 2.8, 8.3, 25.0, and 75.0 mg/L for *D. magna* and control, 1.85, 5.6, 16.7, 50.0, and 150.0 mg/L for *M. macrocopa*). The chronic toxicity tests with *D. magna* and *M. macrocopa* were conducted following OECD guideline 202 (OECD, 1993) and Oh (Sorin Oh, 2007, Development of a standard 7 d chronic toxicity test method using indigenous aquatic macroinvertebrate *M. macrocopa*, Seoul National University Master of Public Health thesis, Seoul, Korea), respectively. Several endpoints related to survival and reproduction were observed following Ji et al. (2008), and the population growth rate was calculated by employing the Euler–Lotka equation (Newman, 2010). Water quality parameters were measured during acute and chronic *D. magna* toxicity test (Supplemental materials, Tables S2 and S3).

2.4. O. latipes 3-month exposure

Long-term 3-month exposure study was conducted employing *O. latipes* following Japanese Ministry of the Environment (2002). Fish were exposed to control, 0.001, 0.01, 0.1, 1, and 10 mg/L of diclofenac. Water quality parameters were measured during the fish exposure (Supplemental materials, Table S5).

2.4.1. Collection of eggs

About 20 pairs of *O. latipes* (body length $\sim 2.5 \pm 1$ cm) were transferred to 1 L beakers containing fresh culture medium, and allowed for mating for at least 7 d. Eggs from all the pairs were pooled and used for experiments when appropriate numbers of eggs (>300) were obtained in a given day. The fish were fed with *Artemia* nauplii (<24 h after hatching) twice daily, and dead fish were removed immediately.

2.4.2. Egg phase

Eggs were exposed to control, 0.001, 0.01, 0.1, 1, and 10 mg/L of diclofenac. Each treatment or control consists of four replicates with 12 eggs each in 50 mL beaker (<12 h after fertilization). Dead embryos were removed daily, and the media were renewed with freshly prepared solutions 3 times a week. Several endpoints including larval survival, hatchability, and time-to-hatch were observed until all surviving embryos were hatched (Nirmala et al., 1999).

2.4.3. Larval-juvenile phase

Newly hatched larvae were transferred to 250 mL beakers immediately, and were kept under exposure to control, 0.001, 0.01, 0.1, 1, and 10 mg/L of diclofenac until 30 days post hatch (dph). The fish in larval-juvenile phase were fed with *Artemia* nauplii (<24 h after hatching) twice a day, and the media was replaced 3 times a week. The dead fish were removed from culture beakers, and the number of mortality was recorded. At the 30 dph, 4 juvenile fish per each treatment group were randomly selected, and were measured for body length and weight to calculate condition factor ($[K]=100 \times$ total weight/total length^3). The surviving juveniles of each treatment group were subsequently transferred to 1 L beakers, and kept in the same exposure condition until 77 dph.

2.4.4. Adult phase

On 77 dph, 3–6 fish among the surviving fish were euthanized by chilling on ice, and then the condition factor was calculated. The fish gonads and livers were also collected and weighed to calculate gonadosomatic index ($[GSI]=100 \times$ gonad weight/body weight) and hepatosomatic index ($[HSI]=100 \times$ liver weight/body weight). Portion of the surviving fish were paired and kept in the same exposure condition for 1 week, and then the eggs spawned were collected. The eggs were separately exposed to the concentration of diclofenac where their parental generation was exposed, and were observed for several endpoints including fertility, hatchability, and time-to- hatch.

2.5. Quantification of vitellogenin protein using enzyme-linked immunosorbent assay (ELISA)

For the measurement of VTG protein level in fish blood plasma, blood samples were obtained from male adult fish (77 dph) of control and each treatment. Blood samples were collected by making an incision of 1–2 mm behind the anus. About 1 μl of blood per fish was gathered in a heparinized 5 μl capillary tube (Marienfeld, Lauda-Königshofen, Germany), and was immediately mixed with an assay buffer to make 50-fold dilution. The samples underwent centrifugation at 8000 rpm for 10 min, and then the supernatant was collected and stored at

Table 1
The nominal concentration and measured concentration of diclofenac obtained from culture media of *D. magna* and *O. latipes*.

Organisms	LOD[a] (mg/L)	Nominal concentration (mg/L)	Measured concentration (mg/L)	
			Before exposure	48 h after exposure
D. magna	0.0003	0	< LOD[a]	< LOD[a]
		0.9	0.9	1.0
		2.8	2.1	2.3
		8.3	6.2	7.0
		25.0	17.6	18.0
		75.0	50.1	59.8
O. latipes	0.0003	0	< LOD[a]	< LOD[a]
		0.001	0.002	0.002
		0.01	0.01	0.01
		0.1	0.1	0.1
		1	0.9	0.7
		10	5.0	4.0

[a] LOD: Limit of detection.