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Original article

Assessment of diclofenac LC50 reference values in juvenile and embryonic stages of the zebrafish (*Danio rerio*)

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Abstract

The aim of the study was to compare the acute toxicity of diclofenac to juvenile and embryonic stages of the zebrafish (*Danio rerio*). Acute toxicity tests were performed on the aquarium fish *Danio rerio*, which is one of the model organisms most commonly used in toxicity testing. The tests were performed using a semi-static method according to OECD guideline No. 203 (Fish, acute toxicity test). Embryo toxicity tests were performed in zebrafish embryos (*Danio rerio*) in compliance with OECD No. 212 methodology (Fish, short-term toxicity test on embryo and sac-fry stages). The results were subjected to a probit analysis using the EKO-TOX 5.2 programme to determine 96hLC50 and 144hLC50 (median lethal concentration, 50% mortality after a 96 h or 144 h interval, respectively) values of diclofenac. The statistical significance of the difference between LC50 values in juvenile and embryonic stages of *Danio rerio* was tested using the Mann-Whitney non-parametric test implemented in the Unistat 5.1 programme. The LC50 mean value of diclofenac was 166.6 ± 9.8 mg/L in juvenile *Danio rerio*, and 6.11 ± 2.48 mg/L in embryonic stages of *Danio rerio*. The study demonstrated a statistically higher sensitivity to diclofenac ($P<0.05$) in embryonic stages compared to the juvenile fish.

Key words: NSAIDs, diclofenac, LC50, zebrafish

Introduction

Many pharmaceuticals are persistent substances which are, because of their biological activity in aquatic systems, recognized as a continuing threat to environmental stability. Chronic ecotoxicity data as well as information on current distribution levels in different environmental compartments unfortunately continue to be sparse and are focused on those therapeutic classes that are more frequently prescribed and consumed. Nevertheless, they indicate the negative impact that these chemical contaminants may have on living organisms, ecosystems and, ultimately, public health (Ottmar et al. 2010, Santos et al. 2010).

Diclofenac (sodium 2-[2-(2,6-dichloroanilino) phenyl]acetate) belongs to the class of drugs called non-steroidal anti-inflammatory drugs (NSAIDs). Diclofenac is an important drug in ambulatory care and is used to reduce pain, inflammation and stiffness caused by many conditions, such as osteoarthritis,

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rheumatoid arthritis, abdominal cramps associated with menstruation, and ankylosing spondylitis (Chan et al. 2001). It is used worldwide and has a production volume estimated to be in the hundreds of tons annually. It is used in the form of tablets, capsules, suppositories, and intravenous solutions, and in ointments and gels for dermal application. It is readily metabolized after oral use, but assimilation is lower after dermal application (Buser et al. 1998). After being used in human therapy, this drug finds its way into municipal sewage treatment plants, where it is not completely eliminated and is often discharged as a contaminant to the receiving waters (Hallare et al. 2004, Glassmeyer and Shoemaker 2005, Kotyza et al. 2009). The predicted environmental concentration (PEC) for Central European surface waters is set at 0.54 µg/L (Hallare et al. 2004). However, Ternes (2001) has reported values of up to 1.2 µg/L. Based on the monitoring of freshwater sources in the Czech Republic, which is done by the Czech Hydrometeorological Institute, we can conclude that in 2009 almost 100% of withdrawn samples tested positive for diclofenac and the concentrations found were between 7 and 18 ng/L. Diclofenac has also been detected in aquatic systems in several other countries, including Germany, Sweden, France, Greece and Italy (Jux et al. 2002, Ferrari et al. 2003, Bendz et al. 2005).

The exact environmental fate, ecotoxicological effects and mechanisms of action of many pharmaceuticals in non-mammalian species are poorly understood (Halling-Sørensen et al. 1998). Diclofenac is no exception, as its total impact on non-target organisms has not yet been identified. Non-target species considered to be most endangered by its action are probably aquatic organisms. This is why fish were used in this study as subjects for the assessment of the acute toxicity of diclofenac. Zebrafish (*Danio rerio*) were chosen in particular because much has already been written about the physiologically normal development and ecotoxicology of this species. The zebrafish is a small, freshwater, aquarium species which is easy to raise and maintain in different environments, has a short generation time, and breeds almost all year round (Hallare et al. 2004). To compare the effects of diclofenac on different life stages of this fish, we performed toxicity tests in both the juvenile and embryonic stages. Eggs of this species are fertilized externally and embryos are completely transparent, allowing one to follow the development of every individual cell (Driever et al. 1994). The early life stage test using zebrafish embryos is currently one of the most widely used tools for investigating detrimental effects of aquatic pollutants in fish. Several authors consider the early life stage to be the most sensitive (Kristensen 1994, Luckenbach et al. 2001), though this may not necessarily be true for all compounds and species. The growing embryos offer many diverse endpoints to determine sublethal effects. The species could serve as an excellent model for studying embryotoxic effects induced by pollutants (Hallare et al. 2004).

The aim of the present study was to determine the acute toxicity of diclofenac to zebrafish (*D. rerio*) (especially 96h LC50 for juvenile and 144h LC50 for embryonic stages) and to compare its toxicity to the juvenile and embryonic stages of this fish.

Materials and Methods

Determination of diclofenac

During the whole course of testing (always before and after the bath change), samples for the determination of diclofenac concentration were regularly withdrawn from test tanks. Diclofenac determination in water samples was performed by high performance liquid chromatography (HPLC) with photometric detection. Water samples were filtered through a 0.45-µm nylon filter (Millipore, Billerica, MA) and used for analysis. The sample volume injected into the HPLC system was 20 µL. Diclofenac was separated by an isocratic elution method with acetonitrile/water 50/50 (v/v) on a Polaris C18-A column (3 µm, 150 × 4.6 mm, Varian, Inc., Palo Alto, CA). The mobile phase flow rate was 1 ml/min, the column temperature was 25°C, and UV detection was performed at 310 nm. Chromatographic analysis was accomplished by means of an Alliance 2695 chromatographic system (Waters, Milford, MA) with a PDA 2996 photodiode array detector (Waters, Milford, MA). Diclofenac was purchased from Sigma-Aldrich (St. Louis, MO). All solvents were of HPLC-grade purity (Chromservis, s.r.o., CZ). The detection limit for diclofenac was 11 ng/mL. The limit of quantification for diclofenac was 37 ng/mL. The coefficient of variation was 4.5%.

Acute toxicity tests

Acute toxicity tests with diclofenac (Sigma-Aldrich, CAS 15307-79-6) were performed on juvenile stages of the aquarium fish *D. rerio* in accordance with OECD guideline No. 203 (Fish, acute toxicity test). The zebrafish were 2-3 months old, weighed 0.4 ± 0.1 g and their mean length was 31 ± 5 mm. The health status of each fish was regularly checked, with

special attention paid to infectious diseases, and no clinical symptoms appeared during the tests performed.

In the experiment, diclofenac was tested in a series of 5 different concentrations constituting an approximate geometric progression. The fish were obtained from a commercial dealer and were kept in 3-l full glass tanks with dechlorinated water prepared from the city water supply system. The zebrafish were divided into 6 groups (including one control group), each containing 10 fish. The tests were carried out using a semi-static method with solution replacement after 24 hours. During the tests, the water temperature, pH value, dissolved oxygen concentration in each of the test tanks, fish behaviour and mortality rate were recorded each day. The temperature of the experimental bath was 22 ± 1°C; fish were kept under the 12-h/12-h light/dark cycle; the dissolved oxygen concentrations did not fall below 60% (89-100%); and the pH ranged between 6.10 and 7.92. The tested concentrations of diclofenac were 100, 130, 160, 190 and 220 mg/L. Due to the low solubility of diclofenac in water, the dissolution of the substance was achieved using an ultrasound device. The amount of diclofenac for each concentration was weighed separately and each of the solutions was also separately prepared. No fish died in the control tanks during the experiments.

Embryonic toxicity tests

Embryonic toxicity tests were performed on embryos of *D. rerio*. These tests were conducted according to OECD guideline No. 212 (Fish, short-term toxicity test on embryo and sac-fry stages).

A series of five ascending concentrations of substance tested was used in the test. The tested concentrations of diclofenac were 5, 10, 15, 20 and 25 mg/L. Dilution of the stock solution was used for the preparation of the concentrations tested. 20 fertilized eggs in a Petri dish were tested at each concentration and in one control. The volume of liquid was 20 mL in each Petri dish. The eggs were placed in the Petri dishes within 8 hours after fertilization at the latest. The tests were terminated after hatching and the absorption of the yolk sack in all individuals in the control dish (96-144 h after placement into the dish). The baths were replaced at 24 h intervals. Early life stage parameters such as egg and embryo mortality, gastrulation, somite formation, movement and tail detachment, pigmentation, heart rate, and hatching success were noted. During the test, the number of dead embryos in individual concentrations was recorded. The mortality rate of the control embryos did not exceed 20%. Test bath temperatures were between 25 ± 0.5°C.

Parameters of diluting water

The basic chemical parameters of diluting water used in the toxicity tests on embryonic and juvenile stages were: $ANC_{4.5}$ 4.2 mmol/L; COD_{Mn} 2.8 mg/L; total ammonia below the limit of determination; NO_3^- 23.48 mg/L; NO_2^- below the limit of determination; Cl^- 18.11 mg/L; $\Sigma\ Ca^{2+} \pm Mg^{2+}$ 14 mg/L.

Statistical analysis

The results (mortality of fish and embryos in individual test concentrations) were subjected to a probit analysis (EKO-TOX 5.2 programme) to determine the LC50 values of diclofenac. The statistical significance of the difference between 96h LC50 values for the juvenile and 144h LC50 values for the embryonic stages of *D. rerio* was calculated using the non-parametric Mann-Whitney test and Unistat 5.1 (Unistat Ltd., GB) software.

Results

The results of the toxicity tests showed that the 96h LC50 value of diclofenac for juvenile *D. rerio* was in the range of 156.8-176.4 mg/L (mean 96h LC50 ± SEM = 166.6 ± 9.8 mg/L). The 144h LC50 value for *D. rerio* embryos was determined to be in the range of 3.68-10.7 mg/L (mean ± SEM = 6.11 ± 2.48 mg/L). A comparison of the diclofenac toxicities for the embryonic and juvenile life stages of *D. rerio* revealed a statistically higher sensitivity ($P<0.05$) of the embryonic stage compared to the juvenile life stage.

Discussion

The results of the embryotoxicity tests showed the inhibition of normal development up to the end of 144 h for all exposed groups. There was a delay in the hatching time among embryos exposed to all concentrations, which corresponds with the results obtained by Hallare et al. (2004), who studied diclofenac toxicity to zebrafish embryos exposed to concentrations of 1-2000 µg/L. Late-hatched embryos (120 h) differed morphologically from normally hatched embryos, displaying signs of hydroedema. Van den Brandhof and Montforts (2010) found

growth retardation, delayed hatching, and yolk sac and tail deformation in concentrations of diclofenac above 1.5 mg/L. In their study 72hEC50 was found to be 5.3 mg/L.

On the basis of the available literature, we can compare diclofenac toxicity to water organisms only with data on lethal concentrations assessed by Nassef et al. (2009) in mature Japanese killifish. They found the 96h LC50 of diclofenac to be 10.1 mg/L, which is comparable to the value obtained in our study for embryos of zebrafish (144h LC50 = 6.11 mg/L).

Tissues of the rainbow trout (*Oncorhynchus mykiss*) exposed to diclofenac concentrations ranging from 1 µg/L to 500 µg/L over a 28 day period were investigated by histopathological methods. The highest concentrations of diclofenac were detected in the liver, followed by the kidneys and the gills. As the most prominent reactions induced by diclofenac in the kidney, a severe accumulation of protein in the tubular cells, macrophage infiltration, and structural alterations (dilation, vesiculation) of the endoplasmic reticulum in the renal tubules were observed. Furthermore, necrosis of endothelial cells in the renal corpuscles had occurred. In the liver, the most striking reactions were the collapse of the cellular compartmentation and glycogen depletion in hepatocytes. Observations made in the gills included pillar cell necrosis and hypertrophy of chloride cells; epithelium lifting had also become evident in the secondary lamellae (Schwaiger et al. 2004).

The possibility of nephrotoxic effects of diclofenac after chronic exposure was described by Revai and Harmos (1999). In concentrations of between 7-15 µg/L, diclofenac exposure induced tubular necrosis in the kidneys of the rainbow trout, and hyperplasia and fusion of the villi in the intestine were detected in concentrations above 1 µg/L. This study demonstrates that sub-chronic exposure to environmental concentrations of diclofenac can lead to its interference in the biochemical functions of fish and to tissue damage, further highlighting concern about this pharmaceutical in the aquatic environment (Mehinto et al. 2010).

From our above-mentioned results we can assume that embryos of *D. rerio* are more sensitive to the tested substance than juvenile fish (Nagel 2002). It is generally stated that early developmental stages of fish (embryos and larvae) are more sensitive to different stimuli compared to juveniles and adults, which has been proven also by many studies with xenobiotics and other chemicals. Kovriznych and Urbancikova (2001) compared LC50 values of 8 different chemical agents assessed in embryonic and acute tests of toxicity performed in *D. rerio* and ascertained different sensitivities to them in these two life stages tested. This difference between the sensitivity of the embryonic developmental stage of fish and juvenile/adult individuals of the same species might be caused by many factors:

– the yet improperly developed enzymatic system in embryos,

– differences in metabolism pathways,

– different processes of absorption of the substance into the organism (Van Leeuwen et al. 1985).

Regarding low environmental concentrations of diclofenac which are detected in surface waters (concentrations of diclofenac in waters are ordinarily in ng/L), we can conclude that the acute toxicity risk of this pharmaceutical for fish is low. In spite of this, we cannot ignore the negative effects of chronic exposure to this substance, which should be the subject of further investigation.

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