Comparison of acute toxicity of ketoprofen to juvenile and embryonic stages of *Danio rerio*

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Abstract

OBJECTIVES: Ketoprofen is a common human medicine from a class of non-steroidal anti-inflammatory drugs (NSAIDs), which is provably detected in surface waters in concentrations ordinarily in $\mu g.L^{-1}$. The aim of this study was to compare the acute toxicity of ketoprofen to embryonic and juvenile stages of aquarium fish – zebrafish (*Danio rerio*).

METHODS: Tests were performed according to the methods of the Organisation for Economic Co-operation and Development (OECD) No. 203 (Fish, acute toxicity test) and OECD No. 212 (Fish, short-term toxicity test on embryo and sac-fry stages).

RESULTS: The results showed (mean ± SD) LC50 value of ketoprofen to be $632.30 \pm 10.10\ mg.L^{-1}$ in juvenile zebrafish and $6.44 \pm 2.22\ mg.L^{-1}$ in embryonic stages of zebrafish. The results revealed statistically significantly higher sensitivity ($p<0.01$) of the embryonic stages of zebrafish to ketoprofen compared to its juveniles. The susceptibility of embryos depends on many factors, especially yet improperly developed enzymatic system in embryos, different ways of the absorption of the substance into the organism or differences in metabolism pathways.

CONCLUSIONS: The acute toxicity of ketoprofen for juvenile stages of zebrafish is low, but the substance seems to be toxic for embryonic stages.

Abbreviations:

$ANC_{4.5}$	- acid neutralizing capacity
CAS	- Chemical Abstracts Service registry number
COD_{Mn}	- chemical oxygen demand
COX	- cyclooxygenase
COX-1	- constitutive cyclooxygenase
COX-2	- inducible cyclooxygenase
NSAIDs	- non-steroidal anti-inflammatory drugs
96h LC50	- median lethal concentration (50% mortality after a 96 h interval)
144h LC50	- median lethal concentration (50% mortality after a 144 h interval)

ORIGINAL ARTICLE

INTRODUCTION

Pharmaceuticals are a class of environmental contaminants that are extensively and increasingly used in human and veterinary medicine (Santos 2010).

Ketoprofen is one of the substances pharmacologically belonging to the class of non-steroidal anti-inflammatory drugs (NSAIDs). The mechanism of effect of non-steroidal anti-inflammatory drugs is the inhibition – either reversible or irreversible – of one or both of the two isoforms of the enzyme cyclooxygenase (COX) (constitutive COX-1 and inducible COX-2) that catalyses the synthesis of various prostaglandins from arachidonic acid (Vane & Botting 1998). Classical NSAIDs inhibit both COX-1 and COX-2 to various degrees, whereas new NSAIDs act more selectively on COX-2, the inducible form responsible for inflammatory reactions. Differences in binding site size are responsible for the selectivity of these drugs (Kurumbail *et al.* 1997; Penning *et al.* 1997; Gierse *et al.* 1999).

Ketoprofen is used mainly in human medicine and its indications are treatment of swelling and mild to moderate pain. This medicine may also be used to treat osteoarthritis and rheumatoid arthritis and painful monthly periods. Ketoprofen is available as a capsule, suppository or tablet, or as a gel to be applied directly on the skin to help relieve muscle and joint pain. The study by Juric *et al.* (2010) pointed to the fact that in high doses ketoprofen can cause acute ischemic stroke, myocardial infarction, and worsening of renal functions in humans. Ketoprofen is rapidly and extensively metabolised in the liver, mainly via conjugation with glucuronic acid (Gierse *et al.* 1999). Approximately 80% of the administered dose of ketoprofen is excreted in the urine in a 24-hour period after the administration, primarily as a glucuronide metabolite.

Pharmaceuticals are usually excreted through faeces and urine in the form of a mixture of metabolites and original parent compounds (Sanderson *et al.* 2003). Subsequently, they enter municipal sewage treatment systems where they are either degraded or adsorbed to sewage sludge, and eventually undergo dilution into surface water. Furthermore, drugs may occur in considerable concentrations in hospital wastewater, wastewater from manufacturers and landfill leachates (Holm *et al.* 1995; Kümmerer 2001; Fent *et al.* 2006). A number of non-prescription drugs and controlled drugs have been found at $ng.L^{-1}$ to $\mu g.L^{-1}$ levels in municipal wastewaters (Boyd *et al.* 2003; Metcalfe *et al.* 2003).

Ketoprofen has been detected in surface waters in Switzerland, Sweden and Germany in concentrations ordinarily in $\mu g.L^{-1}$ (Ternes 1998; Tixier *et al.* 2003). The results of monitoring by the Czech Hydrometeorological Institute (freshwater sources in the Czech Republic in 2007) showed that 1 of 5 samples was positive for ketoprofen, with the concentration found being $0.000053\ mg.L^{-1}$. Although various cases of detection of pharmaceuticals in water are described in the literature, the complete impact of ketoprofen on non-target organisms has not yet been discovered. There is no relevant information on its mechanism of action and the consequences of exposure in aquatic organisms, which are probably the most endangered by its action.

For this reason, we used zebrafish (*Danio rerio)* as a representative of water organisms for the assessment of acute toxicity of ketoprofen. The zebrafish is a model organism according to the guidelines of the Organisation for Economic Co-operation and Development (OECD) (Macova *et al.* 2009; Plhalova *et al.* 2009). Their embryos are completely transparent, allowing one to observe the development of each individual cell (Driever *et al.* 1994). For this reason, and also in view of their sensitivity to tested substances, embryos of *D. rerio* are the organisms most often used in toxicity tests (Luckenbach *et al.* 2001).

The aim of this study was to assess acute toxicity of ketoprofen in juvenile (96h LC50) and embryonic (144h LC50) stages of zebrafish and to compare their sensitivity to this compound.

MATERIAL AND METHODS

Embryonic toxicity tests

Embryonic toxicity tests with ketoprofen (Sigma-Aldrich, CAS 22071-15-4) were performed on embryos of the aquarium fish *D. rerio* in accordance with OECD guidelines No. 212 (Fish, short-term toxicity test on embryo and sac-fry stages). Five series of 5 ascending concentrations of the tested substance were used in the test. The tested concentrations were 1, 3, 6, 9 and $12\ mg.L^{-1}$. Twenty fertilised eggs in a Petri dish were tested in each concentration and in a control dish. The eggs were placed into the Petri dish within 8 hours after fertilisation 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 evaluative parameters such as egg and embryo mortality, gastrulation, somite formation, movement and tail detachment, pigmentation, heart beating, and hatching success were noted. The mortality at individual concentrations was noted during the test. The mortality rate of the control embryos did not exceed 20%; test bath temperatures were $25 \pm 0.5\ °C$ as required by the method used.

Acute toxicity test

Acute toxicity tests were performed on juvenile stages of *D. rerio*. These tests were conducted according to OECD guidelines No. 203 (Fish, acute toxicity test). In this test a semi-static method with solution replacement after 24 hours was utilised. The experimental fish were 2–3 months old, weighed 0.4 ± 0.1 g, and their total length was 31 ± 5 mm. The fish were obtained from a commercial dealer and were kept in 3-L full glass tanks with a 12-h/12-h light/dark cycle. The organisms were

exposed to 5 series of progressive concentrations of ketoprofen (550–750 mg.L^{-1}) for 96 hours. We used 10 fish for each concentration and for the control group in each test series. The water temperature, pH and oxygen saturation of water were recorded every 24 hours, as was fish mortality. The temperature of the experimental bath was 22 ± 1 °C, the dissolved oxygen saturation did not fall below 60% (76–100%) and the pH was between 6.35 and 8.16. The tested concentrations were 550, 600, 650, 700 and 750 mg.L^{-1}. No fish died in the control tanks during the experiments. Due to the low solubility of ketoprofen in water, the dissolution of the substance had to be performed using an ultrasound device.

Water quality parameters

The basic chemical parameters of diluting water used in both of these tests on embryonic and juvenile stages were: $ANC_{4.5}$ 4.20 mmol.L^{-1}; COD_{Mn} 2.80 mg.L^{-1}; total ammonia below the limit of determination (<0.04 mg.L^{-1}); NO_3^- 23.48 mg.L^{-1}; NO_2^- below the limit of determination (< 0.02 mg.L^{-1}); Cl^- 18.11 mg.L^{-1}; Σ Ca ± Mg 14 mg.L^{-1}.

Determination of ketoprofen

Ketoprofen determination in water samples was performed by high performance liquid chromatography (HPLC) with photometric detection. Samples were filtered through 0.45-μm nylon filter (Millipore, Billerica, MA) and used for analysis. The sample volume injected into the HPLC system was 20 μL. Ketoprofen 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^{-1}, column temperature was 25 °C, and UV detection was performed at 275 nm. The chromatographic analysis was accomplished by means of Alliance 2695 chromatographic system (Waters, Milford, MA) with an PDA 2996 photodiode array detector (Waters, Milford, MA). Ketoprofen standard was purchased from Sigma-Aldrich (St. Louis, MO). All solvents were of HPLC-grade purity (Chromservis, s.r.o., CZ). Detection limit for ketoprofen was 19 ng.mL^{-1}. Limit of quantification for ketoprofen was 64 ng.mL^{-1}. The coefficient of variation is 4.1%. In all toxicity tests, the concentration of ketoprofen after 24 h was above 80% of the dosed initial concentration.

Statistical analysis

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

RESULTS AND DISCUSSION

In our tests, we monitored the toxic effects of ketoprofen – a drug from the NSAIDs class, which is detected in surface waters (Ternes 1998; Tixier *et al.* 2003). These tests were performed on the embryonic and juvenile life stages of *D. rerio*.

The 96h LC50 ketoprofen value for juvenile *D. rerio* was (mean ± SD) 632.30 ± 10.10 mg.L^{-1} and the 144h LC50 for embryos was 6.44 ± 2.22 mg.L^{-1}.

The results of toxicity tests with ketoprofen showed statistically significantly higher sensitivity (p<0.01) of the embryonic stage compared to the juvenile life stage. Embryotoxicity tests also showed inhibition of the normal development of the tested organisms until the end of 144 h for all exposure groups. Studies by Kovriznych and Urbancikova (2001) with chemicals have proven that early developmental stages of fish (embryos and larvae) are more sensitive to various stimuli when compared to juveniles and adults. This difference might be caused by the as yet improperly developed enzymatic system in embryos, different ways of the absorption of the substance into the organism, differences in metabolism pathways or the presence of the barrier function of the chorion sheath in embryos which may make it impossible for some substances to reach target organs or to reach them in concentrations sufficient to induce harmful effects (Van Leeuwen *et al.* 1985).

Noticeable hydroedema was observed in embryos exposed to the concentration of ketoprofen higher than 6 mg.L^{-1}. Furthermore, the toxicity of ketoprofen was manifested by the immobilisation of embryos in ketoprofen concentrations of 9 mg.L^{-1} and higher. This observation (hydroedema and immobilisation) corresponds with the results obtained by Hallare *et al.* (2004), who studied diclofenac (also belonging to the NSAIDs class) toxicity to embryos exposed to its concentrations of 1–2000 μg.L^{-1}.

The permeability of the embryo sheaths of *D. rerio* changes depending on the age and the phase of ontogenetic development of the embryos (Hagedorn *et al.* 1997; Gellert & Heinrichsdorf 2001). Gellert and Heinrichsdorf (2001) observed the influence of spawn age on the sensitivity to the effects of sewage waters. Embryos exposed to the tested solution at the age of less than 1 hour after fertilisation were seen to be the most sensitive.

Due to the lack of information on ketoprofen toxicity tests performed on other aquatic organisms, it is possible to compare the obtained results only with the data known for other pharmaceuticals from the same pharmacological group. In general, the acute lethal dose for adult fish ranged between 100–1000 mg.L^{-1} for paracetamol, naproxen and ibuprofen (Fent *et al.* 2006), which is in agreement with our results.

Henschel *et al.* (1997) performed standard acute toxicity tests with paracetamol, salicylic acid, clofibrinic acid and methotrexate using zebrafish embryos as

experimental organisms. According to this paper, fish embryos are the most sensitive to NSAIDs, especially to salicylic acid (EC50=37 mg.L^{-1}).

Concentrations of ketoprofen detected in surface waters are ordinarily in μg.L^{-1} (Tixier *et al.* 2003; Ternes 1998), so based on our results and the acute lethal concentrations of ketoprofen obtained we can conclude that the acute toxicity risk of this pharmaceutical for fish is low.

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