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The effect of tramadol hydrochloride on early life stages of fish

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ABSTRACT

The aim of this study was to perform the fish embryo acute toxicity test (FET) on zebrafish (*Danio rerio*) and the early-life stage toxicity test on common carp (*Cyprinus carpio*) with tramadol hydrochloride. The FET was performed using the method inspired by the OECD guideline 236. Newly fertilized zebrafish eggs were exposed to tramadol hydrochloride at concentrations of 10; 50; 100 and 200 μg/l for a period of 144 h. An embryo-larval toxicity test on *C. carpio* was performed according to OECD guideline 210 also with tramadol hydrochloride at concentrations 10; 50; 100 and 200 μg/l for a period of 32 days.

Hatching was significantly influenced in both acute and subchronic toxicity assays. Subchronic exposure also influenced early ontogeny, both morphometric and condition characteristics and caused changes in antioxidant enzyme activity. The LOEC value was found to be 10 μg/l tramadol hydrochloride.

1. Introduction

The increasing consumption of pharmaceuticals as well as their incomplete removal during wastewater treatment processes is the primary sources of their residues in surface waters. Bioactive compounds can reach the aquatic environment in many ways and have considerable effects on aquatic biota.

Tramadol hydrochloride is a synthetic, centrally acting analgetic drug related to codeine and morphin showing opioid and non-opioid properties (De Leo et al., 2009). It has been reported that 15 — 35% of tramadol is excreted unchanged via urine. Kasprzyk-Hordern et al. (2009) found the average concentration of tramadol in raw sewage in the UK to be >30 μg/l. They observed no tramadol removal in WWTP with biological cleaning using trickling filter beds. However, up to 40% of tramadol in WWTP was removed using activated sludge. In contrast, Wick et al. (2009) found tramadol at a concentration of 0.24 μg/l in WWTP influent and 0.23 μg/l in effluent in Germany. Tramadol hydrochloride is used in both human and veterinary medicine for the relief of acute and chronic pain, although its use to treat anxiety and depression has also been documented (Vazzana et al., 2015). It possesses a weak agonist action to μ-opioid receptor and inhibits the reuptake of serotonin and norepinephrine (Raffa et al., 1992).

Fish have been accepted as vertebrate representatives for the aquatic environment in acute as well as chronic toxicity tests and are also used to monitor the quality of effluents and surface waters (Lammer et al., 2009). The Fish acute toxicity test (FAT) is a mandatory step in the environmental hazard and risk assessment of chemicals. However, its ecotoxicological relevance is questionable. The value of LC_{50} may vary among different species and short-term exposure to high concentrations of toxicant in nature is not expected. The only exception may be represented by acci-

Abbreviations: EFSA, European Food Safety Authority; FAT, fish acute toxicity test according to guideline OECD 203; FET, fish embryo acute toxicity test according to guideline OECD 236; FCF, Fulton's condition factor; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S-transferase; HPLC-ESI–MS/MS, high performance liquid chromatography coupled with electro-spray ionization-tandem mass spectrometry; ISO, International Organization for Standardization; LC_{50}, lethal concentration that kills 50% of population of a specific test animal in a specified period; LOEC, lowest observed effect concentration; MDA, malondialdehyde; OECD, Organisation for Economic Cooperation and Development; Pf, post fertilization; ROS, reactive oxidative species; SGR, specific growth rate; TBARS, thiobarbituric acid reactive substances; WWTP, wastewater treatment plant.

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dental spills. In accordance with the reduction, refinement and replacement philosophy (the 3Rs; Russell and Burch, 1959) fish embryos are considered a replacement or refinement method since these developmental stages are not legislatively protected (Directive 2010/63/EU) (EU, 2010) and are likely to experience less pain or suffering (EFSA, 2005). Athough the Fish embryo acute toxicity test (FET) suggested by Schulte and Nagel in 1994 was designed as an alternative to the Fish acute toxicity test (FAT), the FET was internationally standardized (ISO 2007; OECD, 2013) and plays an important role in chemical toxicity testing. Besides the LC_{50} determination, sub-lethal endpoints such as completion of gastrula, formation of somites, development of eyes, spontaneous movement, heartbeat/blood circulation, pigmentation and oedema may be recorded to indicate the mode of action of toxic compounds (Nagel, 2002).

Fish play a critical role in aquatic food webs and are an important food source for humans. The effects of some analgesic drug residues on fish have already been reported by various authors (Stepanova et al., 2013; Zivna et al., 2013; Stancova et al., 2015; Zivna et al., 2015). However, there is in general still a lack of information describing the chronic effect of drug residues on aquatic vertebrates and the possible mixture toxicity of the residues. That is why the subchronic and chronic effects of pharmaceutical residues should also be studied. The critical development of tissue and organs in embryos and larvae can be easily disrupted by exposure to toxic compounds. The early life stages of fish are generally regarded as the most sensitive to toxic agents and are ideal models for determining responses to environmental contaminants (Hostovsky et al., 2012; Stepanova et al., 2013; Zivna et al., 2015).

The aim of this study was to perform the fish embryo acute toxicity test on zebrafish (*Danio rerio*) and the early-life stage toxicity test on common carp (*Cyprinus carpio*) with tramadol hydrochloride and assess its effect on the early life stages of the fish. The concentrations were selected in accordance with the environmenal concentrations (Kasprzyk-Hordern et al., 2009; Wick et al., 2009).

2. Material and methods

2.1. Fish embryo acute toxicity (FET) test with Danio rerio

The fish embryo toxicity test on zebrafish (*D. rerio*) was performed using the modified method inspired by OECD guideline 236 (Fish Embryo Acute Toxicity (FET) Test). Newly fertilized zebrafish eggs, at the latest at the 16 cell stage, were exposed to tramadol hydrochloride (Sigma-Aldrich, Czech Republic; chemical purity ≥99%) in concentrations of 10 (established with respect to the reported environmental concentration - Kasprzyk-Hordern et al., 2009; Wick et al., 2009); 50; 100, and 200 μg/l for a period of 144 h at 26 ± 1 °C. Eggs were distributed to well plates with 48 wells on each plate. There were 60 eggs for each experimental concentration and 60 eggs in dilution water as a control. The test was performed in triplicate. Dilution water was prepared according to ISO 7346 (ISO, 1996). The test was performed in triplicate. The solutions of tested chemicals and dilution water were renewed after 72 h. During the test, the concentrations did not fall below 80% of their nominal values. Lethal endpoints were recorded according to OECD guideline 236 (OECD, 1992). The observation included coagulation of embryos, lack of somite formation, non-detachment of the tail and lack of heartbeat. Additional, sub-lethal endpoints such as hatching rate, formation of somites, development of eyes, spontaneous movement, hearbeat/blood circulation, pigmentation and oedema were recorded to indicate the mode of action of the toxic compound. The assay also included screening for developmental disorders to indicate teratogenic effects according to Nagel (2002): malformations of the head, otoliths, tail and heart, modified structure of the chorda, scoliosis and deformity of yolk.

2.2. Fish, early-life stage toxicity test with Cyprinus carpio

2.2.1. Experimental protocol

The Embryo-larval toxicity test on common carp (*C. carpio*) was performed according to the OECD guideline 210 (Fish, Early-life Stage Toxicity Test) (OECD, 1992). Eggs were obtained from Rybnikarstvi Pohorelice a.s. (Czech Republic) and produced according to the standard methods of artificial reproduction (Kocour et al., 2005). Fish eggs were collected 12 h post-fertilization and groups of 100 eggs were transfered to 900 ml crystallization dishes. The crystallization dishes were arranged in 5 groups with every tested group in triplicate. The first group (300 eggs) was exposed to the tramadol hydrochloride (Sigma-Aldrich, Czech Republic; chemical purity ≥99%) at a 10 μg/l concentration. Three groups were exposed to concentrations of 50, 100 and 200 μg/l of tramadol hydrochloride. The fifth group was exposed to tap water as control. During the test, concentrations of tested substance did not fall below 80% of their nominal values. The semistatic method was used, with twice daily bath replacement. The temperature, pH, and oxygen saturation were recorded daily. Hatching and survival were observed twice a day and dead embryos and larvae were recorded and removed. Larvae were fed *ad libitum* twice a day with freshly hatched *Artemia salina* from day 6. The test was completed after 32 days. Embryos, larvae and juveniles were sampled on days 6, 14, 21, 28 and 32 (completion of the test).

Fish were fixed in 4% formalin. Samples were taken in order to record developmental stage, morphometric and condition characteristics (total length, body weight, Fulton's condition factor, specific growth rate and inhibition of specific growth), and morphological anomalies.

Ten samples were also taken from each group in order to evaluate antioxidant enzyme activity and lipid peroxidation; these were stored at −85 °C until analysis and 15 specimens were taken and fixed in 10% formalin for histopathological examination.

2.2.2. Water parametres

The temperature of the water ranged from 19 to 22 °C and pH was between 7.4 and 8.2. The level of dissolved oxygen did not fall below 70%. Values for the chemical parametres of the tap water were: acid neutralization capacity ($ANC_{4.5}$)—1.0–1.2 mmol/l; chemical oxygen demand (COD_{Mn})—1.1–1.3 mg/l; total ammonia—below the limit of determination (<0.04 mg/l); nitrates—11.7–13.2 mg/l; nitrites—below the limit of determination (<0.01 mg/l); Cl^-—15.3–16.7 mg/l and Σ Ca + Mg—3.06 mmol/l.

2.2.3. Determination of developmental stages

The developmental stages were determined according to Penaz et al. (1983); nine embryonic (E1–E9), six larval (L1–L6), and two juvenile (J1–J2) stages of common carp were described.

2.2.4. Morphometric and condition characteristics

The total length (TL) was measured stereomicroscopically using a micrometer to 0.01 mm; weight (w) was measured to 0.1 mg. Fulton's condition factor was calculated at each sampling time according to:

$$FCF = \frac{w \times 100}{l^3}$$

where w is weight in g and l is the total length in mm.