Ecotoxicology and Environmental Safety 164 (2018) 297–304

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Chronic effects of carbamazepine on zebrafish: Behavioral, reproductive and biochemical endpoints

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ARTICLE INFO

Keywords:
Human pharmaceutical
Danio rerio
Behavior
Biochemical biomarkers
Histology

ABSTRACT

Carbamazepine (Cbz), one of the most prescribed pharmaceuticals in the world is often detected in surface waters and sediments. However, few studies addressed its chronic effects in fish. In the present study, *Danio rerio* adults were exposed for 63 days to Cbz (0 - control, 10 μg L^{-1} - concentration found in effluents, and 10,000 μg L^{-1} – 5% of LC_{50} at 72 h). Assessed endpoints were: feeding behavior, growth rate, number of eggs produced and their viability, histological alterations in female gonads, and biochemical biomarkers associated with antioxidant defenses (catalase - CAT, and glutathione *S*-transferase – GST activities), neurotransmission (acetylcholinesterase activity - AChE) and metabolism (lactate dehydrogenase - LDH). Cbz exposure increased the total time for food intake but did not affect *D. rerio* growth. Although the total number of eggs was not affected by Cbz exposure, the eggs viability was significantly impaired. Exposure to Cbz caused alterations in the female gonads follicular stages. In terms of biochemical endpoints, CAT activity in liver and gills, was sensitive to the pharmaceutical exposure presenting a decreased activity. AChE activity was induced in the head (both concentrations) and muscle (10,000 μg L^{-1}). GST activity was increased in gills (both concentrations) but inhibited in the intestine. Concerning LDH, enzymatic activity was increased in the liver and decreased in muscle and gills. Several of the above-mentioned effects can be directly linked with effects at population level (e.g. feeding behavior) and occurred at environmental concentrations (the lowest concentration tested), thus serious concerns regarding risks posed by Cbz residues to fish populations arise with this study.

1. Introduction

Pharmaceuticals are currently considered emerging contaminants of concern (Santos et al., 2010) mainly due to their increased production, consumption and presence in the environment allied with a biological active nature and potential noxious effects in the living organisms (Palacios-Rosas and Castro-Pastrana, 2017). Most of these compounds and their metabolites are not efficiently removed or biodegraded in the sewage treatment plants (Martins et al., 2012). Once in the environment pharmaceuticals residues may undergo bioaccumulation (Deblonde et al., 2011) and/or act on aquatic organisms, especially fish which have highly conserved physiological features compared to humans (Kreke and Dietrich, 2008; LaLone et al., 2013), for whom drugs have been designed. Carbamazepine (Cbz) is an anticonvulsant prescribed for the treatment of psychomotor epilepsy, bipolar disorder, and trigeminal neuralgia (Calcagno et al., 2016). It is known to interact with potassium and sodium channels and several signaling pathways (Ayano, 2016) and to modulate voltage gated sodium channels that will decrease the neuronal activity (Galus et al., 2014). In the liver, the main biotransformation organ, Cbz is converted into Cbz 10,11- epoxide and other derivatives (Villanueva et al., 2018). Cbz has a high distribution and abundance in the aquatic environment (Oropesa et al., 2016; Pires

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https://doi.org/10.1016/j.ecoenv.2018.08.015
Received 28 May 2018; Received in revised form 19 July 2018; Accepted 2 August 2018

et al., 2016), being found in wastewater treatment plants effluents, surface waters, and soils (Oliveira et al., 2015). Only 10% of Cbz is removed in wastewater treatment plants (Chen et al., 2014), presenting a low degradation rate in the environment (Pires et al., 2016) with a permanency time in the aquatic environment of around 82 days (Brandão et al., 2013). It can also be bioaccumulated and bioconcentrated (Oropesa et al., 2016).

Carbamazepine has been widely detected in waste waters and surface waters. For instance, a study performed in Republic of Korea monitored influents and effluents from municipal, hospital, livestock and pharmaceutical waste water treatment plants. Maximum concentrations of Cbz found in these effluents were 21.0, 14.4, 10.2 and 150 $\mu g\,L^{-1}$ respectively (Sim et al., 2011). In the "EU Wide Monitoring Survey of Polar Persistent Pollutants in European River Waters", Cbz was one of the most frequently detected contaminants in the 122 river analyzed with a highest maximum concentration found of 12 $\mu g\,L^{-1}$ (Loos et al., 2009).

Although some studies have been performed in the last years to understand lethal and sublethal effects of Cbz on aquatic organisms like algae, cladocerans and fish (Parker, 2015; Oropesa et al., 2016) there is still a lack of knowledge concerning chronic effects (Deblonde et al., 2011). The reported chronic effects in fish include decreased embryo production, irregularities in oocytes, somatic stromal tissue and decreased plasma sex steroids in adult zebrafish (*Danio rerio*) (Galus et al., 2013); decreased motility and sperm velocity and decreased superoxide dismutase, glutathione peroxidase and glutathione reductase activity and lipid peroxidation in sperm of the common carp (*Cyprinus carpio*; Li et al., 2010a); lipid peroxidation in the brain and decreased superoxide dismutase and glutathione reductase, with glutathione peroxidase and CAT presenting a non-linear response over time with an increase followed by a decrease in their activities (Li et al., 2010b).

Therefore, the aim of the study was to perform a multi-level evaluation of the chronic effects of Cbz on adult zebrafish focusing on growth, reproduction, feeding behavior, biochemistry in gills, liver and intestine, genotoxicity on erythrocytes and histopathology in female gonads. The working hypothesis is that chronic exposure to carbamazepine will induce biochemical alterations leading to behavioral and reproductive effects.

2. Materials and methods

2.1. Test chemicals and chemical analysis

Carbamazepine (Cbz) (CAS 298-46-4) was purchased from Sigma-Aldrich. All other reagents were analytical grade.

A stock solution was prepared by dissolving 200 mg of Cbz in 4 L of culture water; dissolution of the compound was promoted by sonicating the solution for 30 min. Test solutions were obtained by dilution of the stock with culture water. Carbamazepine samples were analyzed to confirm the stability of Cbz under the experimental conditions. Thus, 5 L aquaria containing 10,000 $\mu g\,L^{-1}$ of test solution, the highest concentration tested, were kept under conditions similar to the experimental tanks with fish (light and temperature) and daily, 100 ml of water from each aquarium were collected for High Performance Liquid Chromatography (HPLC Shimadzu-Prominence) following the method described by Demirkaya and Kadioğlu (2005) (See details in Suppl. Material, Fig. S1, Tables S1 and S2).

2.2. Test organisms

Sexually mature fish zebrafish (*D. rerio)*, of six months, were purchased from ZAIA (Brasilia) and acclimated for 40 days to laboratory conditions. The fish average weight was of 0.51 (± 0.013) g for females and 0.34 (± 0.008) g for males. Culture water was obtained by reverse osmosis and the conductivity adjusted to 550 (± 100) μS, by adding salt "Aquarium Systems" (USA). Water temperature was kept at 26.0 (± 1) °C, pH at 7.5 (± 0.5), and dissolved oxygen equal or above 99% saturation. A 12:12 h (light: dark) photoperiod cycle was maintained. Fish were fed twice a day with commercially diet (TetraMin fish food, EUA).

2.3. Experimental design

The test procedure generally followed OECD guideline 215 (OECD, 2000) and was performed under conditions similar to acclimation.

Males and females were randomly distributed into nine experimental tanks, containing 5 L of the test solution (nominal concentrations: 0, 10 or 10,000 $\mu g\,L^{-1}$ of Cbz). Three replicates (with 10 fish each) were used per treatment. The lowest Cbz concentration tested, 10 $\mu g\,L^{-1}$, was selected based on a concentration reported in a wastewater treatment plant in lake Paranoá (Gunthert et al., 2014) - 11.3 $\mu g\,L^{-1}$. The highest concentration, 10,000 $\mu g\,L^{-1}$, corresponds to approximately 5% of Cbz lethal concentration value (LC_{50}) after 72 h of exposure (Van den Brandhof and Montforts, 2010). Animals were exposed for 63 days and fed once a day with a quantity of TetraMin fish food (EUA) corresponding to 2% of the fish weight in the aquarium. Test media was renewed every three days. Water quality parameters were kept within the ranges described in Section 2.2.

Fish weight (Section 2.3.1) and feeding behavior (Section 2.3.2) were evaluated at days 21, 42 and 63 of exposure. At days 23, 27, 35, 42, 47, 54 and 61, fish were allowed to reproduce to assess reproductive output (number of eggs and its viability). Zebrafish reproductive behavior can be observed, in their natural environment, at dawn. Thus, to obtain eggs, before the lights turned on in the lab fish were transferred from the exposure vessels to aquaria with culture water, marbles and plants and left in these conditions for mating for two hours after the lights turned on. Fish were then returned to the exposure aquaria, marbles (which were used to avoid predation of the eggs by fish) were removed and eggs carefully collected with the help of a net and washed in culture water. Approximately 2.5 h after egg collection they were examined under a stereomicroscope to determine their total number and condition (fertilized/viable or not). Eggs were considered fertilized and viable if they were in the expected developmental stage for that observation time (blastula stage according to Kimmel et al., 1995).

At the 63rd day of exposure, fish were sacrificed, peripheral blood collected and smears prepared for micronuclei and other nuclear abnormalities assessment (12 animals; Section 2.3.3). Head, gills, liver, muscle, and intestine of each fish were isolated and frozen in microtubes containing phosphate buffer, pH 7.4 for biochemical analyses (21animals; Section 2.3.4). These samples were stored at −80 °C until analysis. Nine female organisms per treatment were used for histology. Animals were sacrificed on ice, the whole-body fixed with Davidson solution during 24 h and preserved in 70% ethanol for histological analysis (Section 2.3.5).

2.3.1. Fish weight determination

The weight of each fish was determined by transferring it, with the help of a net, to a glass beaker containing approximately 60 ml culture water placed in a digital scale (0.001 g). Before weighing the fish were fasted for 24 h.

2.3.2. Feeding behavior

Feeding behavior was assessed by transferring from each tank 5 fish to an aquarium containing culture water and by adding 6 granules of TetraMin, based on the methods described by Domingues et al. (2016). The time taken until the first feeding action and for the total intake of food (up to a maximum of 20 min - min) was recorded.

2.3.3. Micronuclei and others nuclear abnormalities assessment

The assessment of micronuclei and other nuclear abnormalities generally followed the methodology established by Hooftman and de Raat (1982) for fish erythrocytes. Approximately 20 μL of peripheral