Environmental Toxicology and Pharmacology 55 (2017) 14–19

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

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

Research Paper

Interaction of erythromycin and ketoconazole on the neurological, biochemical and behavioral responses in crucian carp

Jianchao Liu[a], Yuanfei Cai[a,b], Guanghua Lu[a,c,*], Xiaoxiang Dan[a], Donghai Wu[a], Zhenhua Yan[a]

[a] Key Laboratory of Integrated Regulation and Resources Development, College of Environment, Hohai University, Nanjing, 210098, China
[b] Wentian College, Hohai University, Ma'anshan, 243031, China
[c] College of Hydraulic and Civil Engineering, XiZang Agricultural and Animal Husbandry College, Linzhi, China

ARTICLE INFO

Keywords:
Pharmaceuticals
Bioconcentration
Behavior
Crucian carp

ABSTRACT

The presence of pharmaceuticals in the aquatic environment has received great attention due to their potential impacts on public health. The single, as well as the combined toxicities of erythromycin (ERY) and ketoconazole (KCZ) on the bioaccumulation, biochemical and behavioral responses, were examined in crucian carp. This study focused on the uptake of contaminants, acetylcholinesterase (AChE) activity in the brain, swimming and shoaling behavior of fish. After 14 days of binary exposure, the addition of KCZ at nominal concentrations of 0.2, 2 and 20 μg/L significantly increased the accumulation of ERY in the brain of the fish and the bioconcentration factor of 2.08 was 2.6-fold higher than that calculated from the ERY-alone exposure. The brain AChE activity was significantly inhibited by ERY and KCZ with a significant correlation with respect to the accumulative concentration of the contaminants. The inhibition rates of swimming activity to KCZ were increased with a corresponding increase in the exposure concentration of KCZ in the single exposure. However, this manner was altered by the combined exposure. In addition, shoaling was significantly enhanced by KCZ-alone exposure, which was significantly correlated with the swimming activity. This study indicates that the mixture of the contaminants may cause endocrine disrupting effects and behavior modification especially in fish with known ecological and evolutionary consequences.

1. Introduction

Pharmaceuticals are a class of environmental contaminants that emerged at the end of the twentieth century. The aquatic environment is one of the most important sinks for these human and veterinary pharmaceuticals, which mainly come from the discharge of sewage treatment plants (Dai et al., 2015). These compounds have become persistent and have emerged as a group of pollutants in the aquatic environment. Results of monitoring show that all classes of pharmaceuticals were detected in river water, seawater, groundwater and drinking water of many countries with concentrations below the μg/L threshold (Chen and Ying, 2015). There is considerable evidence to show that the exposure to environmental pharmaceuticals can cause a series of adverse effects on homeostasis, development and reproduction in vertebrates and invertebrates, leading to a deviation from the normal endocrine function (Godoy et al., 2015).

Among the pharmaceuticals, anti-inflammatory drugs, antibiotics, β-blockers and azoles are the most commonly detected in the aquatic environment. Erythromycin (ERY; $\log K_{ow}$ = 3.06, aqueous solubility = 2.0 g/L) has been used since the 1950s and is part of the macrolide group of antibiotic. Relatively higher concentrations of ERY were detected in surface water and treated effluent up to 1.7 μg/L (Kolpin et al., 2002) and 6.0 μg/L (Kümmerer, 2009) respectively. Amongst the river organisms, it seems that the blue-green algae are the most sensitive to the direct toxicity of ERY (González-Pleiter et al., 2013). This justifies why ERY was selected as one of the 'watch list' of priority pollutants (EU Decision, 2015). Moreover, ERY can cause high mortality to *Daphnia magna*, elevated CYP450 catalytic activity, inhibited acetylcholinesterase (AChE) activity and powerful genotoxic effect on fish (Burkina et al., 2015; Ji et al., 2012; Rocco et al., 2012). Ketoconazole (KCZ; molecular mass is 531.44), as an antifungal pharmaceutical of dermal/topical application, leads to a lower absorption of 5–10% via skin, which may result in large amounts of its residue in the aquatic environment. KCZ shows a pH-dependent solubility profile, which has an excellent solubility at acidic pH below 3 but decreases to only 2 mg/L at pH 7–8 (Adachi et al., 2015). KCZ have been detected in STPs, surface water and sludge at maximal concentrations up to 0.19 μg/L, 7.5 ng/L and 1800 ng/g, respectively (Chen and Ying, 2015), and showed bioaccumulation potential in wild fish (Liu et al., 2015). *In vivo* and *in vitro* inhibition studies have suggested that KCZ

* Corresponding author at: Key Laboratory of Integrated Regulation and Resources Development, College of Environment, Hohai University, Nanjing, 210098, China.
E-mail address: ghlu@hhu.edu.cn (G. Lu).

http://dx.doi.org/10.1016/j.etap.2017.08.002
Received 27 February 2017; Received in revised form 1 August 2017; Accepted 4 August 2017
Available online 07 August 2017

acts as a potent inhibitor of liver CYP1A enzymatic activity and AChE activity in fish (Burkina et al., 2015). Once KCZ enters the environment, it might lead to endocrine disruption effects on the non-target organisms via acting as a competitive inhibition of a wide range of CYP enzyme expression and then act synergistically to elevate the bioaccumulation and/or adverse effects of other organic contaminants towards aquatic organisms when they are co-present.

The AChE enzyme regulates acetylcholine, which is one of the most widely distributed neurotransmitters in vertebrates. Inhibition of AChE disrupts the nervous system as accumulating the neurotransmitter, resulting in deleterious effects including death (Rhee et al., 2013). The US EPA (1998) suggests that 20% inhibition rate of AChE activity can be considered as a clear toxicological effect, which can subsequently lead to changes in behaviors, including reduced swimming performance, retarded intrauterine growth, altered social behavior and greater predation risk (Renick et al., 2016). Disruption of these sequences before completion is likely to result in detrimental behavioral alterations. Thus, they are benefited by concurrent consideration on both the physiological and ecological indicators of toxicity (Scott and Sloman, 2004). Moreover, different classes of pharmaceuticals have been detected simultaneously in environmental compartments, and which in a mixture may act additively, synergistically or antagonistically. Thus, the combined pollution of these chemicals in waters may result in toxicokinetic interaction in aquatic organisms, and lead to unpredictable biological effects (Wang et al., 2011). More specifically, the neurological and biochemical mechanisms and behavioral consequences underlying their interactive effects require further investigation.

The aims of this study were to investigate the bioaccumulative, neurological and behavioral effects of the antibiotic ERY and antifungal agent KCZ in crucian carp to provide a better understanding of their interactive effects. We measured the AChE activity and bioaccumulation of the pharmaceuticals in the brain of the fish and behavioral endpoints, including swimming activity and shoaling. The relationships between the physiological responses and altered behaviors of the fish were investigated to present a comprehensive evaluation of the two pharmaceuticals.

2. Materials and methods

2.1. Chemicals

KCZ (purity > 99%), ERY (purity > 98%), Acetylthiocholine iodide (ATChI), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and tricaine methanesulfonate (MS222) were purchased from Sigma-Aldrich (Flanders, New Jersey, USA). Acetonitrile and methanol (HPLC grade) were obtained from Merck Serono Co., Ltd. (Darmstadt, Germany). Water was purified using a Milli-Q integral water purification system (Millipore, Milford, MA, USA).

2.2. Fish culture and exposure

Crucian carp is an important economic species and has been demonstrated to be a very sensitive species to study the toxic effects of pollutants on aquatic organisms (Chen et al., 2012). Crucian carp weighing 30.2 ± 2.6 g were purchased from the Nanjing Institute of Fishery Science (Nanjing, China). In the laboratory, the fish were fed every day with commercial fish food (6% of body weight/day). According to OECD guideline 305 (OECD, 2012), the exposure water's quality was checked daily and maintained at conditions suitable for crucian carp (Water temperatures 20 ± 1 °C; pH 7.4 ± 0.3; DO > 6.0 mg/L and $CaCO_3$ 110.5 ± 3.5 mg/L). Feces and uneaten food were removed every day by suction. A daily 12/12-h light/dark photoperiod cycle was used throughout the experiment.

Based on the maximum concentrations of 0.19 μg/L for KCZ and 1.7 μg/L for ERY detected in the aquatic environment and the reported PNEC value of 0.28 μg/L for KCZ toward *Pseudokirchneriella subcapitata* (Minguez et al., 2016), the fish were randomly assigned to seven pharmaceutical exposure groups, including alone exposure of ERY (2 μg/L) and KCZ (0.2 μg/L (KCZ1), 2 μg/L (KCZ2) and 20 μg/L (KCZ3), and binary exposure ERY in combination with KCZ (ERY + KCZ, 2 μg/L + 0.2 μg/L, 2 μg/L + 2 μg/L and 2 μg/L + 20 μg/L, named as CO-K1, CO-K2 and CO-K3). One dechlorinated water blank control group and one solvent control group (0.01% methanol) were maintained in parallel to the treatment groups. The semi-static exposures were renewed every 24 h with an array of nominal test solutions. The test solutions were prepared by the dilution of stock solutions with dechlorinated water. For each treatment, six fish (i.e., one fish tested for enzyme assays and five fish pooled for chemical analysis) were studied at each time point and exposure concentration. Also, each treatment was replicated three times simultaneously. The fish were not fed during the exposure to prevent the release of bile from the gall bladder to the duodenum. Water was randomly sampled in triplicate from each group every day.

2.3. Sample extraction and chemical analysis

Detailed protocols for the extraction, quality assurance and quality control are provided in the Supplemental Material (SM). In brief, water samples were extracted using Oasis HLB cartridges (200 mg, 6 mL, Waters, USA). Tissue samples were extracted using a Dionex ASE 350 pressurized liquid extraction system (Thermo Fisher, Germering, Germany) and concentrated in a Büchi R200 rotary evaporator (Labortechnik, Flawail, Switzerland). Then, the concentrated solution was subjected to further cleanup using the freezing-lipid technique. Finally, the extracts were evaporated under a stream of nitrogen and reconstituted with 1 mL of acetonitrile, and 50 μL of the 1 mg/L internal standard solution was added. The extracted solutions from the water and the fish tissue samples were analyzed by ultra-high performance liquid chromatography tandem triple quadrupole mass spectroscopy (UPLC/MS/MS). The optimal conditions for the analyte monitoring are summarized in Table S1.

2.4. Enzyme assays

After 3, 7 and 14 days of exposure, the fish were collected from each treatment at each sampling time, anaesthetized with MS222 (100 mg/L), and sacrificed by cervical transection. The brain tissue was removed, collected and immediately washed with 0.15 M KCl. Subsequently, the tissue was blotted with filter paper and immediately frozen in liquid nitrogen. The brain specimens were homogenized in 1:9 (w:v) ice-cold buffer (0.1 M, pH 7.2, triton 0.1%) and centrifuged for 20 min (10,000*g*) at 4 °C. The activity of AChE was determined at 405 nm using a microplate reader (Molecular Device Versamax, USA), as described by Guilhermino et al. (1996). Protein concentrations were determined using the method developed by Bradford (1976). Detailed protocols are available in the SM.

2.5. Behavioral analyses

At the end of 14 days of exposure, behavioral traits including shoaling and swimming activity were assayed according to the approach described by Xie et al. (2016). Within shoaling and swimming activity, the experimental tank (30 cm high × 30 cm wide × 60 cm long) was divided lengthwise into three compartments (two small compartments of 15 cm and one large central compartment of 30 cm) separated by two transparent glass partitions. The bottom of the central compartment was equally divided into four segments (Fig. S1). One of the smaller compartments held four similar-sized fish as a shoal. An individual fish was introduced to the center of the large compartment and allowed to acclimate for 10 min, followed by a 600-s video recording of its movements from above. To assess shoaling behavior, the