Environmental Toxicology and Pharmacology 51 (2017) 114–123

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

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

Ecotoxicity of veterinary enrofloxacin and ciprofloxacin antibiotics on anuran amphibian larvae

Paola M. Peltzer[a,b,*], Rafael C. Lajmanovich[a,b], Andres M. Attademo[a,b], Celina M. Junges[a,b], Carla M. Teglia[b,c], Candela Martinuzzi[a], Lucila Curi[a,b], María J. Culzoni[b,c], Hector C. Goicoechea[b,c]

[a] Laboratorio de Ecotoxicología, Facultad de Bioquímica y Ciencias Biológicas (FBCB), Universidad Nacional del Litoral (UNL), Santa Fe, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina
[c] Laboratorio de Desarrollo Analítico y Quimiometría (LADAQ), Cátedra de Química Analítica I, (FBCB-UNL), Santa Fe, Argentina

ARTICLE INFO

Article history:
Received 4 July 2016
Received in revised form 27 January 2017
Accepted 29 January 2017
Available online 4 February 2017

Keywords:
Antibiotics
Amphibian
Development
Biomarkers
Pharmaceuticals

ABSTRACT

The ecological risks posed by two β-diketone antibiotics (DKAs, enrofloxacin, ENR and ciprofloxacin, CPX), characterized by their long persistence in aqueous environments and known deleterious effect on model organisms such as zebrafish were analysed using *Rhinella arenarum* larvae. Sublethal tests were conducted using environmentally relevant concentrations of both ENR and CPX (1–1000 $\mu g\,L^{-1}$) under standard laboratory conditions for 96 h. Biological endpoints and biomarkers evaluated were body size, shape, development and growth rates, and antioxidant enzymes (glutathione-S-transferase, GST; Catalase, CAT). Risk assessment was analysed based on ration quotients (RQ). The size and shape measurements of the larvae exposed to concentrations greater than 10 $\mu g\,L^{-1}$ of CPX were lower compared to controls (Dunnett post hoc $p < 0.05$) and presented signs of emaciation. Concentrations of 1000 $\mu g\,L^{-1}$ of CPX induced GST activity, in contrast with inhibited GST and CAT of larvae exposed to ENR. Risk assessments indicated that concentrations greater than or equal to10 $\mu g\,L^{-1}$ of CPX and ENR are ecotoxic for development, growth, detoxifying, and oxidative stress enzymes. It is suggested that additional risk assessments may provide evidence of bioaccumulation of CPX and ENR in tissues or organs of amphibian larvae by mesocosm sediment test conditions. Finally, intestinal microbiome studies should be considered to establish the mechanisms of action of both antibiotics.

1. Introduction

Over the past decades, the scientific community has been alerted by the increasing levels of veterinary pharmaceuticals recorded in freshwaters, including groundwater and sources of drinking water (Farré et al., 2008; Oggier et al., 2010; Rico et al., 2013). Pharmaceuticals reach the aquatic systems through several pathways, such as direct disposal of domestic surplus drugs, inadequate processing in the effluent treatment plants (Li and Randak, 2009) or incomplete removal mechanisms (Van Doorslaer et al., 2014). In general, 10–90% of pharmaceuticals and their metabolites are excreted via urine and feces (Kümmerer et al., 2000; Zhang et al., 2014, 2015). Besides their applications to livestock, poultry or swine feedlots, pharmaceuticals are widely used in aquaculture (Rico et al., 2014a). The continuous entry of pharmaceuticals into the aquatic environment from wastewater effluents or leaching and runoff of agricultural soils amended with manure, even at low concentrations, may pose long-term risks to aquatic and terrestrial organisms (Klavarioti et al., 2009; Martini et al., 2012; Rico et al., 2014b). In surface waters, pharmaceuticals and their metabolites generally include several pharmaco-therapeutic classes, such as antibiotics, antipyretics, anti-inflammatory drugs, β-blockers, lipid regulators, hormones, antidepressants, and anesthetics (Kümmerer, 2009).

In the '70s, the first evidence on pharmacologically active compounds (clorphibric acid) in aquatic systems was reported (Hignite and Azarnoff, 1977), but the occurrence of pharmaceuticals in the environment became an emerging concern only in the mid-1990s, when new analytical technologies were accessible (Heberer and Stan, 1997; Zuccato and Castiglioni, 2009). There are severe concerns over this bioactive compound role in enhancing antibiotic resistance among pathogenic bacteria (Homem and Santos, 2011). Fluoroquinolones, classes of commonly used β-diketone antibiotics (DKAs) persist for long time in the aquatic environment (Qin and

* Corresponding author at: FBCB-UNL, Paraje El Pozo s/n, 3000 Santa Fe, Argentina. Tel.: +54 0342 4575215x158.
E-mail address: paolapeltzer@hotmail.com (P.M. Peltzer).

http://dx.doi.org/10.1016/j.etap.2017.01.021
1382-6689/

Liu, 2013; Wang et al., 2014) due to their massive dose and frequent application of DKAs in human and livestock (Zhang et al., 2016). This environmental problem can exacerbate bacterial resistance in considerable proportion (Redgrave et al., 2014). Enrofloxacin (ENR) and ciprofloxacin (CPX) are the most used drugs in human (since the 1980s) and veterinary (since the 1990s) medicine to treatment of urinary tract, enteric, low respiratory tract, and bone infections, being mainly active against Gram-negative bacteria (López-Cadenas et al., 2013).

During the last century, different technologies were developed to remove fluoroquinolones from different aquatic systems (Van Doorslaer et al., 2014; Alcaráz et al., 2015). However, their removal is complex and they can be found in numerous water matrices worldwide at concentrations that range among ng L^{-1} to mg L^{-1} (Reinstorf et al., 2008). For instance, Watkinson et al. (2009) found 1.30 μg L^{-1} CPX concentration in water bodies of Australia, whereas Gibs et al. (2013) reported CPX residues (0.077 μg L^{-1}) in aquatic systems located downstream to the wastewater treatment plant discharges of New Jersey. Higher concentrations at concentrations above 14 mg L^{-1} of CPX were detected in areas with no or poor wastewater treatment (Larsson et al., 2007, Fick et al., 2009).

Therefore, in an ecotoxicological analysis regarding to emerging pollutants it is important to include acute effects and sub-lethal effects on non-target organisms (Rico et al., 2014a). ENR and CPX, affect growth and reproduction of cyanobacterium *Microcystis aeruginosa*, green algae species *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata* and duckweed *Lemna minor* at concentrations ranging from 1.96 to 53 μg L^{-1} (Robinson et al., 2005). Moreover, in crustacean *Daphnia magna*, zebrafish *Danio rerio* and fathead minnow *Pimephales promelas* have limited toxicity with no-observed-effect concentrations (NOEC) at 10 mg L^{-1} (Robinson et al., 2005; Iannacone and Alvariño, 2009; Plhalova et al., 2014; Dalla Bona et al., 2015). In addition, Wang et al. (2014) and Zhang et al. (2016) observed changes in both creatine kinase activity and creatine concentration, swimming behaviour, and severe histopathological changes of zebrafish heart tissue. To date, knowledge about mode of action of fluoroquinolones in amphibians is limited (Howard et al., 2010). ENR and CPX, pose risks to sensitive aquatic organisms that have identical and/or similar target molecules as antibiotics are developed to target-specific molecular pathways (Martins et al., 2012; Santos et al., 2013), and generally the model organisms use for investigate their ecotoxicity were performed on exotic species (Krull and Barros, 2012).

The present study approached the risk of ENR and its main metabolite CPX at relevant environmental concentrations on biological endpoints of amphibian larvae based on exposition (ecotoxicity assays at sublethal concentrations), and effects (morphological development and growth rates; GST and CAT activities).

2. Materials and methods

2.1. Stock and test solutions

ENR and CPX were purchased from Sigma–Aldrich (Steinheim, Germany). All standards were of analytical grade (purity >95%). Standard stock solutions of ENR and CPX at 1000 mg L^{-1} were prepared in methanol and stored at −20 °C. Based on the reported environmental concentrations of both antibiotics in lentic aquatic systems (MEC, 8.77 μg $L^{-1}{}_{ENR}$; 7.49 μg $L^{-1}{}_{CPX}$; Wei et al., 2012), where amphibians usually use to reproduce, several test solutions were prepared by taking appropriate aliquots of the standard stock solutions, evaporating the methanol with N_2, and re-suspending in the corresponding volume of water having a final concentrations of 1, 10 μg L^{-1} (MEC solutions), 100 and 1000 μg L^{-1}. Test solutions were prepared in 200 ml of dechlorinated tap water (DTW), whereas reference or control assay consisted in 200 ml of DTW (Alcaráz et al., 2015). Record of fluoroquinolones in aquatic environments in Argentina are possible due to their area the most used veterinary medicine (García et al., 2006), and traces were found in preliminary water sampling at 1 μg L^{-1} concentration. Despite the widely used in veterinary (feedlots of swine, poultry and livestock) no regulation and no treatment plants exists for washed off and poultry manures in Argentina.

2.2. Study species and Acute Toxicity Test Design

Eggs and embryos of the South American common toad *R. arenarum* (Anura: Bufonidae) were selected as model test organisms. Three surface egg strings of *R. arenarum* were collected from temporary ponds (31°40′29″ S–60°20′13″ W, protected reserve, Entre Ríos Province, Argentina) in November 2014; these sites are considered as unpolluted. This anuran species has a Neotropical distribution from southern Brazil, Argentina (south of Chubut Province), Uruguay and Bolivia (Kwet et al., 2004). It is frequently occur in different habitats comprising vegetated areas, ponds, wetlands, monocultives, and urban lands. This toad species is listed as "Least Concern" IUCN (2010), and it is used as sentinel species in numerous studies in Argentina (Junges et al., 2013; Lajmanovich et al., 2014). In addition, the quite fast development of aquatic life-stages (1–3 weeks until metamorphosis) is beneficial to laboratory work.

Egg strings of various cohorts (50 cm of each string) were randomly mixed, to genetic homogenization before adding to experimental microcosms, and they were maintained under lab conditions (24 ± 2 °C, 12/12-h photoperiod cycle) until they reached the development stages St 23–25 (Gosner stage, 1960, larvae with complete opercula) to perform toxicity test.

Due to lack of information in the literature about the effects of those antibiotics on sentinel and native anuran species, the first step was to elucidate the direct toxicity on *R. arenarum* larvae. Short-term static tests (96 h) were conducted using 200 ml sterile plastic recipients (85 mm diameter, 110 mm height).

Larvae (Stage 26) were exposed to 96 h to concentrations of ENR and CPX (1 μg L^{-1} and 10 μg L^{-1} –measured environmental concentrations – and 100, and 1000 μg L^{-1}) and a negative control (with dechlorinated tap water, DTW).

ENR and CPX treatments were made in triplicate with ten tadpoles per aquarium ($n = 30$). Average tadpole size (snout-tail tip) was 26 ± 1.3 mm and weight was 0.02 ± 0.05 g. The water temperature and pH were 24 °C and 6.5, respectively. A photoperiod consisted of 16 h light (>100 Lx)/8 h dark segments for each day, to simulate the photoperiod expected in environment and prevent photolysis of antibiotics (Babić et al., 2013). Survival, presence of faeces in the bottom of each aquarium (qualitative scale, low enough faeces < 40% of the aquarium bottom; medium: covering = 50% of the aquarium bottom, high: faeces cover more than 60%), temperature, pH, and dissolved oxygen were recorded daily with standard digital instruments and Aquamerk® kits, respectively. Larvae were fed 0.2 g of boiled lettuce and a similar amount was added at 48 h if all food were ingested. No differences in larvae mortality were found within replicates during the study period (Fisher's exact test $p > 0.05$); and therefore, we pooled data ($N = 30$) from replicates of each antibiotic and control treatments for all analyses. The pooled data based on mortality rather than development and growth rates were used because mortality was follow each day and is the most common parameter to pooling data in replicate treatments (Peltzer et al., 2013).

2.3. Biological endpoints

Larvae were treated according to ASIH et al. (2004) guidelines and with approval of Facultad de Bioquímica y Ciencias Biológicas