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Bioconcentration, metabolism, and biomarker responses in freshwater fish *Carassius auratus* exposed to roxithromycin

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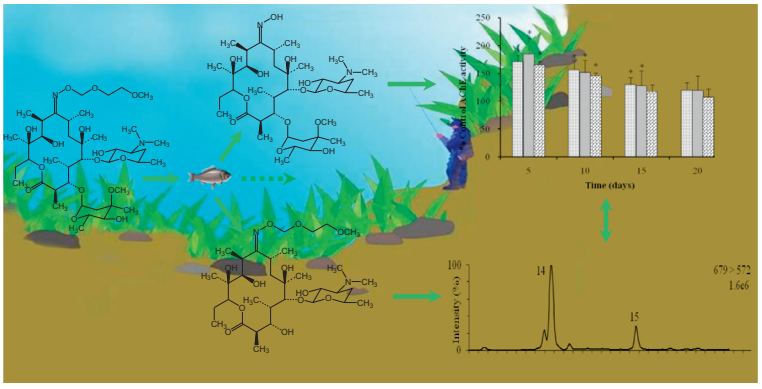
HIGHLIGHTS

- ROX in water could accumulate in crucian carp, particularly in liver and bile.
- Seventeen metabolites were detected in bile.
- A plausible scheme for biotransformation pathways of ROX in fish was depicted.
- ROX could induce change of biomarkers, which related to ROX or its metabolites.

GRAPHICAL ABSTRACT



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ABSTRACT

To investigate the distribution, bioconcentration, metabolism, and biomarker responses of macrolide antibiotic roxithromycin (ROX) in fish, crucian carp (*Carassius auratus*) were exposed to various concentrations of ROX (4, 20, and 100 μg L^{-1}) for 20 d. The ROX content in different tissues was quantified using UPLC/MS/MS. The liver exhibited the highest ROX concentration followed by the bile, gills, and muscle tissues. After 15 d of exposure to different concentrations of ROX, the bioconcentration factors were 2.15–38.0 in the liver, 0.950–20.7 in the bile, 0.0506–19.7 in the gill, and 0.0439–13.8 in the muscle; these results were comparable to the estimated BCF values. The metabolites formed in the bile were identified based on metabolic identification in human bile. Additionally, the biomarkers, including acetylcholinesterase in the brain, as well as 7-ethoxyresorufin O-deethylase and superoxide dismutase in the liver changed significantly after 5, 10, 15, and 20 d of exposure ($P < 0.05$). Our results suggest that ROX can accumulate and be metabolized in fish; therefore, interactions between ROX or its metabolites and the biological systems may induce biochemical disturbances in fish.

1. Introduction

Numerous xenobiotics, including pharmaceutical compounds, are continuously released into the environment due to various human activities (Buchberger, 2011). Effluents from sewage treatment plants (STPs) are a major known source of pharmaceuticals in aquatic environments (Chang et al., 2010). The frequent detection of pharmaceuticals in aquatic environments has elicited concern in both the public and scientific communities regarding the potential effects of these compounds on non-targeted organisms. The multiple pharmaceuticals were reportedly present in fish collected from effluent-dominated rivers, marine farms, and the effluents of STPs (Choi et al., 2008; Liu and Wong, 2013).

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Roxithromycin (ROX) is a semi-synthetic macrolide antibiotic widely used to treat respiratory tract, urinary and soft tissue infections by binding irreversibly to the subunit 50S of the bacterial ribosome. ROX ($\log K_{ow} = 2.75$) is lipophilic, and its pK_a is 8.8 at 20 °C (Beausse, 2004). The broad use of ROX has contributed to its presence in aquatic environments worldwide. ROX has been detected at levels up to 1 μg L^{-1} in the effluent streams of STPs (Kümmerer, 2009), and the removal efficiency of ROX was below 37% (Gao et al., 2012); lower concentrations (0.56 μg L^{-1}) have been found in surface water. In a few cases, ROX has been detected in trace amounts in drinking water (Kleywegt et al., 2011). Pharmaceutical compounds are designed to target specific metabolic and molecular pathways in human and animals. Numerous effects on non-targeted systems can occur due to chronic exposure to low concentrations of these bioactive substances. ROX might have strong growth inhibitory effects at the μg L^{-1} level on freshwater green alga (*Pseudokirchneriella subcapitata*); the lowest observed effective concentration was 40 μg L^{-1} (Yang et al., 2008). Moreover, ROX and its metabolites could express certain potency to interact with cytochrome P450 (CYP450) in rat and man (Yamazaki et al., 1996). In addition, as an ether-oxime derivative of erythromycin, ROX may also interact with acetylcholinesterase (AChE) since oxime is reactivator of inhibited AChE (Worek et al., 2004). Information regarding the bioaccumulation, metabolism and biological effects of ROX in fish has not been reported in literature.

The uptake and bioconcentration of pharmaceuticals in different tissues are often monitored to characterize their potential impacts on exposed organisms (Ashauer et al., 2012). Togunde et al. (2012) found that the unmodified parent compounds or metabolites detected in bile may be used as biomarkers for pharmaceutical uptake by fish exposed to these emerging contaminants in aquatic environments. In addition, AChE, 7-ethoxyresorufin O-deethylase (EROD) and superoxide dismutase (SOD) were useful biomarkers for describing the integrated toxicological effects of pharmaceuticals (Li et al., 2012). However, no studies have investigated the interactions between the bioconcentrations and the metabolism of ROX in biological systems with freshwater fish.

The current work aims to investigate the distribution and bioconcentration of ROX in different *Carassius auratus* tissues, as well as the ROX metabolites in bile. The activities of acetylcholinesterase (AChE), a biotransformation phase I enzyme (EROD), and an antioxidant defense enzyme (SOD) were utilized to evaluate the biochemical effects of ROX on fish and relate them to ROX's bioconcentration and metabolism.

2. Materials and methods

2.1. Materials and fish exposure

The standards, solvents, and chemicals employed in this study are provided in Supplemental material (SM). Immature crucian carp (60 ± 15 g, 16 ± 2 cm) were purchased from the Nanjing Institute of Fishery Science (Nanjing, China).

All healthy fish were acclimatized to laboratory condition for 14 d in dechlorinated municipal water and fed every other day with OSI freshwater aquarium pellet food (6% of body weight/day) up to 4 d before the experiment began. The exposure water's quality was checked daily and maintained at conditions suitable for crucian carp (temperature 18 ± 1 °C; pH 7.2 ± 0.2; DO 6.0 ± 0.5 mg L^{-1}; and $CaCO_3$ 116.3 ± 3.5 mg L^{-1}) over the 20 d of exposure. A daily 12/12-h light/dark photoperiod cycle was used throughout the experiment.

The fish were randomly assigned to different ROX exposure groups and kept in glass aquaria at a ratio of approximately 1.5 g L^{-1} fish/water. The fish were not fed during the exposure to prevent the release of bile from the gall bladder to the duodenum. Sewage was removed every day by suction. The semi-static exposures were always aerated and renewed every 24 h with the nominal concentrations (4, 20 and 100 μg L^{-1}) of ROX. The test solutions were prepared by diluting stock solutions with dechlorinated municipal water. A dechlorinated municipal water blank control and a solvent control (0.01% methanol) were included in the experimental design. For each treatment, six fish (*i.e.*, one fish was tested for enzyme assays, and five fish were pooled for chemical analysis) were studied at each time point and exposure concentration; each treatment was replicated three times simultaneously.

Water samples were collected from each group before and after the water renewal every day. After 5, 10, 15 and 20 d of exposure, the fish were collected from each treatment, anaesthetized with MS222 (100 mg L^{-1}), and killed by cervical transection. Their brain, muscle, liver, gill and bile tissues were removed and collected immediately. All tissues were washed with 0.15 M KCl, blotted with filter paper, and immediately frozen in liquid nitrogen (−80 °C).

2.2. Enzyme assays

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 AChE activity was determined according to the method described by Guilhermino et al. (1996). The liver samples were homogenized in 1:19 (*w*:*v*) ice-cold buffer (0.25 M sucrose, 0.1 M Tris–HCl, 1 mM EDTA, pH 7.4) and centrifuged for 15 min (10 000 g) at 4 °C. The EROD activity was determined according to the method described by Lu et al. (2009). The SOD activity was determined according to the method described by Marklund and Marklund (1974). The protein concentrations were determined using the method developed by Bradford (1976) with bovine serum albumin as the standard. Detailed protocols are available in the SM.

2.3. Sample preparation and extraction

The sample preparation and extraction procedures were developed based on EPA Method 1694 (USEPA, 2007) with some modifications. The experimental details are provided in the SM. Briefly, a water sample was removed and subjected to a traditional solid phase extraction (SPE) method. Fish tissue samples were extracted using acetonitrile with sonication. The extraction solution was degreased using *n*-hexane, and further cleanup was carried out using the SPE method. Finally, the extracts were evaporated to dryness under a stream of nitrogen, reconstituted with 1 mL methanol, and analyzed by UPLC/MS/MS.

2.4. Instrumental analysis

ROX was quantified using an Agilent 1290 Ultra-high performance liquid chromatography (Agilent, Waldbronn, Germany). Detection was performed using an Agilent 6460 Triple Quad-liner mass spectrometer equipped with an electrospray ionization source (ESI). The screening for possible metabolites was conducted by extracting the MS data at the exact mass of known metabolites from literature. Method detection limits (MDL) and limits of quantitation (LOQ) for the target analytes in the fish tissue samples were calculated. The MDL and LOQ were 0.25–0.35 and 0.80–1.2 ng g^{-1} or ng mL^{-1}, respectively. The mean percent recoveries of ROX in the different matrices and concentrations are presented in Table SM-1, ranging from 83.13% to 102.44%. Further details can be found in the SM.