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The bioaccumulation and biotransformation of synthetic estrogen quinestrol in *crucian carp*

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ABSTRACT

The occurrence and fate of endocrine disrupting chemicals (EDCs) in aquatic species have attracted close attention during the last decades. In this study, the bioaccumulation and biotransformation of synthetic estrogen quinestrol, one of the typical EDCs, in the plasma and liver of *crucian carp*, were investigated by a newly developed and validated reversed-phase high performance liquid chromatography with fluorescent detection method. *Crucian carp* were exposed to quinestrol in concentration of 2, 10, 50, 100 μg/L (5.49, 27.43, 137.17, 274.34 nmol/L) for 60 days. After 60 days' exposure, the concentrations of quinestrol found in liver and plasma were in the range of 0.25–0.69 mg/kg and 0.19–0.30 mg/L respectively, positively correlated with the exposure concentrations ranged 2–100 μg/L (5.49–274.34 nmol/L). There was a negative correlation between the bio-accumulation ratios and the exposure concentrations of quinestrol. 17α-Ethinylestradiol was also found in liver and plasma, and the concentrations were 0.02–0.19 mg/kg and 0.37–0.96 mg/L, respectively. The results indicated that quinestrol can be accumulated and transformed to 17α-ethinylestradiol in *crucian carp*. Moreover, exposure to quinestrol caused oxidative damages to *crucian carp* and the content of malondialdehyde increased in all treatment concentrations.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) have been extensively used for decades for pharmacy purposes (Daughton and Ternes, 1999; Kolpin et al., 2002; Boxall et al., 2012). Of these, natural or synthetic estrogens, known as the most potent endocrine disrupting chemicals (EDCs), are members of a group of chemicals which may exert adverse physiological effects on human and animals at very low concentrations (Auger et al., 1995; Xu et al., 2008; Soares et al., 2009). Due to their extensive use and poor removal, estrogens are eventually discharged into aquatic environments (Colucci et al., 2001; Svenson et al., 2003; Braga et al., 2005). These estrogens can be found in sewage treatment plant effluents, surface and ground waters in the range of parts-per-trillion to parts-per-million, which poses a risk on aquatic species (Hill and Janz, 2003; Johnson et al., 2005; Zhang et al., 2007; Bayen et al., 2013). Studies have demonstrated that the estrogens can be also accumulated in fish and other aquatic animals, transformed into other substances, exerting estrogen activity (Lai et al., 2002a,b; Khanal et al., 2006; Della et al., 2008; Gaulke et al., 2008; Fick et al., 2010). Exposure of marine animals to estrogens often leads to biochemical disturbances and enhances reactive oxygen species (ROS) production with oxidative damage (Gül et al., 2004; Ferreira et al., 2005). Malondialdehyde (MDA) has been frequently used as a biomarker for lipid peroxidation in many studies, the content of MDA reflects the influence of pollutants on living organisms (Del Rio et al., 2005; Valavanidis and Vlachogianni, 2010). Some estrogens, such as 17α-ethynylestradiol (EE2), can cause reproductive impairment as well as abnormal content of MDA in animals (Sissan et al., 1994; Parrott and Blunt, 2005; Hussein and Abdel-Gawad, 2010; Tompsett et al., 2013).

Quinestrol is a kind of synthetic estrogen used in contraceptive and hormone replacement therapy and occasionally for treating breast cancer and prostate cancer (Lotvin and Berman, 1970; McGonigle et al., 1994). It has a similar chemical structure with

Abbreviations: BARs, bioaccumulation ratios; EDCs, endocrine disrupting chemicals; EE2, 17α-ethinylestradiol; HPLC, high performance liquid chromatography; MDA, malondialdehyde; PPCPs, pharmaceuticals and personal care products; ROS, reactive oxygen species; RSD, relative standard deviation.

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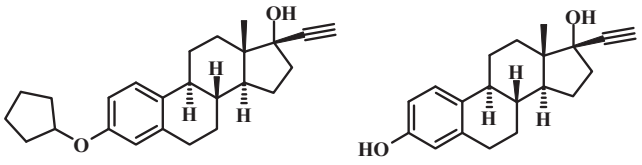

Quinestrol (Quin) 17¦Á-ethinylestradiol (EE2)

Fig. 1. The chemical structures of quinestrol and EE2.

EE2 and is thought to be a kind of typical EDCs in the environment (Fig. 1) (Tang et al., 2012). Quinestrol can enter the aquatic environment from the discharge of municipal wastewater effluent and its other application (Hanselman et al., 2003; Fu et al., 2013). The half-life periods of quinestrol in water with different pH were in the range of 148.1–316.6 days and the degradation rates within 90 days in tap, pond, river water were in the range of 49.0%, 63% and 77.0%, respectively (Tang et al., 2010), indicating that quinestrol may exert toxic effects on aquatic animals for quite a long time in its original form. However, literature concerning the eco-toxicity of EDCs indicates a lack of studies on the possible influences of quinestrol on non-target organisms. (Colborn et al., 1993; Foster and Mcintyre, 2002; Vandenberg et al., 2012) Therefore, it is essential to investigate the occurrence and fate of quinestrol in aquatic animals.

The aim of this study was to investigate whether quinestrol can be bioaccumulated, biotransformed or exert toxic effects on aquatic animals. *Crucian carp* was chosen as the experimental animal as it is a kind of widespread freshwater fish, readily available throughout the year in temperate zone and subtropic zone, easy to culture, and sensitive to polluted water (Yi et al., 2007). After 60 days' exposure of quinestrol in the concentration range of 2–100 μg/L (5.49–274.34 nmol/L), the concentrations of quinestrol and EE2 in the plasma and liver of *crucian carp* were determined by a novel liquid chromatography method. The change of MDA production in plasma was also detected to assess the impact of quinestrol on the lipid peroxidation in *crucian carp* and EE2 (10 μg/L, equal to 33.74 nmol/L) is a reference reagent in this part.

2. Materials and methods

2.1. Chemicals and reagents

Quinestrol (≥98%) and 17α-ethynylestradiol (≥98%), rhodamine B (≥98%), trichloroacetic acid (≥99%), and hydrazine hydrate (80%) were analytical grade reagent and purchased from Sinopharm Chemical Reagent Beijing Co., Ltd. Methanol (HPLC grade) was purchased from J.T. Baker (Phillipsburg, NJ) and ultrapure water was obtained by a Milli-Q water purification system (Millipore, Billerica, MA). All the other reagents were of analysis grade and purchased from Sinopharm Chemical Reagent Beijing Co., Ltd.

Quinestrol stock solutions of 5000 mg/L in acetone were prepared for the exposure of quinestrol in the fish tanks. Quinestrol and EE2 solutions of 500 mg/L in acetonitrile, MDA solution of 10 mmol/L in methanol were also prepared for the HPLC determination. These stock solutions were stored at 4 °C.

2.2. Instruments

A HPLC system, consisting of two LC-10ATvp pumps and an RF-10AXL fluorescent detector (Shimadzu, Japan), was applied for the separation and analysis of the quinestrol and EE2. A reversed-phase kromasil ODS C_{18} column (250 mm × 4.6 mm, 5 μm) and chromatograph solution light chemstation for LC system was employed and fixed to obtain and process chromatographic data.

2.3. Fish care and treatment

Juvenile *crucian carp* (32.0 ± 5.0 g, 10.0 ± 1.0 cm) in both genders were purchased from Tongzhou Aquarium (Beijing, China). All fish were cultured in dechlorinated water (temperature 18 ± 5 °C, pH 7.2 ± 0.3, DO 7.0 ± 0.4 mg/L) with a 12-h light: 12-h dark regime for a minimum of 15 days prior for use, and fed with frozen adult brine shrimp every morning. After acclimatization, fish were exposed to quinestrol in a series of concentrations (2, 10, 50, 100 μg/L, equal to 5.49, 27.43, 137.17, 274.34 nmol/L), EE2 (10 μg/L, equal to 33.74 nmol/L) for 60 days, with an untreated group for control and the proportion of the solvent acetone should be ≤0.1‰ in the fish tank, each treatment and control group were in 5 replicates (Yi et al., 2007). The experiment was carried out using a static-renewal regimen, 50% of the exposure solution was replaced daily to ensure the stabilization of test substance concentrations (Peters et al., 2007; Yi et al., 2007).

2.4. Sample preparation

In the present study, the plasma was selected as the analysis matrixes. After 60 days' exposure, venous blood samples (about 1.0 mL) were collected from subaortic caudal vein of *crucian carp* ($n = 5$). Blood samples were placed in pre-cooled ethylenediaminetetraacetic acid (EDTA)-vacutainer tubes and immediately centrifuged at 2500 g for 10 min at 4 °C. The supernatant liquid (Plasma) was collected and stored at −80 °C for the analysis. For the determination of quinestrol and EE2, the plasma of 200 μL was diluted to 1 mL with double-distilled water and centrifuged at 2500 g for 5 min. The supernatant was decanted into 10 mL amber ampoules and 5 mL organic solvent (hexane:isoamyl alcohol, 90:10, v/v) was added (Tang et al., 2010). The sample was vortex-mixed for 1 min and centrifuged at 2500 g for 5 min to aid layer separation of the aqueous and organic solvents, then frozen at −20 °C for 1 h. Finally, the organic layer was transferred into a 5 mL amber ampoule. The organic solvent was evaporated under a stream of nitrogen at 55 °C until dry. The residue was reconstituted with 80% acetonitrile into 1 mL and vortex-mixed for 1 min. The resulting solution was filtered through a 0.22 μm filter membrane and injected in the chromatographic system.

After 60 days' exposure, the livers of *crucian carp* ($n = 5$) were also carefully dissected from adipose tissue and pancreas, rinsed with physiological salt water, and weighed. Then the livers were placed in ice-cold 0.25 M sucrose containing 10 mM Tris-HCl buffer, pH 7.5, and 0.1 mM EDTA and homogenized with 6 vol. of the same sucrose medium in a Potter-Elvehjem glass homogenizer using a glass pestle (Vaglio and Landriscina, 1999). The homogenates were centrifuged at 1850 g for 15 min at 4 °C and stored at −70 °C for analysis. All the above operations were carried out below 4 °C. Then the liver sample was added with 60% acetonitrile, vortex-mixed for 1 min, processed with ultrasonic treatment of 100 W, and deposed for 12 h. Then the total solution was added with 0.1 g anhydrous magnesium sulfate and 0.05 g sodium chloride, vortex-mixed for 1 min, centrifuged at 5000 g for 10 min. After that, 1.0 mL of the total solution was transferred to another tube, added with 0.01 g anhydrous magnesium sulfate, 0.05 g PSA and 0.01 g neutral alumina, vortex-mixed for 1 min, and centrifuged at 6250 g for 10 min (Anastassiades et al., 2003; Payá et al., 2007). The resulting solution was filtered through a 0.22 μm filter membrane and injected into the chromatographic system for the determination of quinestrol and EE2.