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Stereoselective effects of ibuprofen in adult zebrafish (*Danio rerio*) using UPLC-TOF/MS-based metabolomics☆

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ABSTRACT

Ibuprofen (IBU), as a commonly used non-steroidal anti-inflammatory drug (NSAID) and pharmaceutical and personal care product (PPCP), is frequently prescribed by doctors to relieve pain. It is widely released into environmental water and soil in the form of chiral enantiomers by the urination and defecation of humans or animals and by sewage discharge from wastewater treatment plants. This study focused on the alteration of metabolism in the adult zebrafish (*Danio rerio*) brain after exposure to R-(-)-/S-(+)-/rac-IBU at 5 μg L^{-1} for 28 days. A total of 45 potential biomarkers and related pathways, including amino acids and their derivatives, purine and its derivatives, nucleotides and other metabolites, were observed with untargeted metabolomics. To validate the metabolic disorders induced by IBU, 22 amino acids and 3 antioxidant enzymes were selected to be quantitated and determined using targeted metabolomics and enzyme assay. Stereoselective changes were observed in the 45 identified biomarkers from the untargeted metabolomics analysis. The 22 amino acids quantitated in targeted metabolomics and 3 antioxidant enzymes determined in enzyme assay also showed stereoselective changes after R-(-)-/S-(+)-/rac-IBU exposure. Results showed that even at a low concentration of R-(-)-/S-(+)-/rac-IBU, disorders in metabolism and antioxidant defense systems were still induced with stereoselectivity. Our study may enable a better understanding of the risks of chiral PPCPs in aquatic organisms in the environment.

1. Introduction

Ibuprofen (IBU), as a commonly used non-steroidal anti-inflammatory drug (NSAID), is frequently prescribed by doctors to relieve pain. It is known as a cyclooxygenase inhibitor that reduces prostaglandins to play a good analgesia role (Day et al., 2000). The production and consumption of IBU have reached up to hundreds of tons per year due to its good therapeutic effects (Heath et al., 2006). It can be directly released into the environment via the discharge of medical waste and dregs. And, IBU also can be expelled to the environment due to its incomplete metabolization in humans or animals, which is a threat to the environment, especially the aquatic environment.

Increasing studies have reported that the global detection rate of IBU is higher than ever before. It was detected in the surface water and sediments of the Turia River Basin (0.18–7.20 μg L^{-1}) and in the sewage and seawater of Spain (N.D.-3.90 μg L^{-1}) and Norway (0.10–20.00 μg L^{-1}) (Carmona et al., 2014; Gracia-Lor et al., 2012; Weigel et al., 2004). Most astonishingly, IBU was detected in irrigation water in southern California (Chen et al., 2013). Therefore, it is necessary to investigate the toxicological effects of chiral IBU.

As a typical model organism, zebrafish has been used to evaluate the potential risks of pollutants in the environmental toxicology field. Previous studies reported that IBU exposure induced decreased hatching rate, growth developmental retardation and other behavioural changes in zebrafish (David and Pancharatna, 2009). And, embryo locomotivity, swimming distance, duration and speed were also affected in zebrafish after IBU exposure (Xia et al., 2017). Besides, exposure to IBU also caused proteomic responses in zebrafish (Adhikari, 2012).

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IBU is a known chiral pharmaceutical which contains two enantiomers, including R-(-)-IBU and S-(+)-IBU. Chiral pharmaceuticals have drawn people's attention due to the differences in toxicity. Increasing studies have been conducted to investigate the stereoselective effects of environmental chemicals. Xu Nana et al. suggested that (-)-PCB95 and (+)-PCB95 induced different metabolic perturbations in zebrafish embryos, indicating the stereoselective effects of two enantiomers of PCB95 (Xu et al., 2016). IBU is clinically used as racemate, so the both of the enantiomers have been detected in the environment (Ali et al., 2009). But, few studies are about the stereoselective effects of IBU. Therefore, it is of great significances to evaluate the stereoselective toxicology of IBU.

In recent years, metabolomics has become an emerging and effective tool to evaluate biological effects in zebrafish after xenobiotics exposure. On the basis of previous studies of untargeted metabolomics, potential metabolites and matched pathways related to physiological mechanisms were identified in adult zebrafish, indicating the effects of pharmaceutical hazards on aquatic organism at a low concentration (De Sotto et al., 2017). Besides, the targeted metabolomics was also used to evaluate the changes of one or several kinds of targeted metabolites in zebrafish based on absolute quantitation in environmental toxicological tests. 22 amino acids were quantitated in to evaluate the stereoselective effects of chiral PCB 91 and chiral PCB 149 using targeted metabolomics, which indicated the metabolic disorders in zebrafish (Chai et al., 2016). Increasing metabolomics studies were conducted in investigating effects of pharmaceuticals and persistent organic pollutants (POPs), but little research is about the stereoselective effects of IBU, which was commonly detected in the aquatic environment. So, metabolomics analysis will be effective in exploring the stereoselective effects of IBU in zebrafish.

The goal of our study was to provide a better understanding of the stereoselective effects of IBU on zebrafish using untargeted metabolomics. Potential biomarkers and related pathways were identified. To enable a better understanding of the results of the untargeted metabolomics, 22 amino acids and the activities of 3 antioxidant enzymes were selected to be quantified and measured by the targeted metabolomics and oxidative stress parameter analysis, respectively. As far as we know, this is the first study to systematically investigate the stereoselective effects of IBU in zebrafish at environmental concentration using metabolomics. The results will provide new insight into the stereoselective effects of chiral IBU on metabolic disorders.

2. Experimental section

2.1. Zebrafish maintenance

Adult AB wild-type zebrafish (*Danio rerio*) were raised and kept at $26 \pm 2\,^{\circ}C$ with a photoperiod of 14 h/10 h (light/dark). The proportion between male and female is 1:1. The total daily food intake was fish feed weighing 2% of the adult zebrafish weight.

2.2. Chemical and reagents

The rac-IBU was purchased from Alta Scientific First Standard® (Tianjin, China), and the R-(-)-IBU and S-(+)- IBU (Valderrama et al., 2009) were obtained by separating the rac-IBU by high-performance liquid chromatography (HPLC). The direct chiral separation of rac-IBU was performed with a Waters 2695 (Waters, USA) HPLC coupled with a Waters 2489 UV/Visible Detector (Waters, USA) on a CHIRALCEL® OJ-H column (4.6 mm × 250 mm, 5 μm particle size) using the isocratic elution with n-hexane/2-propanol (98/2), and the UV detection was performed at 254 nm. The two enantiomers were separated completely in 10 min at the flow rate of 1 mL min^{-1}.

The quantities and purities (99.0%) of the IBU enantiomers were determined using HPLC. The rac-/R-(-)-/S-(+)-IBU were dissolved in acetone in the following experiments.

The methanol and acetonitrile for the HPLC-MS/MS were purchased from Merck (Darmstadt, Germany), the HPLC-grade *n*-hexanes and 2-propanol were supplied by Fisher (Belgium, Canada), and the chloroform was purchased from Duksan (Ansan, Korea). The MS-grade formic acid (>99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was obtained from a Milli-Q system (Bedford, MA, USA).

2.3. Exposure experiments

This study conformed to the Chinese legislation and was approved by the independent animal ethics committee of the Institute of Quality Standards and Testing Technology for Agro-Products.

The adult zebrafish were randomly divided into 12 60-L glass aquariums containing 50 L of the exposure solution. The exposure concentration at 5 μg L^{-1} was selected based on the detection concentration in the environment. The same volume of acetone was diluted in the control samples which were below 0.01%. Half of the exposure media in our study was renewed for each exposure at 24-h intervals during the uptake experiment.

The used water was collected at each renewal period to determine the actual concentrations of IBU in the exposure water after the exposure period. The duration of the exposure experiment was 28 days. During the exposure period, the exposure conditions were maintained stably according to The Zebrafish Book. Five zebrafish were selected from the exposure samples and control samples at 7 th, 14 th, 28 th days to analyze enzyme activities. At the end of 28 days, five adult zebrafish were selected for the metabolomics analysis.

All the zebrafish selected for the metabolomics analysis and enzyme activity analysis were anaesthetized with MS-222 on ice to death. The brain tissues used for the enzyme activity analysis were immediately collected and treated on ice. For the metabolic analysis, the brain tissues were stored at $-80\,^{\circ}C$ before the sample preparation.

2.4. Determination of actual IBU concentration in the exposure media

An analysis of the actual concentrations of IBU in the exposure media was performed on a HPLC-MS/MS. MS spectrometry analysis was performed on an Agilent 6470 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, USA) in negative (ESI^-) multiple reaction monitoring mode (MRM) with the IBU quantification transition of m/z 205 > 161.2. The gas temperature and the capillary voltage were maintained at 300 °C and 3.5 kV, respectively, and the fragmentor voltage and collision energy were 70 V and 3 V, respectively.

An Agilent 1290 Infinity II LC system (Agilent Technologies, Santa Clara, USA) was used. The IBU was separated by a gradient elution programme on a 150 × 2.1 mm, 3.5 μm Zorbax Eclipse XDB-C18 column (Agilent Technologies, Santa Clara, USA) with water (A) and methanol (B) as follows: 0–2 min, 10% B; 2–6 min, 10%–90% B; 6–8 min, 90% B; 8.1–10 min, 90%–10% B. The column temperature was maintained at 25 °C, and the injection volume and the mobile phase flow rate were 2 μL and 0.4 mL min^{-1}, respectively.

Water samples were twice-filtered through a 0.2 μm GHP filter (Waters, Milford, USA) before the HPLC-MS/MS analysis.