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Identification and dose dependency of ibuprofen biliary metabolites in rainbow trout

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HIGHLIGHTS

- Rainbow trout were exposed to the anti-inflammatory drug ibuprofen and the biotransformation of the drug was studied.
- Several phase I and phase II metabolites were identified in bile samples by the use of various LC–MS/MS techniques.
- A high capacity for biotransformation of ibuprofen was evident.

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ABSTRACT

The biotransformation of the anti-inflammatory drug ibuprofen (IBF) was studied by exposing rainbow trout (*Oncorhynchus mykiss*) to IBF via intraperitoneal (i.p.) injection, and via water at four (0.17, 1.9, 13 and 145 μg L^{-1}) exposure levels for 4 d. Following exposure, the bile was collected and analyzed by LC–MS/MS methods. The identification of the formed metabolites in i.p. injected fish bile was based on the exact mass determinations by a time-of-flight mass analyzer (Q–TOF–MS) and on the studies of fragments and fragmentation patterns of precursor ions by ion trap mass analyzer (IT-MS). In addition to unmetabolized IBF, several phase I and phase II metabolites were found in the bile. The main metabolites were acyl glucuronides and taurine conjugates of IBF and of hydroxylated IBFs. The bioconcentration factors (BCF_{bile}), defined as the ratio of the sum of IBF and its metabolites in fish bile to the concentration of IBF in water, was determined following enzymatic deconjugation and was found to range from 14000 to 49000. The highest BCF_{bile} was found at the lowest exposure concentration (0.17 μg L^{-1}). The results show that rainbow trout has a high capacity for biotransformation of IBF, and the exposure of fish to sub μg L^{-1} concentrations of IBF can be determined by the analyses of the biliary metabolites of the compound.

1. Introduction

The anti-inflammatory drug ibuprofen (IBF) is an extensively used pharmaceutical. In 2010, approximately 113 tons of IBF was used in Finland within the population of 5.4 million persons (Finnish Medicines Agency FIMEA, 2012). Although the elimination of IBF in wastewater treatment plant (WWTP) processes is typically over 90% (Ternes, 1998; Buser et al., 1999; Lindqvist et al., 2005; Quintana et al., 2005), still easily measurable concentrations of IBF and its metabolites can be found in recipient waters (Halling-Sørensen et al., 1998; Ternes, 1998; Buser et al., 1999; Weigel et al., 2004; Lindqvist et al., 2005; Vieno et al., 2005; Daneshvar et al., 2010). Due to the continual discharges of IBF to the environment, the risks possessed by the drug on aquatic organisms need to be assessed.

IBF is a chiral compound and it is on the market mainly as a racemic mixture (*rac*-IBF). The inactive enantiomer, *R*-IBF, has shown to go through chiral inversion *in vivo* in human and the active *S*-IBF enantiomer is formed (Kaiser et al., 1976; Tan et al., 2002). In addition, a higher amount of *S*-IBF than *R*-IBF has been found in WWTP influents and in surface waters (Buser et al., 1999). The major mammalian metabolites of IBF excreted via urine are the phase I metabolites 2-hydroxyibuprofen (2-OH-IBF) and carboxyibuprofen (carboxy-IBF), which is formed via 3-hydroxyibuprofen (3-OH-IBF), and their corresponding phase II glucuronide conjugates (Tan et al., 1997, 2002). Taurine conjugation has been shown to be a minor (1.5% of the dose) metabolic pathway (Shirley et al., 1994). The metabolism of IBF is enantioselective and favors the *S*-forms (Shirley et al., 1994; Tan et al., 1997, 2002).

Recently, we have shown that rainbow trout accumulates the anti-inflammatory drugs diclofenac (Kallio et al., 2010) and naproxen (Brozinski et al., 2011) together with their metabolites in the bile when the trout are exposed to low μg L^{-1} levels of the

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compounds in aquaria. The main metabolites detected in trout bile were hydroxylated diclofenacs, their glucuronide conjugates, and the glucuronide conjugate of naproxen. The glucuronides accounted for on average 80–90% of the amount of the metabolites (Kallio et al., 2010; Brozinski et al., 2011). Anti-inflammatory drugs and antidepressants have been detected in blood, tissues and bile collected from fish exposed to WWTP effluents or wild fish caught from water receiving WWTP effluents (Brooks et al., 2005; Brown et al., 2007; Ramirez et al., 2007, 2009; Fick et al., 2010; Lahti et al., 2012; Brozinski et al., 2013).

In the current study, the biotransformation of IBF in rainbow trout was investigated. The structures of the formed metabolites were determined by the application of various LC–MS/MS methods. The biotransformation of IBF by the hepatobiliary system of fish was investigated over a wide range of exposure concentrations in aquaria. The apparent bioconcentration factors (BCF_{bile}), defined as the ratio of the sum of IBF and its metabolites in fish bile to the concentration of IBF in water, was determined in enzymatically deconjugated samples by triple-quadrupole mass analyzer (QqQ–MS) operated in multiple reaction monitoring (MRM) mode.

2. Material and methods

2.1. Chemicals

Racemic ibuprofen (IBF, >98%, CAS 15687-27-1), β-glucuronidase/aryl-sulfatase isolated from *Helix pomatia* (prod. No. G7770, CAS 9001-45-0) and tricaine methane sulfonate (MS222, CAS 886-86-2) were purchased from Sigma–Aldrich (Steinheim, Germany). *Rac*-2-hydroxyibuprofen (2-OH-IBF, >98%, CAS 51146-55-5), 1-hydroxyibuprofen (1-OH-IBF, >98%, mixture of diasteomers, CAS 53949-53-4), carboxyibuprofen (carboxy-IBF, >98%, mixture of diasteomers, CAS 15935-54-3) and 1-*β*-*O*-acyl glucuronide of IBF (>98%, mixture of diasteomers, CAS 115075-59-7) were purchased from Toronto Research Chemicals Inc. (North York, Canada). The internal standard ibuprofen-D3 (D3-IBF, >98%, 99 atom% D, CAS 121662-14-4) was obtained from Fluka (Buchs, Switcherland). Taurine conjugate of IBF (tau-IBF) was synthesized according to Shirley et al. (1994) and its purity was checked with LC–MS and ^{1}H NMR. The water used in the LC–MS analysis was purified using a Millipore Simplicity 185 system (Millipore S.A.S., Molsheim, France).

2.2. Fish exposures to ibuprofen

Rainbow trout (*Oncorhynchus mykiss*) were purchased from the hatchery of Hanka Taimen Oy (Hankasalmi, Finland, 0.5-year-old) and from the Finnish Game and Fisheries Research Institute (Laukaa, Finland, 1-year-old). Before the experiments, fish were acclimatized to laboratory conditions in continuously changing non-chlorinated artesian well water (pH 7.6 ± standard deviation, SD 0.3 or 7.0 ± 0.2, temperature 11.6 ± SD 0.04 °C or 9.9 ± SD 0.2 °C, respectively for 0.5 and 1-year-old-fish) for one week in minimum. The 0.5-year old fish were fed twice a day and 1-year old fish every other day (ca. 0.5% of fish biomass, Vital Plus, Rehu Raisio, Raisio, Finland). Feeding was stopped 3 d before the experiments to ensure the availability of bile (fish is suspected to empty the gall bladder soon after feeding).

Three 1-year-old juvenile rainbow trout (average weight 58 ± SD 11 g and length 17 ± SD 1 cm) were anesthetized with MS222 (100 mg L^{-1}), and subsequently IBF (0.52 mg IBF/100 g in 1:1 ethanol/0.7% NaCl solution) was intraperitoneally (i.p.) injected to the abdominal cavity of the fish. Three control fish were injected with 100 μL/100 g in 1:1 ethanol/0.7% NaCl solution. Fish were allowed to recover in clean reference water and after 24 h the injections were repeated. Water temperature in the aquarium (490 L) was kept at 9.8 ± SD 0.3 °C, the oxygen concentration was 12.1 ± SD 0.3 ppm and pH 7.6 ± SD 0.1. After 2 d of the first injection, the fish were immobilized and biles were collected, frozen in liquid nitrogen and stored at −80 °C prior to analysis.

The dose dependency of the hepatobiliary excretion was studied at IBF nominal concentration levels of 0, 0.1, 1.0, 10 and 100 μg L^{-1}. At each level, nine 0.5-year-old rainbow trout (average weight 8.9 g and length 9.8 cm) were used. A periodic water replacement system was used, where 20% of the water (total volume 500 L) was changed daily. Water temperature was 11.6 ± SD 0.04 °C, its oxygen concentration was 12.4 ± SD 0.06 mg L^{-1} and pH 7.6 ± SD 0.3. Following the exposure, the gall bladders were collected from immobilized fish, frozen in liquid nitrogen and stored at −80 °C prior to analysis. Legal permission to conduct the experiments described above was issued by the national authority (ESLH-2007-06053/Ym-23).

2.3. Sample preparation

2.3.1. Identification of metabolites

Three bile samples (50 μL) obtained from the i.p. dosed rainbow trout were diluted with 500 μL of 5% acetonitrile (ACN) in 0.01 M ammonium acetate. The solutions were analyzed by LC–MS using an ion trap mass analyzer (IT-MS) and a time-of-flight mass analyzer (Q–TOF–MS). Also, additional 15 μL of a pooled fish bile sample obtained from trouts exposed to 145 μg L^{-1} of IBF in aquaria (see below) was diluted with 100 μL of 2% ACN in 0.01 M ammonium hydroxide and was analyzed by Q–TOF–MS.

2.3.2. Exposures in aquaria

Due to the small size of the rainbow trouts in aquaria exposures, nine bile samples per five exposure concentration were pooled to five samples containing each a total volume of 60–100 μL of bile. *β*-Glucuronidase/aryl-sulfatase (600 U) isolated from *Helix pomatia* was dissolved in 200 μL of 1 M acetate buffer (pH 5). This solution and 200 μL of an 1 M acetate buffer solution containing 250 ng of internal standard (D3-IBF) were added to the pooled bile sample. The obtained solution was incubated for 4 h at 37 °C at condition where glucuronide and sulfate metabolites are known to be hydrolyzed (Lahti et al., 2012; Brozinski et al., 2013).

Following the hydrolysis, the pooled samples were diluted with 1 mL with deionized water (pH 2) and the compounds of interests were purified by solid phase extraction as in Brozinski et al. (2013). The Oasis HLB 3 cm^2 (60 mg; Waters, Milford, MA, USA) cartridges were conditioned with 3 mL methanol and 3 mL water (pH 2). After the sample loading, the cartridges were washed with 1 mL water (pH 2) and 1 mL 20% methanol and the compounds of interest were eluted with 1 mL 80% methanol in 2% NH_4OH. The extracts were evaporated to dryness under a stream of nitrogen, re-dissolved in 300 μL of 2% ACN in 0.01 M ammonium hydroxide and analyzed immediately by LC–MS (QqQ–MS).

Water samples (25–100 mL depending on the IBF exposure concentration) were taken twice per day from each aquarium (before and after water change) and the concentration of IBF was determined. Following the addition of the internal standard (200 ng of D3-IBF) and adjustment of pH to 2, the water samples were introduced to Oasis HLB 3 cm^2 (60 mg) cartridges (Waters, Milford, MA, USA), which has earlier been preconditioned with 2 mL of hexane, 2 mL of acetone, 3 mL of methanol and 3 mL of water at pH 2. IBF was eluted from the cartridges with 4 × 0.5 mL of methanol. The extracts were evaporated to dryness under a stream of nitrogen, redissolved in 500 μL of 30% ACN in 0.01 M ammonium acetate and analyzed by LC–MS (QqQ–MS).

2.4. LC–MS methods

The chromatographic separations were performed on an XBridge C18 column (3.5 μm, 2.1 × 50 mm) equipped with a guard column of the same brand (3.5 μm, 2.1 × 10 mm; Waters, Milford, MA, USA). The mobile phase consisted of 0.01 M ammonium acetate