

# Metabolite profiling of carbamazepine and ibuprofen in *Solea senegalensis* bile using high-resolution mass spectrometry

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**Abstract** The widespread occurrence of pharmaceuticals in the aquatic environment has raised concerns about potential adverse effects on exposed wildlife. Very little is currently known on exposure levels and clearance mechanisms of drugs in marine fish. Within this context, our research was focused on the identification of main metabolic reactions, generated metabolites, and caused effects after exposure of fish to carbamazepine (CBZ) and ibuprofen (IBU). To this end, juveniles of *Solea senegalensis* acclimated to two temperature regimes of 15 and 20 °C for 60 days received a single intraperitoneal dose of these drugs. A control group was administered the vehicle (sunflower oil). Bile samples were analyzed by ultra-high-performance liquid chromatography–high-resolution mass spectrometry on a Q Exactive (Orbitrap) system, allowing to propose plausible identities for 11 metabolites of CBZ and 13 metabolites of IBU in fish bile. In case of CBZ metabolites originated from aromatic and benzylic hydroxylation, epoxidation, and ensuing *O*-glucuronidation, *O*-methylation of a catechol-like metabolite was also postulated. Ibuprofen, in turn, formed multiple hydroxyl metabolites, *O*-glucuronides, and (hydroxyl)-acyl glucuronides, in addition to

several taurine conjugates. Enzymatic responses after drug exposures revealed a water temperature-dependent induction of microsomal carboxylesterases. The metabolite profiling in fish bile provides an important tool for pharmaceutical exposure assessment.

**Keywords** Ibuprofen · Carbamazepine · Fish metabolites · Carboxylesterases · Bile · High-resolution mass spectrometry

## Introduction

During the last few years, the presence of pharmaceuticals in riverine fish has been reported, demonstrating that long-term exposure of aquatic organisms to pharmaceutical residues emitted from wastewater treatment plants may result in bioaccumulation [1–3]. Besides, marine species in coastal areas may also be exposed to pharmaceuticals through effluent discharges, waste disposal, and the use of drugs in aquaculture. The impact of this exposure, however, has received very little attention, and studies reporting the detection of human drug residues in wild marine fish species are very scarce [4–6]. This is surprising because the occurrence of pharmaceuticals in estuaries and potential contaminated areas has raised concerns about potential adverse effects on the species living on them. The first report on the detection of pharmaceuticals in wild marine species dates back to 2009 [7]. In this study, diazepam was measured in marine flatfish collected near ocean discharges of municipal wastewater effluent. More recently, Álvarez-Muñoz et al. reported the presence of several pharmaceuticals, including metoprolol, azithromycin, and venlafaxine, in the different trophic levels of the food chain from coastal areas [4]. Other authors reported the presence of carbamazepine (CBZ) [8, 9] in different marine species being

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this compound one of the pharmaceutical compounds more frequently reported in marine species.

Through the gills, as one of the major ports of entry, drugs are taken up from the water reaching the bloodstream. Upon passage through the liver, hepatic detoxification systems recognize these xenobiotics and may bring about their transformation by phase I and II metabolizing steps into more readily excretable metabolites [10]. In fact, over 60% of clinically used drugs are metabolized by CYP-mediated (mostly CYP3A4) phase I metabolism [11]. Another phase I enzyme carboxylesterases (CbEs) are involved in the hydrolysis of many administered drugs and pro-drugs as well as endogenous compounds [12]. As yet, only the metabolism in rainbow trout, representing a model freshwater species frequently used in environmental studies, has been investigated using the three nonsteroidal anti-inflammatory drugs: naproxen [13], diclofenac [14], and ibuprofen (IBU) [15] as substrates. In all instances, the metabolic pathways were studied under controlled laboratory conditions with exposure through either spiked tank water or intraperitoneal administration of the test compounds. In case of naproxen, the analysis of the bile proved the acyl glucuronides of the parent compound and its demethylated metabolite to be the major biotransformation products [13]. Detailed profiling of bile samples from the diclofenac experiments revealed a substantial overlap with the metabolic reactions observed in humans, including 4'- and 5-hydroxylations, acyl glucuronidation, sulfation and glucuronidation of hydroxyl metabolites, and hydroxyl-acyl glucuronidation [14]. Rainbow trout dosed with ibuprofen, in turn, yielded, apart from the hydroxylated metabolites, (hydroxylated) acyl glucuronides, and ether glucuronides of hydroxy-IBU, also a number of taurine conjugates [15] although these were deemed of minor relevance.

In contrast to metabolism studies carried out with juvenile or adult fish, alternative test systems have been described, exploring the use of zebrafish at larval or embryonic stage for elucidating the biotransformation of human drugs. Following exposure of larvae to  $^{14}\text{C}$ -radiolabeled IBU, high-resolution mass spectrometry (HRMS) allowed to identify hydroxy-IBU as a major metabolite while additional drug-related material accounting for a relatively low fraction of radioactivity could not be identified [16]. In contrast, a comprehensive characterization of the metabolites of clofibrac acid was accomplished in extracts of zebrafish embryos [17]. Besides common reactions including oxygenation, glucuronidation, sulfation, and combinations thereof, a series of less frequently observed conjugative reactions was reported involving the enzymes acyl-CoA synthetase, *N*-acetyl transferase, carnitine acyl transferase, and catechol-*O*-methyltransferase (COMT) [15].

Taken together, the findings from laboratory exposure studies provide strong evidence for fish having an inherent capability for metabolizing pharmaceuticals using enzymatic

pathways very similar to those involved in the hepatic clearance of drugs in the human body. Nonetheless, the rate of metabolic conversion in fish may vary largely between substrates as suggested by the quantitative results from in vitro studies obtained with trout liver S9 fractions [18] and trout liver spheroids [19]. Comparison of intrinsic clearance values and depletion rate constants, respectively, for sets of environmentally relevant drugs demonstrated extensive biotransformation in some cases while under the assay conditions, other drugs were resistant to metabolic conversion. In both these model systems, the anticonvulsant carbamazepine, representing one of the most frequently monitored drugs in the aquatic environment, proved to be metabolically stable. Conversely, recent data published on the occurrence of CBZ and two of its human oxidative metabolites, 2-hydroxy-CBZ and CBZ-epoxide, in *Gambusia affinis* and *Jenynsia multidentata*, having been captured in a wastewater-impacted Argentinian river, suggested biotransformation in these species [20]. Subsequent exposure experiments with *J. multidentata* at laboratory scale provided further support for this hypothesis by identifying both metabolites in various organs.

Given the still fragmentary information on the metabolic pathways of CBZ in fish, we set out to conduct a comprehensive characterization of its biotransformation by analyzing bile from the flatfish Senegalese sole (*Solea senegalensis*) as a species of great economic importance in fisheries and aquaculture. In addition to the dosing experiments of this marine species with CBZ, bile was harvested and profiled for drug metabolites after administration of IBU. The findings obtained for the marine fish were contrasted with those reported for rainbow trout inhabiting freshwater environments [15, 21]. The work presented here was carried out as part of a broader study on the biological interactions of human drugs with hepatic enzymes in fish [22].

## Experimental

### Chemical reagents

IBU ( $\geq 98\%$ ), 2-hydroxy-IBU (99.2%), IBU acyl glucuronide (98%), CBZ ( $\geq 99\%$ ), IBU- $\text{d}_3$  (99.4%), and CBZ- $\text{d}_{10}$  (99.2%) were purchased from Sigma-Aldrich (Madrid, Spain) and 2-hydroxy-CBZ (99.3%) and 3-hydroxy-CBZ (99.4%) from Toronto Research Chemicals (Toronto, Canada). All organic solvents used in LC analysis were Chromasolv LC grade or equivalent. Ultra-pure water and LC/MS grade formic acid were purchased from Sigma-Aldrich (Munich, Germany). LC grade acetonitrile was purchased from Riedel de Hen (Steinheim, Germany). KCl (99%), dithiothreitol (99%), ethylenediaminetetraacetic acid (EDTA; 99.5%), 4-nitrophenyl

acetate (99%), and phenanthroline (reagent grade) were purchased from Sigma-Aldrich (Madrid, Spain).

### Lab-scale experiments

*S. senegalensis* were obtained and maintained as in Solé et al. [23]. Juveniles were acclimated in tanks for a period of 2 months at water temperatures of 15 and 20 °C in order to assess the influence of temperature on the formation of the metabolites. After the adaptation phase, the fish received a single intraperitoneal injection of IBU at a dose of 10 mg/kg ( $n = 12$ ) or CBZ at a dose of 1 mg/kg ( $n = 12$ ). Control animals ( $n = 12$ ) were administered the carrier (sunflower oil at 1  $\mu\text{L/g}$  b.w.). After injection, the fish were fasted and sacrificed 48 h later. Their organs were dissected, and liver and bile were immediately frozen in liquid nitrogen and stored at  $-80$  °C until analysis. Livers were also weighed to calculate gross morphometric indexes (see Electronic Supplementary Material (ESM) Table S3). Handling and sacrifice of the fish was done according to the national and institutional regulations of the Spanish Council for Scientific Research (CSIC) and Directive 2010/63/EU.

### Bile sample analysis

Ten-microliter aliquots of the bile samples were diluted with 150  $\mu\text{L}$  of acetonitrile/0.1 N HCl (1:3) to ensure the solubility of the metabolites and to inhibit their pH-dependent degradation. The latter was important as at elevated pH, acyl glucuronides (expected for IBU) are susceptible to chemical hydrolysis and to acyl migration. The diluted samples were centrifuged, and the supernatants were analyzed by ultra-high-performance liquid chromatography (UPLC)-HRMS for comprehensive drug metabolite profiling.

### UPLC/ESI-MS/MS analysis

Chromatographic separation was performed on a Waters Acquity BEH C18 column ( $50 \times 2.1$  mm, 1.7  $\mu\text{m}$  particle size) equipped with a guard column ( $5 \times 2.1$  mm) of the same packing material and connected to a Waters Acquity liquid chromatograph. The mobile phases for IBU in negative mode were (A) 20 mM of ammonium acetate and (B) acetonitrile. The chromatographic run in these neutral conditions (pH 6.8) was started at 95% A. After 0.5 min of isocratic elution at 95% A, the proportion of A was linearly decreased to 5% within 15 min. The percentage of A was then maintained at 5% for 2 min, and the initial mobile phase composition was reestablished within 1.0 min followed by a 2.0-min equilibration step at 95% A.

For acid conditions, mobile phase A was 0.1% formic acid and the chromatographic run for CBZ in the positive ion mode started at 90% A. After 0.5 min of isocratic elution at 90% A,

the proportion of A was linearly decreased to 10% within 6.5 min. The percentage of A was then maintained at 10% for 1.0 min, and the initial mobile phase composition was reestablished within 1.0 min followed by a 2.0-min equilibration step at 90% A. The flow rate was 300  $\mu\text{L}/\text{min}$ , and the injection volume was 10  $\mu\text{L}$ . Exact mass measurements of IBU, CBZ, and their metabolites were carried out in full scan and product ion scan mode on a Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with a heated electrospray ion (HESI) source. Negative and positive ion modes were used for the analysis of the IBU and CBZ samples, respectively. The source parameters were as follows: spray voltage of  $\pm 3000$  V, sheath gas flow of 40 L/h, auxiliary gas flow of 10 L/h, capillary temperature of 350 °C, and heater temperature of 300 °C. The Orbitrap mass analyzer was operated at a resolving power of 70,000 (FWHM) in full scan mode and of 17,500 (FWHM) in the MS<sup>2</sup> mode. External mass calibration for positive and negative ESI modes was conducted weekly for the mass range of  $m/z$  50–2000 by infusing a Pierce LTQ Velos ESI Positive and Negative Ion calibration solution from Thermo Fisher Scientific. MS data acquisition and processing was done using the software package Xcalibur 2.2. The differential analysis software Sieve 2.1 was used to aid in the detection of metabolites. The chromatographic alignment allows the determination of relatively intensive peaks formed through higher injections of drugs. Detected peaks were elucidated through MS/MS accurate measurements obtained in data-dependent mode. This approach is helpful to overcome the lack of authentic standards to propose metabolite identities.

### Hepatic biochemical determinations

Liver samples ( $\approx 1$  g) were homogenized in ice-cold buffer phosphate (100 mM, pH 7.4), containing 150 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM phenanthroline, 0.1 mg/mL trypsin inhibitor, and 1 mM EDTA in a 1:4 ( $w/v$ ) ratio between sample weight and volume using a Polytron blender. The obtained homogenate was centrifuged at consecutive steps of 10,000g for 30 min and 100,000g for 60 min at 4 °C to obtain the microsomal pellet which was resuspended in the homogenization buffer containing 20% glycerol in a 2:1 ( $w/v$ ) ratio. Microsomal protein content was determined by Bradford's method [24] adapted to microplate format and using the Bradford Bio-Rad Protein Assay reagent and bovine serum albumin as the standard (0.05–0.5 mg/mL). The absorbance was read at 595 nm. All protein determinations were performed in triplicate.

Carboxylesterase activity was measured in microsomes using a kinetic mode in 96-well microplates [25]. Twenty-five microliters of appropriately diluted microsomes was incubated with 200  $\mu\text{L}$  4-nitrophenyl acetate as the substrate (1 mM of 4-NPA final concentration) in phosphate buffer

(50 mM, pH 7.4) for 5 min at 25 °C. Formation of 4-nitrophenol was read at 405 nm in a Tecan Infinite 200 microplate reader. Carboxylesterase activity was measured in triplicate, and results were expressed as nanomoles/minute/milligram protein.

## Statistics

Student's *t* test was applied to detect differences between metabolites formed at the two temperatures and between enzyme activities at a given temperature with respect to the carrier control.

## Results and discussion

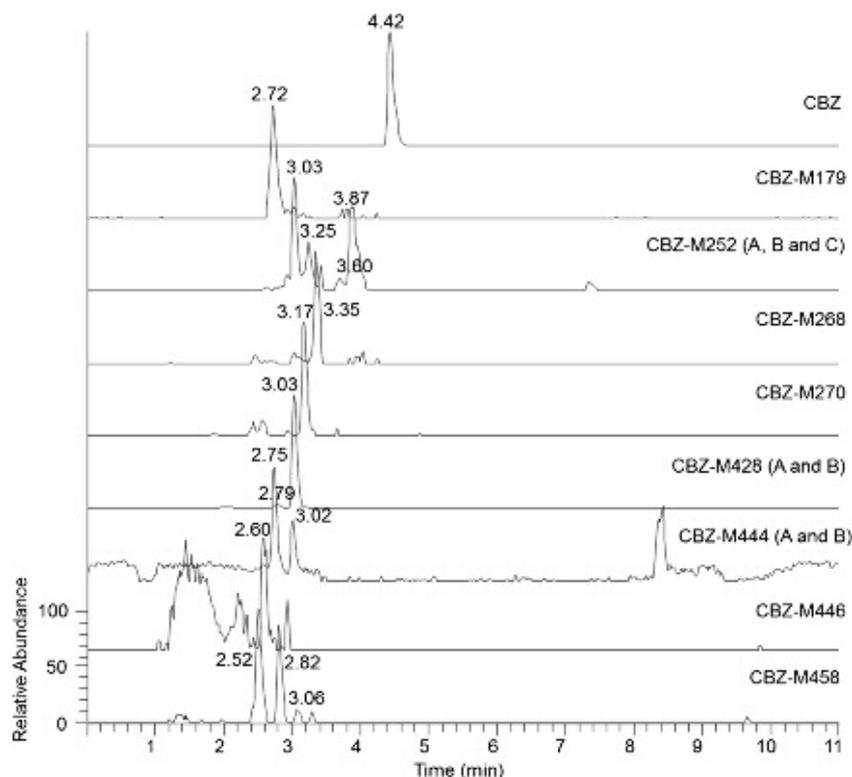
### Detection and identification of drug metabolites

In order to facilitate a thorough and automated search for drug-related metabolites in a complex matrix such as the bile samples, differential analysis Sieve 2.1 software was used. By comparing the aligned total ion chromatograms of the samples from vehicle-treated animals with those collected from fish treated with CBZ or IBU (Figs. 1 and 2), the presence of compounds unique to the drug-exposed group was indicated. After the chromatographic alignment, the software provided a list of candidates with accurate mass, retention time, and several ratios of differences between treated and control samples.

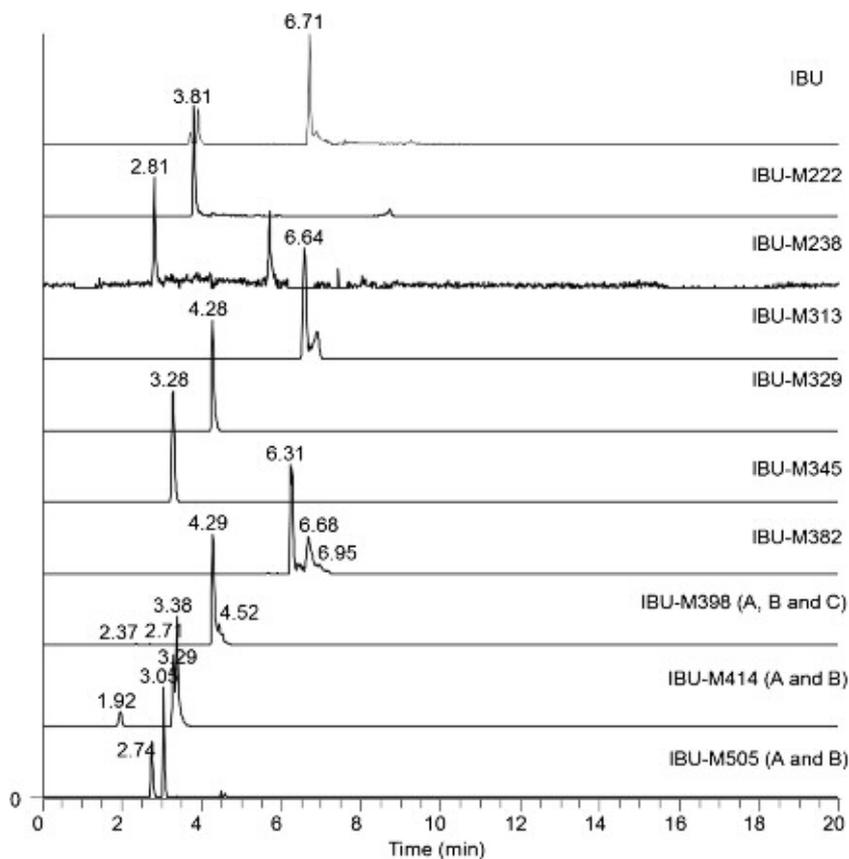
Finally, the most intensive and relevant peaks were checked manually to obtain all the available information, including their MS/MS spectra. This comparative approach allowed us to filter the most relevant metabolites in this complex biological matrix. In the resulting list of potential drug metabolites, each hit was characterized by exact mass, chromatographic retention time, and peak intensity. Based on the knowledge about common phase I and phase II metabolic reactions and combinations thereof, appropriate filters for the minimum and maximum number of the atoms (H, C, N, O, and S) were defined and applied to the aforementioned drug metabolite list. This produced elemental formulae alongside the relative mass errors between the experimentally measured values and the ones calculated for the respective elemental composition. This procedure allowed to propose the type of enzymatic reactions involved in the biotransformation of CBZ and IBU. The subsequent examination of the product ion spectra of the metabolites provided further support for the proposed metabolites. The identification of characteristic fragment ions helped determine the site of metabolism in the case of positional isomers originating from regioselective conversions.

The proposed metabolites of CBZ and IBU are listed in Tables S1 and S2 in the ESM, respectively, in the order of increasing mass, and the postulated pathways leading to their formation are depicted in Figs. 3 and 4. Since in this work only a few metabolites were available as authentic standards for ultimate confirmation of the metabolite identities, in most instances, the exact positions of the hydroxyl groups—and any

**Fig. 1** Extracted ion chromatograms (XIC) of CBZ metabolites detected by HRMS analysis of the diluted bile of *Solea senegalensis*



**Fig. 2** Extracted ion chromatograms (XIC) of detected IBU metabolites by direct analysis of the treated bile sample



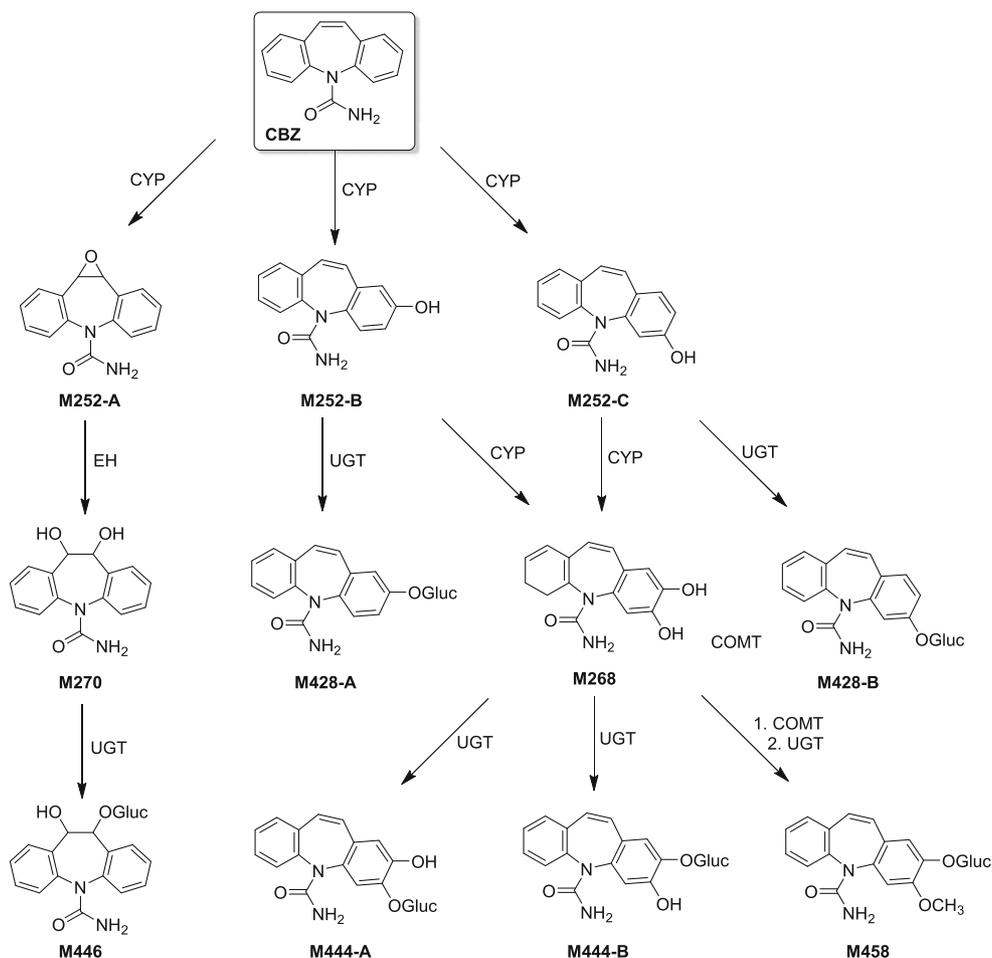
subsequent phase II reaction at those sites—could not be pinpointed. While this is a well-known limitation in the interpretation of ESI-MS/MS data in drug metabolism studies, it does not constitute an obstacle for elucidating the metabolic pathways and their respective enzymes. For the identification of the metabolites, the product ion spectra of the parent compounds CBZ and IBU, as well as those of their metabolites, were recorded to determine plausible elemental compositions and structures of the fragment ions (Fig. 5).

In case of CBZ (see ESM Table S1), three monooxygenated metabolites were detected (M252-A, M252-B, and M252-C) of which the first one was assigned as corresponding to the epoxidation of the double bond in the seven-membered central ring. That the other two metabolites were the product of aromatic hydroxylations occurring at adjacent positions in one of the phenyl rings is discussed further on (Fig. 3). The structures of the two hydroxyl metabolites were confirmed with authentic standards (ESM Table S1). Two further phase I metabolites were the doubly hydroxylated CBZ-M268 and the metabolite CBZ-M270 (Fig. 3). Once oxidative reactions introduced suitable handles for ensuing conjugative reactions, hydroxylated metabolites yielded the corresponding glucuronides. By this route, CBZ-M428-A and its isomer CBZ-M428-B were formed whereas the two metabolites (CBZ-M444-A and CBZ-M444-B) were the products of glucuronidations of dihydroxylated metabolites

(ESM Table S1). The identification of M-444 based on its mass spectrum is depicted in Fig. 5C. The two metabolites with ion mass 445.1245 break easily in the ESI source to yield their precursor drug ( $m/z$  269.0923). This neutral loss of 176 Da is diagnostic of glucuronides. Upon collision-induced dissociation of the protonated dihydroxylated CBZ molecule, fragment ions were detected at  $m/z$  226 corresponding to the neutral loss of  $\text{NH}_3$  and  $\text{HNCO}$  (43 Da) (Fig. 5C). A third hydroxy-glucuronide (CBZ-M446) was proposed to arise from uridine-diphospho-glucuronosyltransferase (UGT)-mediated conjugation of CBZ-M270 (Fig. 3) which was in line with the order of elution from the LC column: M446 displayed weaker retention than the two M444 isomers just as M268 eluted before M270.

Besides the hydroxylations and epoxidation, belonging to the category of phase I reaction, and the subsequent conjugations with glucuronic acid as a phase II-type reaction (Fig. 3), the elemental formula of the heaviest detected metabolite, M458, clearly indicated a gain of  $\text{CH}_2$  with respect to M444. This methylation, likewise falling into the class of conjugations, was most likely the product of COMT-mediated conversion, thus requiring a substrate with two hydroxyl groups on adjacent carbon atoms within an aromatic ring, namely M268 (Fig. 3). Unlike the various hydroxylation and glucuronidation reactions with their inherent introduction of polar groups and thus an overall increase in hydrophilicity, the

**Fig. 3** Proposed metabolic pathway of carbamazepine in *Solea senegalensis* based on the HRMS analysis of bile. Annotations on arrows indicate an enzyme likely involved in metabolic reaction: catechol-*O*-methyltransferase (COMT), cytochrome P450 (CYP), epoxide hydrolase (EH), and uridine-diphospho-glucuronosyltransferase (UGT). Positions of glucuronide in M444-A and M444-B are inferred from chromatographic retention times (see ESM Table S1)

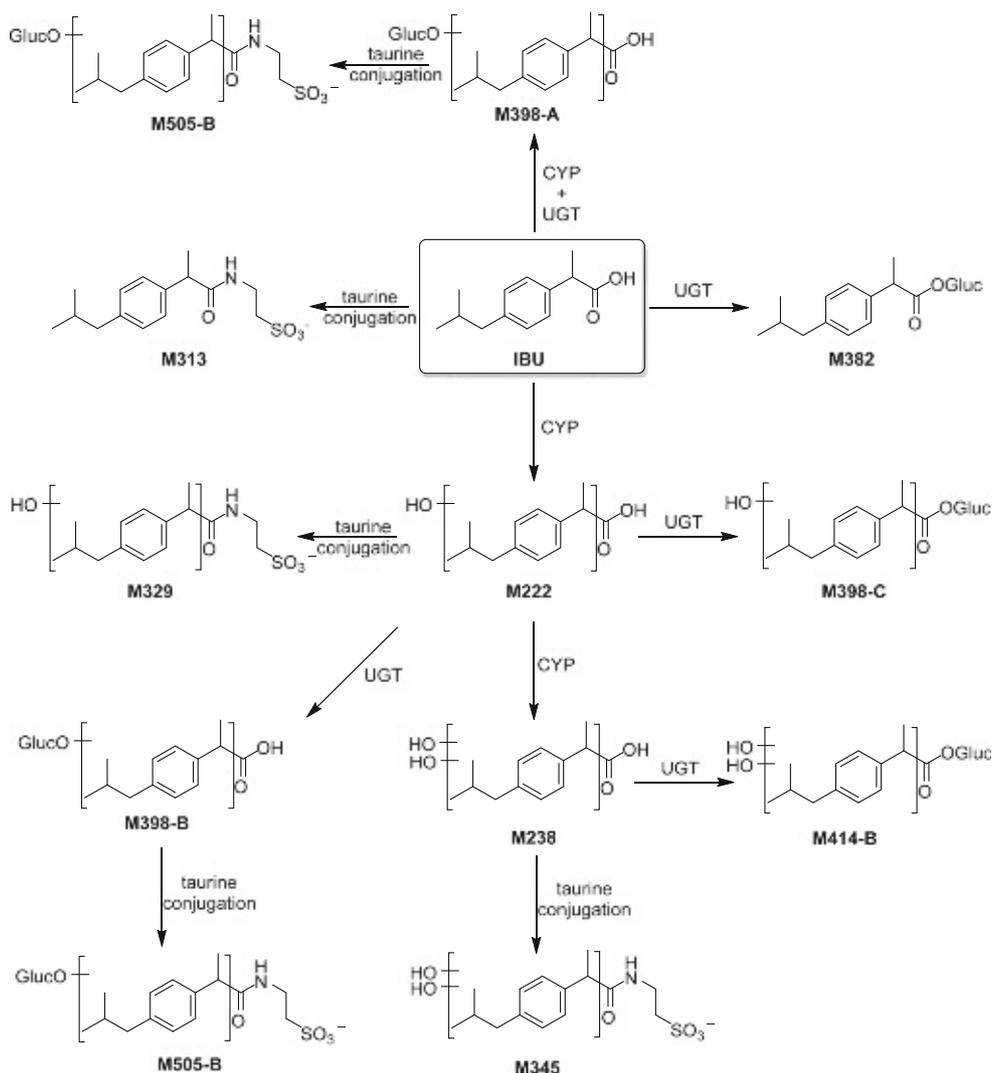


generation of an *O*-methyl metabolite from a phenol generally produces a more lipophilic entity. Taking into account that the secretion of hepatic metabolites into bile occurs against a concentration gradient, suitable transporter proteins at the membrane separating the hepatocyte from the bile canaliculus are required. With specific transporters in place to recognize glucuronized species and thus to promote their secretion, the methylation was likely to precede the conjugation with glucuronic acid in M458. Consequently, it was produced from M268 with subsequent glucuronidation. In fact, the importance of glucuronides in the elimination of CBZ from fish was reflected in the relative abundances of the oxidative vs. conjugated metabolites (Table 1): the biotransformation product with the highest relative abundance by far was M428-B, likely corresponding to the 3-hydroxy-glucuronide (Fig. 3).

With respect to the functional groups in the chemical structure of IBU, the presence of a carboxyl group in this acidic compound made it directly amenable to phase II reactions, in particular to UGT-catalyzed glucuronidation (Fig. 4). In addition, IBU features a series of chemically diverse C–H bonds (aromatic and benzylic; primary and secondary alkyl), rendering it attractive for CYP-mediated oxygenations. As compiled in Table S2 (see ESM), one mono- and one dihydroxylated

metabolite (M222 and M238) were detected. In contrast to CBZ where the glucuronidation of the (di)hydroxylated metabolites involved the formation of an ether bond, transfer of glucuronic acid to the carboxyl group IBU molecule produced the acyl glucuronide (M382), i.e., an ester bond. The observation of a partially resolved peak cluster of M382 in the extracted ion chromatogram ( $m/z$  381.1558) instead of a single peak with a defined retention time (see ESM Table S1) is typical of acyl glucuronides because they are subject to acyl migration yielding positional isomers [26]. Taking advantage of this characteristic behavior, the visual inspection of the peak profiles allowed to easily discriminate between the hydroxy-glucuronides bearing the glucuronic acid on the OH group and the isobaric acyl glucuronides. A second useful trait for telling apart *O*-glucuronides from acyl glucuronides was the large difference in retention times: owing to the presence of two COOH groups, the former were more polar and thus exhibit faster elution as compared to the acyl glucuronides in which the carboxy group is masked by the glucuronic acid (Fig. 4). Applying these principles, two hydroxylated *O*-glucuronides (M398-A and M398-B) were proposed while M398-C corresponded to the hydroxyl-acyl glucuronides. Similarly, M414-A (1.92 min) is assigned to be the *O*-

**Fig. 4** Proposed metabolic pathway of ibuprofen in *Solea senegalensis* based on the HRMS analysis of bile. Annotations on arrows indicate an enzyme likely involved in metabolic reaction: cytochrome P450 (CYP) and uridine-diphosphoglucuronosyltransferase (UGT)

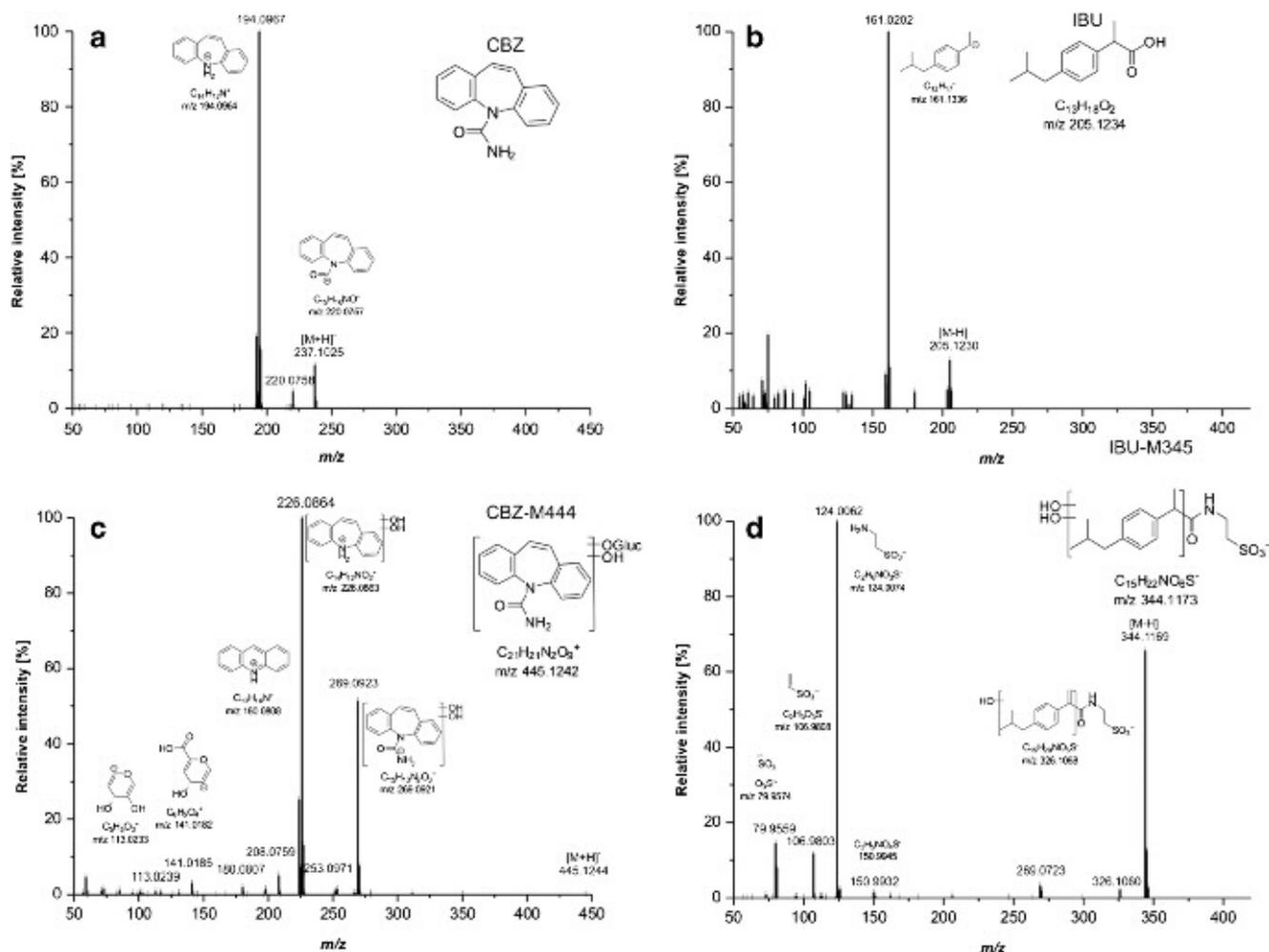


glucuronide of a dihydroxylated IBU whereas the isomeric acyl glucuronides of a dihydroxylated IBU (M414-B) were eluted at 3.29–3.38 min. Apart from substrate oxygenation and (subsequent) glucuronidation, a third category of metabolites could be unequivocally identified as originating from conjugation of the carboxyl group with taurine ( $\text{NH}_2\text{CH}_2\text{CH}_2\text{SO}_3^-$ ). This comprised the series of M313 as the direct conjugate, its hydroxyl derivative M329, and its dihydroxylated form (M345) (Fig. 4). Given the decrease in chromatographic retention times due to the presence of additional hydroxyl group in the metabolite structures, it appeared plausible to postulate that M329 (4.28 min) and M345 (3.28 min) were formed by taurine conjugation of M222 (3.81 min) and M238 (2.81 min), respectively. In the ESI-MS/MS spectrum of IBU-M345, the deprotonated taurine is detected at  $m/z$  124.0062 (Fig. 5D). Two further isomeric metabolites of minor intensity (M505-A and M505-B) were detected in the bile samples as a pair of unusual diconjugates

bearing a taurine group at the carboxyl group as well as an *O*-glucuronide (Fig. 4).

### Qualitative analysis

Based on the qualitative analysis, 11 and 13 metabolites were detected for CBZ and IBU, respectively, in bile samples from *S. senegalensis*. The CBZ and IBU metabolites were identified as a combination of accurate mass measurements of the molecular ions and fragment ions detected in MS/MS experiments (see ESM, Tables S1 and S2 as well as in Table S4 and S5). Since authentic standards of most of the CBZ and IBU metabolites were not available, relative metabolite abundances were calculated as the ratio of the analyte MS peak area to the total peak areas of all metabolites and intact parent compound. Taking into account relative areas, the fraction of unmetabolized CBZ was much higher than that of IBU, indicating limited capacity of hepatic enzymes in *S. senegalensis* to biotransform CBZ. This



**Fig. 5** ESI-MS/MS spectra of CBZ (A) and IBU (B) and their metabolites CBZ-M444 (C) and IBU-M345 (D)

is, in stark, contrast to the hepatic disposition in humans where more than 95% of the absorbed dose is transformed to a large number of metabolites [27]. Nonetheless, the overlap in metabolite identities of CBZ between human and the studied fish is surprisingly large. Although one of the principal metabolic reactions for CBZ and IBU was glucuronidation of the hydroxylated

compound, IBU formed also taurine conjugates of hydroxylated IBU. In comparison to the findings in rainbow trout [15], previously not reported metabolites of IBU include an ether glucuronide and a taurine conjugate of the dihydroxylated substrate (M414-A and M345, respectively), as well as an acyl glucuronide of dihydroxy-IBU (M414-B).

**Table 1** Biological, hepatosomatic index (HSI) [(liver weight / total weight) × 100], and microsomal activities of juvenile *Solea senegalensis* injected with vehicle (sun flower oil) and the drugs ibuprofen and carbamazepine

Group	Vehicle control		Carbamazepine-treated		Ibuprofen-treated	
	15	20	15	20	15	20
Temperature (°C)	15	20	15	20	15	20
N (male/female)	6 (1:5)	6 (4:2)	6 (2:4)	6 (5:1)	6 (2:4)	6 (4:2)
Length (cm)	25.3 ± 1.21	24.9 ± 1.24	26.2 ± 1.47	27.2 ± 1.72	26.3 ± 0.52	26.0 ± 1.87
Weight (g)	252.7 ± 36.1	254.8 ± 34.5	286.3 ± 37.2	264.8 ± 36.9	273.7 ± 31.8	253.0 ± 26.4
HSI	0.97 ± 0.08	0.81 ± 0.07	0.90 ± 0.08	0.62 ± 0.11	0.89 ± 0.06	0.77 ± 0.15
CbE (p-NPA)	39.1 ± 1.4	17.1 ± 2.2	68.6 ± 7.8**	17.7 ± 2.1	55.8 ± 5.7*	18.1 ± 2.2

Data are expressed as mean ± SEM ( $n = 6$ ).  $t$  test contrasts were made between each treatment and its temperature control

\* $p < 0.05$

The relative peak intensities of IBU, CBZ, and their detected metabolites in fish bile were measured at two water temperatures. Although no pronounced differences were observed in the relative intensities of the metabolites (ESM Tables S1 and S2), the effect of water temperature on hepatic enzyme activity is discussed in the next section.

### Biological effects on detoxification enzymes

There is proved evidence of metabolism of CBZ and IBU in fish at both temperatures by the action of phase I CYP and phase II UDPGT enzymes as revealed by the identity of the metabolites formed. The total amount of metabolites detected in bile after 48 h of injection was not clearly seen as temperature dependent as only one metabolite of IBU and three of CBZ were significantly higher at 15 °C (ESM Tables S1 and S2). By contrast, the enzymatic responses on CYP-dependent activities and CbEs were only traceable at 15 °C but not at 20 °C (Table 1) [22]. An effect of rising temperature alone over these and other hepatic enzymes has been recently published for these same fish species with higher activities displayed at lower temperatures as a result of acclimation [28]. More recently, the combined effect of temperature and these same drug exposures on some of these enzymes and other metabolic and oxidative stress parameters has also been published [22]. In addition to former observations, both injections [IBU ( $p < 0.05$ ) and CBZ ( $p < 0.01$ )] increased microsomal CbE activity. As an elevation of CYP3A4-mediated BFCOD activity also occurred with IBU ( $p < 0.05$ ) and CBZ (albeit not significant), while CYP1A-mediated EROD activity was not affected [22], it is plausible that induction of CYP3A4 and CbE may occur by the same mechanism such as via the nuclear receptor PXR as revealed in fish as well as in mammals [11, 29]. It could also be hypothesized that the lower response after IBU injection, despite the dose assayed was higher, could be due to the inhibition of CbEs by NSAID glucuronides formed as revealed in humans [30]. To the best of our knowledge, this is the first report on CbE induction by these two pharmaceutical drugs in fish although an induction by other drugs such as gemfibrozil, nonylphenol, and ethinylestradiol was formerly reported in vivo in the same fish species [31]. IBU and CBZ have also revealed to inhibit in vitro microsomal CbE activity in several marine species [28], and the lipid regulators simvastatin and fenofibrate interfere in vitro with microsomal CbE of either adults or juveniles of a related species *Solea solea* [28, 32]. Due to the importance of this enzyme family in drug and prodrug metabolism as well as its endogenous role in lipid homeostasis, we recommended its potential use as marker of drug exposures.

### Conclusion

In the present study, we have identified 13 metabolites of IBU and 11 metabolites of CBZ in fish bile. The structures of metabolites have been proposed using HRMS and HRMS/MS data. In particular, the combination of mass accuracy and the fragmentation patterns of metabolites and parent compounds allowed proposing plausible structures for each metabolite. The set of generated IBU metabolites was similar to those reported in a previous work on the metabolism of IBU in rainbow trout under similar experimental conditions [15]. Three new IBU metabolites were identified to be formed as ether glucuronide and a taurine conjugate of the dihydroxylated substrate (M414-A and M345, respectively), as well as an acyl glucuronide of dihydroxy-IBU (M414-B). Regarding CBZ, the current work constitutes the first study on the identification of fish metabolites of CBZ (13 metabolites). We evaluated also the relative peak areas of CBZ, IBU, and their metabolites in the fish exposed to two water temperatures. No quantitative/qualitative differences in metabolism of CBZ and IBU were observed at the two temperature regimes in terms of metabolite formation; however, enzyme activities differed significantly at the two acclimation temperatures. This reveals that after 48 h of drug injections, the hepatic response to the drugs was only traceable at the lowest temperature despite similar metabolism achieved under both rearing conditions as revealed by biliary in which the metabolites formed. In sum, the findings of the present study enabled us to gain further insight into the metabolism of two pharmaceuticals (CBZ and IBU) in the marine fish and by extension to other pharmaceuticals detected in the aquatic environment.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

**Statement that the studies have been approved by the appropriate ethics committee along with the full name of the ethics committee** The study was conducted in accordance with the ethical regulations of the ethical committee of the SCSIE-University of Valencia for animal experimentation and European directive (210/63/UE).

One of the co-authors holds a certificate that regulates the use of animals for experimental and other scientific purposes.

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