# Uptake and Metabolism of Human Pharmaceuticals by Fish

— A Case Study with the Opioid Analgesic Tramadol

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Environmental Science &amp; Technology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID</td>
<td>es-2017-03441k.R1</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>n/a</td>
</tr>
</tbody>
</table>
| Complete List of Authors: | Tanoue, Rumi; Ehime University, Center for Marine Environmental Studies (CMES)  
Margiotta-Casaluci, Luigi; Brunel University London, Institute of Environment, Health and Societies  
Huerta, Belinda; Brunel University London, Institute of Environment, Health and Societies  
Runnalls, Tamsin; Brunel University, Institute for the Environment  
Nomiyama, Kei; Ehime University, Center for Marine Environmental Studies (CMES)  
Kunisue, Tatsuya; Ehime University, Center for Marine Environmental Studies (CMES)  
Tanabe, Shinsuke; Ehime University, Center for Marine Environmental Studies (CMES)  
Sumpter, John; Brunel University, Institute for the Environment  
|
Uptake and Metabolism of Human Pharmaceuticals by Fish

— A Case Study with the Opioid Analgesic Tramadol

Rumi Tanoue†‡*, Luigi Margiotta-Casaluci†, Belinda Huerta‡, Tamsin J. Runnalls†,
Kei Nomiyama†, Tatsuya Kunisue†, Shinsuke Tanabe†, John P. Sumpter†

† Centre for Marine Environmental Studies, Ehime University, 2-5 Bunkyo-cho, Matsuyama,
Ehime 790-8577, Japan.
‡ Institute of Environment, Health and Societies, Brunel University, Uxbridge, Middlesex, London,
UB8 3PH, United Kingdom.

*Address correspondence to:
Rumi Tanoue, Ph.D.
Centre for Marine Environmental Studies (CMES), Ehime University, 2-5,
Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan.
TEL/FAX: +81-89-927-8162
E-mail address: rumi.tanoue.lw@gmail.com
ABSTRACT

Recent species-extrapolation approaches to predict the potential effects of pharmaceuticals present in the environment on wild fish are based on the assumption that pharmacokinetics and metabolism in humans and fish are comparable. To test this hypothesis, we exposed fathead minnows to the opiate pro-drug tramadol and examined uptake from the water into the blood and brain, and metabolism of the drug into its main metabolites. We found that plasma concentrations could be predicted reasonably accurately based on the lipophilicity of the drug, once the pH of the water was taken into account. The concentrations of the drug and its main metabolites were higher in the brain than in the plasma, and the observed brain/plasma concentration ratios were within the range of values reported in mammalian species. This fish species was able to metabolise the pro-drug tramadol into the highly active metabolite O-desmethyl tramadol and the inactive metabolite N-desmethyl tramadol in a similar manner to mammals. However, we found that concentration ratios of O-desmethyl tramadol to tramadol were lower in the fish than values in most humans administered the drug. Our pharmacokinetic data of tramadol in fish help bridge the gap between widely available mammalian pharmacological data and potential effects on aquatic organisms, and highlight the importance of understanding drug uptake and metabolism in fish to enable the full implementation of predictive toxicology approaches.
INTRODUCTION

It is now well known that many different human pharmaceuticals are present in the rivers of essentially all countries. This is a consequence of them, and their metabolites, being excreted by people into the sewage system and not being completely removed by wastewater treatment.\textsuperscript{1–4} Many of these pharmaceuticals can be found in aquatic organisms such as fish.\textsuperscript{5–10} Due to the impracticality of testing all human pharmaceuticals\textsuperscript{11,12} on fish and other aquatic organisms, which is a consequence of the large amount of time and resources that would be required to do so, as well as the ethical issues around conducting those ecotoxicity tests, the way forward has to be to reach a stage where it is possible to accurately predict the degree of threat these biologically-active molecules (and their metabolites) pose to aquatic organisms. Key steps in making those reliable predictions are understanding the occurrence and concentrations of the pharmaceutical in the aquatic environment and factors influencing the degree of its uptake, distribution, and metabolism in the organism (pharmacokinetics), as well as being able to predict the likely effects (pharmacodynamics). It is well known that the uptake of pharmaceuticals is mainly influenced by their physicochemical properties,\textsuperscript{13–17} and that the likelihood of effects is governed primarily by the presence or absence of the drug target.\textsuperscript{18} However, the steps between these two factors – namely internal distribution and metabolism in fish – are much less well understood. Understanding whether or not fish metabolise pharmaceuticals, and if so to what, is crucial to accurate predictions of risk, for a number of reasons. One is that many pharmaceuticals are detoxified (i.e., made inactive) by metabolism: the parent molecule is active, but the metabolites are not. Another is the opposite: some pharmaceuticals (the so-called pro-drugs) are inactive until they are metabolised, with the efficacy of the drug dependent on metabolism of the parent molecule into the pharmacologically active molecule(s). Finally, the possibility exist that fish, and other aquatic organisms, might metabolise pharmaceuticals differently to humans; if they did, different metabolites, which could have unique pharmacological activities, and hence lead to unanticipated effects, could be produced. Hence, if the read-across hypothesis\textsuperscript{13,19} is to be maximally useful in aiding the prediction of the effects of pharmaceuticals on non-target
species such as fish, drug metabolism in these species needs to be understood. This study was designed to improve our knowledge of the uptake and metabolism of pharmaceuticals by fish through studying how they metabolise the pro-drug tramadol.

Tramadol, which is an opioid analgesic, is widely used to treat moderate to severe pain, acting as an agonist of μ-opioid receptors as well as being a serotonin and noradrenaline reuptake inhibitor. As tramadol is only partially removed in conventional wastewater treatment plants (WWTPs), the compound has frequently been detected in surface waters around the world, at concentrations in the range of ng L\(^{-1}\) to low µg L\(^{-1}\). Some researchers have reported more than ten times higher concentrations of tramadol in South Wales (United Kingdom) than other regions — the highest concentrations were 98 µg L\(^{-1}\) and 7.7 µg L\(^{-1}\) in wastewater effluents\(^{20}\) and surface waters\(^{26}\), respectively. Likewise, extremely high concentrations of tramadol ranging from 10 to 100 µg L\(^{-1}\) were found in surface water from Cameroon.\(^{27}\) In widely used biodegradability tests from the OECD series, tramadol was characterized as a “not readily biodegradable” substance.\(^{28}\) A dissipation half-life of 49 days in degradation experiments with a bench-scale flume was previously reported.\(^{29}\)

The present study aimed at investigating the partitioning of tramadol between test water and fish blood, and between blood and brain in a teleost fish, the fathead minnow (\textit{Pimephales promelas}), after 23–24 days of chronic waterborne exposure to tramadol. An additional aim was to investigate tramadol metabolism in fish by measuring the active metabolite \(O\)-desmethyl tramadol (T-M1) and the inactive metabolite \(N\)-desmethyl tramadol (T-M2), and to compare metabolism of tramadol in fish and mammalian species. In humans, metabolism of tramadol is mainly mediated by cytochrome P450 (CYP) 2D6, which transforms tramadol into the active metabolite T-M1, and by CYP2B6 and CYP3A4, which transforms tramadol into the inactive metabolite T-M2.\(^{30}\) Considering the existing uncertainties around the evolutionary aspects and functional diversity of these enzymes (especially CYP2 subfamilies) in teleost fish,\(^{31}\) generating novel drug-metabolism data in fish can provide important information on the functional conservation of these metabolic
properties across species, and support species-extrapolation and predictive pharmacology/toxicology approaches. In order to facilitate data interpretation, we also quantified the metabolism of the antidepressant fluoxetine in the same species, and used it as a reference. Fluoxetine is metabolised to the equipotent active metabolite norfluoxetine through $N$-demethylation by CYP 2D6, 2C9, 2C19, and 3A4 in humans.  

**MATERIALS AND METHODS**

**Chemicals.** Tramadol hydrochloride was purchased from Sigma-Aldrich (Dorset, UK) with purity higher than 99% (product number 42965-5G-F, lot number BCBN4547V). Fluoxetine hydrochloride was purchased from Sigma-Aldrich (Dorset, UK) with purity higher than 99.9% (product number PHR1394, lot number LRAA1901). N, N-dimethylformamide (DMF) was obtained from Fisher Scientific (Loughborough, UK). Liquid chromatography–mass spectrometry grade methanol, acetonitrile, acetic acid, and ammonium acetate (98%) were purchased from Wako Chemicals (Osaka, Japan). Ultrapure water (Milli-Q water) was obtained using a Direct-Q3 water purification system (Millipore, Japan). Oasis HLB cartridges (30 mg, 1 mL) were purchased from Waters (Milford, MA, USA). Analytical certified solution standards of cis-tramadol hydrochloride, $O$-desmethyl-cis-tramadol hydrochloride, $N$-desmethyl-cis-tramadol hydrochloride, fluoxetine hydrochloride, and norfluoxetine oxalate, were purchased from Sigma–Aldrich (St. Louis, MO, USA). Internal standard (IS) solutions of tramadol-$^{13}C$-$d_3$ hydrochloride, $O$-desmethyl-cis-tramadol-$d_6$ hydrochloride, fluoxetine-$d_6$ hydrochloride, and norfluoxetine-$d_6$ oxalate, were purchased from Sigma–Aldrich (St. Louis, MO, USA).

**Test Fish.** Adult fathead minnows (*Pimephales promelas*), approximately seven months old, 2.6 ± 0.53 g average weight, and 5.4 ± 0.30 cm average length, were supplied from breeding stocks maintained at Brunel University London, UK. Ten days before the beginning of chemical dosing, sexually mature males were transferred into the flow-through systems for acclimation to the test
conditions. Fish were fed three times per day: once with adult brine shrimp (Tropical Marine Centre, Gamma irradiated), and twice with flake food (King British Tropical flake food), throughout the experiment. This study was carried out under Project and Personnel Licences granted by the UK Home Office, which follows the United Kingdom Animals (Scientific Procedures) Act 1986, and the European Animal Directive 2010/63/EU.

Experimental Design. A preliminary short-term exposure test with two different pH conditions was performed before the chronic 23–24 days exposure test, to assess the effect of water pH on uptake of tramadol by fish. Thirty-two fish were transferred into 2 glass tanks (n = 16 in each tank) which were filled with 20 L of thermostatically-heated dechlorinated tap water adjusted to pH 8.1 ± 0.09 (short-term treatment A) or 8.5 ± 0.09 (short-term treatment B) by addition of 0.1 M NaOH. Water concentrations of tramadol in tanks were nominally set at 100 µg L\(^{-1}\) by adding 4 mL of tramadol solution (500 mg L\(^{-1}\) in DMF: Milli-Q water (1:4, v/v)) to 20 L of dechlorinated tap water. Test water in tanks was partially renewed (50%) every 12 h, resulting in approximately 70% daily replacement. Four fish were sampled from each tank at 12, 24, 48, and 72 h, for analysis of tramadol in plasma.

The 23–24 days chronic exposure was carried out using a continuous flow-through system comprising twelve 20.5 L glass tanks (dimensions: 45(l) x 24(w) x 19(d) cm). The test was run at a photoperiod of 16 h light: 8 h of dark, with 20 min dawn/dusk transition periods. During the experiment, the temperature of water, pH, and dissolved oxygen concentrations were maintained at 25 ± 1°C, 7.8 ± 0.19 (7.5–8.2), and 6.0–8.0 mg L\(^{-1}\), respectively. Thermostatically-heated dechlorinated tap water flowed into 12 glass mixing chambers at a rate of approximately 167 mL min\(^{-1}\) (10 L h\(^{-1}\)), which supplied approximately 12 tank volumes per day to each test tank. The same mixing chambers also received stock solutions containing test chemicals delivered via peristaltic pumps at a rate of approximately 33 µL min\(^{-1}\) (2 mL h\(^{-1}\)). The stock solutions containing test chemicals were prepared every four days in amber bottles with DMF: Milli-Q water (1:4, v/v) as a
carrier solvent. The final DMF concentration in the test water was approximately 0.004%. Six exposure treatment groups: water dilution control (WDC), solvent control containing 0.004% of DMF (SC), 1 µg L$^{-1}$ (TGf1), 10 µg L$^{-1}$ (TGf10), and 100 µg L$^{-1}$ (TGf100) of tramadol, and 100 µg L$^{-1}$ of fluoxetine (FGf100), were prepared. Each treatment group had two replicates (2 tanks). Eight males were randomly allocated to the glass tanks, giving a total of 16 fish per treatment. The concentrations of chemicals in the test water were chosen to cover both environmentally and pharmacologically relevant concentrations. The highest water concentration (100 µg L$^{-1}$) was chosen in order to produce fish plasma levels of tramadol proximate to the human therapeutic plasma concentration range (100–300 ng mL$^{-1}$)$^{33}$. Water samples (1 mL) were collected in polypropylene tubes on day-0, 1, 2, 4, 7, 10, 14, 16, 19, and 22 from all tanks, to measure the water concentrations of test chemicals.

After the 23–24 days chemical exposure, all fish were individually anaesthetised with an aqueous solution of ethyl 3-aminobenzoate methanesulfonate (0.5 g L$^{-1}$ of MS-222 at pH 7.5), according to UK Home Office regulations. Fish blood was taken from the caudal vein using heparinized capillary tubes. Blood samples were centrifuged (8000 × g, 5 min, 4°C) to obtain plasma samples. The plasma samples were stored at −80°C until chemical analysis. Standard length and body weight of fish were measured, and then fish brain was collected, weighed, snap-frozen in liquid nitrogen, and stored at −80°C until subsequent analysis.

**Chemical analysis.** Detailed information on analytical procedures for identification and quantification of tramadol and its metabolites T-M1 and T-M2, as well as fluoxetine and its metabolite norfluoxetine in water, plasma, and brain samples, can be found in the Supporting Information (SI). Briefly, 10 to 20-fold diluted water samples were directly injected in an instrument described below. Plasma samples (10 µL) were subjected to protein precipitation with IS solution, acetate buffer, and methanol. Following centrifugation, an aliquot of the supernatant was diluted with Milli-Q water. Totally 20 to 500-fold diluted plasma extracts were directly
injected in an instrument described below. Brain samples (14–25 mg) were homogenized in IS solutions, acetate buffer, methanol, and acetonitrile, subsequently subjected to protein precipitation and ultrasonically extraction. Following centrifugation, an aliquot of the supernatant was diluted with Milli-Q water. The water-diluted sample was loaded onto an Oasis HLB cartridge, the analytes retained in the cartridge were then eluted with methanol. The solvent was evaporated and the contents were reconstituted in methanol: Milli-Q water (4:6, v/v). Totally 20 to 2000-fold diluted brain extracts were injected in an instrument described below. Instrumental analysis was performed on an ultra-high-performance liquid chromatograph system (UFLC XR, Shimadzu, Japan) coupled to an AB Sciex Qtrap 5500 mass spectrometer (Applied Biosystems Sciex, Tokyo, Japan) operating in electrospray ionization (ESI) positive mode with multiple reaction monitoring (MRM).

**Quality Assurance and Control (QA/QC).** Target compound concentrations were determined by an isotope-dilution method. IS-corrected recovery rates of target compounds in plasma and brain of fish were determined by triplicate analyses of target compound-free fish tissues spiked with target compounds at 6 concentrations ranging from 0.1 to 10000 ng mL⁻¹ plasma or ng g⁻¹ brain. Method detection limits (MDLs) were calculated from the standard deviation (SD) of nine replicate injections of the fortified tissue extracts (at the lowest concentrations spiked). IS-corrected recovery rates, precision, and MDLs for plasma and brain samples are shown in Supporting Information (Table S2 and S3). IS-corrected recovery rates ranged between 85.2% and 126%, with relative standard deviations less than 15%.

**Prediction of Tramadol Concentration in Fish Plasma.** A fish plasma model (FPM) has been proposed as a screening technique to estimate potential risk of pharmaceuticals to wild fish.¹⁹,³⁴,³⁵ In the FPM, drug plasma concentration is predicted using the theoretical partition coefficient between water and fish blood based on chemical lipophilicity. The predicted fish plasma concentration is then compared with the widely available effective drug concentrations in human
plasma (human therapeutic plasma concentration ranges). However, the original fish blood–water partitioning model was developed based on empirical data for neutral organochlorine compounds such as polychlorinated biphenyls (PCBs) that are relatively stable in animal tissues. The original fish blood–water partition coefficient was described by the following equation

\[ P_{BW} = (10^{0.73 \log K_{ow}} \times 0.16) + 0.84 \]  

where \( P_{BW} \) is the equilibrium blood–water partition coefficient, \( K_{ow} \) is the octanol–water partition coefficient. This equation was developed using empirical data from rainbow trout, and the terms of 0.16 and 0.84 represent the organic (lipids and proteins) and aqueous fractions in rainbow trout whole blood, respectively.

In recent years, pH-dependent octanol–water partition coefficient (\( D_{ow} \)) has been proposed as an alternative to \( K_{ow} \), to more accurately reflect partitioning for ionisable pharmaceuticals.\(^5,37–44\) By using \( D_{ow} \) as a parameter, various authors have successfully predicted the plasma drug concentrations within a log unit deviation for fluoxetine\(^40\), sertraline\(^38\), propranolol\(^45\), and oxazepam\(^39\). On the other hand, liposome–water partition coefficient (\( K_{lipw} \)) has been expected to be a more accurate descriptor than \( K_{ow} \) to estimate bioconcentration of ionisable chemicals in organisms.\(^46–49\) The \( K_{lipw} \) can be converted into pH-dependent liposome–water partition coefficient (\( D_{lipw} \)).\(^46\)

In the present study, steady-state plasma bioconcentration factors (\( BCF_{plasma} \): fish plasma/water concentration ratios) of tramadol in fathead minnows were predicted by the concept of \( P_{BW} \) described above, based on an assumption that the tramadol concentration in fish plasma and whole blood is approximately equal, as blood-to-plasma ratio was previously determined at 1.09 \( \pm \) 0.02 in healthy male volunteers.\(^50\) The aqueous fraction in the whole blood of fathead minnow (87.6%) is slightly higher than that in rainbow trout (83.9%).\(^51\) Thus, the original equation was modified based on the difference in blood composition between rainbow trout and fathead minnow, and the following equations were obtained.

\[ BCF_{plasma} = (10^{0.73 \log K_{ow}} \times 0.12) + 0.88 \]
BCF\text{plasma} = (10^{0.73\log D_{ow}} \times 0.12) + 0.88 \quad (3)

BCF\text{plasma} = (10^{0.73\log D_{lipw}} \times 0.12) + 0.88 \quad (4)

The pH-dependent \( D_{ow} \) was calculated by

\[ D_{ow} = f_{\text{neutral}} \times K_{ow \ (\text{neutral})} + f_{\text{ion}} \times K_{ow \ (\text{ion})} \quad (5) \]

where \( f_{\text{neutral}} \) and \( f_{\text{ion}} \) are the fractions of the neutral and ion species at the study pH, respectively, and \( K_{ow \ (\text{neutral})} \) and \( K_{ow \ (\text{ion})} \) are the respective \( K_{ow} \) values. The relationship between \( f_{\text{ion}} \) and \( f_{\text{neutral}} \) is defined by

\[ f_{\text{ion}} = f_{\text{neutral}} \times 10^{pH - pK_a} \quad (6) \]

It was assumed that \( \log K_{ow \ (\text{ion})} \) is 3.5 log units lower than the corresponding \( \log K_{ow \ (\text{neutral})} \). Mean pH values measured in each tank were used for the calculation. Although various programs have been developed to predict \( \log K_{ow} \), each program uses different algorithms. Given the uncertainty of calculating \( \log K_{ow} \), we used the lowest and the highest \( \log K_{ow} \) values (2.45 and 3.01, respectively) from databases and reported pKa value of 9.41 to predict BCF\text{plasma}. Physicochemical properties of tramadol were summarized in Table S4 in the supporting information. The pH-dependent \( D_{lipw} \) was calculated by

\[ D_{lipw} = f_{\text{neutral}} \times K_{lipw \ (\text{neutral})} + f_{\text{ion}} \times K_{lipw \ (\text{ion})} \quad (7) \]

where \( f_{\text{neutral}} \) and \( f_{\text{ion}} \) are the fractions of the neutral and ion species at the study pH, respectively, and \( K_{lipw \ (\text{neutral})} \) and \( K_{lipw \ (\text{ion})} \) are the respective \( K_{lipw} \) values. The \( K_{lipw \ (\text{neutral})} \) was calculated with the PP-LFER equation\textsuperscript{53} for \( \log K_{lipw \ (\text{neutral}, 25^\circ C)} = 0.48 + 0.55L - 0.95S - 0.05A - 4.02B + 1.65V \) and chemical parameters taken from the UFZ-LSER database v 3.1\textsuperscript{54}. The \( K_{lipw \ (\text{ion})} \) was calculated with COSMOmic extended for the description of charged organic chemicals via the implementation of the membrane bilayer potential.\textsuperscript{37,46} Eventually, measured tramadol concentrations in plasma of fathead minnows were compared with the concentrations predicted by the BCF\text{plasma} estimated using three different chemical lipophilic parameters (\( K_{ow}, D_{ow}, \) and \( D_{lipw} \)), i.e. equations (2), (3), (4). Mean tramadol water concentration measured in each tank was used for the prediction.
Statistical Analysis. Normal distribution and homogeneity of variance were tested with Shapiro–Wilk and Levene’s tests, respectively. For data with normal distribution and variance homogeneity, parametric tests were applied. If the data did not show a normal distribution, nonparametric tests were applied. Eventually, comparing $BCF_{\text{plasma}}$ values between short-term treatment A and B, non-parametric Mann-Whitney-Wilcoxon rank sum tests were conducted. Non-parametric Kruskal–Wallis test followed by Steel-Dwass test were performed to compare the $BCF_{\text{plasma}}$ from different water pH conditions. For assessing the relationship between the concentration of tramadol in plasma and brain, nonparametric Spearman’s rank correlation coefficients were calculated. Parametric one-way ANOVA followed by a Tukey’s HSD test was conducted to compare brain/plasma tramadol concentration ratios among treatments. For assessing the relationship between plasma fluoxetine levels and norfluoxetine/fluoxetine concentration ratios, nonparametric Spearman’s rank correlation coefficients were calculated. A $p$-value of <0.05 was considered statistically significant. All statistical analyses were conducted using the open source statistical software R 3.3.2 GUI 1.68 Mavericks build (7288) (http://www.r-project.org/).

RESULTS AND DISCUSSION

Preliminary Experiment to Assess the Influence of Water pH on Fish Uptake of Tramadol.

Measured concentrations of tramadol in test water were $96 \pm 3.9 \mu g \ L^{-1}$ and $93 \pm 4.4 \mu g \ L^{-1}$ for short-term treatment A and B, respectively. Time-course of tramadol concentrations in fish plasma are presented in Figure 1 (A). Although mean plasma concentrations in both treatment A (at pH 8.1) and B (at pH 8.5) increased with the exposure time, the data from 24 h to 72 h did not show significant changes. Assuming near steady-state condition, measured plasma data from 24, 48 and 72 h were all used to calculate $BCF_{\text{plasma}} (24\text{–}72h)$. Comparing $BCF_{\text{plasma}} (24\text{–}72h)$ values between treatment A (median: 1.4, $n = 12$) and treatment B (median: 1.8, $n = 12$), a statistically significant difference was observed ($p = 0.019$). Even if plasma tramadol concentrations did not reach steady-state conditions, it can be speculated that $BCF_{\text{plasma}} (24\text{–}72h)$ values reflected the difference in
fish uptake rates between treatment A and B. For ionisable chemicals, it is well known that ionization can reduce their uptake into organisms owing to a decrease in their lipophilicity and accompanying membrane permeability. As tramadol is a weakly basic compound and has a pKa value of 9.41 (amino group), theoretically, 95% and 89% of tramadol are considered to be positively charged at pH 8.1 and 8.5, respectively.

**Tramadol and Fluoxetine Concentrations in Test Water for 23–24 Days exposure.** None of the targeted chemicals were detected in any control (WDC and SC) samples. Tramadol was not detected in any fluoxetine-treated water samples, and fluoxetine was not detected in any tramadol-treated water samples. Tramadol water concentrations (mean ± SD, n = 18) measured throughout the experiment for TG-1, TG-10, and TG-100 treatment were 1.1 ± 0.053 µg L⁻¹, 9.9 ± 0.65 µg L⁻¹, and 98 ± 5.2 µg L⁻¹, respectively. Fluoxetine water concentration (mean ± SD, n = 18) measured throughout the experiment for the FG-100 treatment was 94 ± 8.5 µg L⁻¹. Measured concentrations were all within ± 20% of the nominal values. Inter-tank variabilities were also within ± 20%.

**Concentrations of Tramadol and Its Metabolite in Fish Plasma After 23–24 Days Exposure.** None of the targeted chemicals were detected in any control (WDC and SC) samples. Tramadol fish plasma concentrations (mean ± SD, n = 16) measured after the 23–24 days chronic exposure for TG-1, TG-10, and TG-100 treatment groups were 1.0 ± 0.32 ng mL⁻¹, 5.9 ± 2.9 ng mL⁻¹, and 46 ± 12 ng mL⁻¹, respectively. Within each treatment, the difference between the minimum and maximum plasma concentrations was up to 4-fold. Plasma tramadol concentrations of all fish exposed to waterborne tramadol at 98 µg L⁻¹ were slightly below the human therapeutic plasma concentration range (100–300 ng mL⁻¹). Active metabolite T-M1 plasma concentrations (mean ± SD, n = 16) measured for TG-10 and TG-100 treatment groups were 0.88 ± 0.60 ng mL⁻¹ and 3.8 ± 0.99 ng mL⁻¹, respectively. All plasma samples in TG-1 treatment group had T-M1 concentrations
below the MDL value (0.14 ng mL$^{-1}$). Plasma T-M1 concentrations (3.8 ± 1.0 ng mL$^{-1}$) of fish exposed to tramadol at 100 µg L$^{-1}$ were approximately 10 times lower than effective plasma T-M1 concentrations (40 ± 30 ng mL$^{-1}$) reported in humans.$^{55}$ Inactive metabolite T-M2 plasma concentrations (mean ± SD, $n = 16$) measured for TG-1, TG-10, and TG-100 treatment groups were 0.48 ± 0.21 ng mL$^{-1}$, 1.2 ± 0.43 ng mL$^{-1}$, and 7.2 ± 1.7 ng mL$^{-1}$, respectively.

**Measured vs. Predicted Fish Plasma Concentrations and BCF$^{\text{plasma}}$ of Tramadol.** Measured plasma concentrations were compared with the concentrations predicted by the FPM (Figure 1 (B)). When pH-dependent chemical lipophilicity ($D_{ow}$ or $D_{lipw}$) was used for the prediction, measured median values were 2–6 times lower than predicted values. When using FPM for estimating the potential risk of pharmaceuticals, an overestimated prediction would not be serious from the viewpoint of precautionary principle. Nevertheless, the disagreement between measured and predicted plasma tramadol concentrations in fathead minnows might be due to differences in the existence form in the blood and/or hepatic clearances, between tramadol and PCBs. In fact, plasma protein binding of tramadol in human was reported to be approximately 20%$^{56}$, while lipid-soluble PCBs can be highly retained in the blood lipids. For the clearance of tramadol, we found its metabolites T-M1 and T-M2, with the concentration ratios of tramadol: T-M1 + T-M2 = 4:1 in plasma of fathead minnows. As it can be presumed that biotransformation of tramadol in fish occurs much faster than PCBs, metabolism of tramadol by fathead minnow is likely involved in the disagreement between measured and predicted plasma tramadol concentrations. Accounting for the protein binding and metabolism, BCF$^{\text{plasma}}$ of tramadol can be predicted by

$$\text{BCF}_{\text{plasma}} = [(10^{0.73\log_{\alpha}} \times 0.12 \times f_{\text{bp}}) + 0.88] \times f_{\text{parent}} (8)$$

where $\alpha$ is $D_{ow}$ or $D_{lipw}$, the terms of 0.12 and 0.88 represent the organic and aqueous fractions in fathead minnow whole blood, respectively, and $f_{\text{bp}}$ is the fraction bound to proteins (value measured in human plasma: 0.20), $f_{\text{parent}}$ is the fraction of parent compound tramadol (tramadol/tramadol + TM-1 + TM-2 concentration ratio in plasma of fathead minnow: 0.80). When
comparing measured tramadol concentrations in plasma of fathead minnow with the concentrations predicted by the $\text{BCF}_{\text{plasma}}$ estimated using the equation (8), only 0.86–1.8 fold differences were observed. In the present study, only 2 metabolites (i.e., TM-1 and TM-2) in fathead minnow were measured and the $f_{\text{parent}}$ of 0.80 was applied as a provisional value. The actual value should be lower than 0.80, because 23 metabolites of tramadol were previously identified in human urine. Additionally, several study have reported that 25–30% of an oral dose is excreted as unchanged drug in the urine of human, whereas 55–60% of an oral dose is excreted as metabolites.

Measured $\text{BCF}_{\text{plasma}}$ of tramadol for 23–24 days exposure are shown in Figure 1 (C). These $\text{BCF}_{\text{plasma}}$ values, ranging from 0.29 to 1.6 for fathead minnows, were similar to or slightly lower than those for rainbow trout (min–max: 2.3–3.3) exposed to treated wastewater in Sweden. Measured $\text{BCF}_{\text{plasma}}$ values were the highest for TG-1 treatment group, followed by TG-10 and TG-100 treatment groups. Combining results from preliminary short-term and 23–24 days chronic experiments, it was found that $\text{BCF}_{\text{plasma}}$ values increased as the water pH increased (Figure 1 (D)). The lowest (median $\text{BCF}_{\text{plasma}}$: 0.44) and the highest (median $\text{BCF}_{\text{plasma}}$: 1.8) values were found at pH 7.6 and 8.5, respectively. Theoretically, 99% and 89% of tramadol are considered to be positively charged at pH 7.6 and 8.5, respectively. Our result supports previous studies, highlighting the importance of taking the water pH influence into account when $\text{BCF}_{\text{plasma}}$ of ionisable chemicals are estimated. From the viewpoint of environmental risk assessment for basic chemicals, using water-based threshold values from in vivo tests at only neutral water pH can lead to underestimation of their actual risks in natural alkaline surface waters, as pointed out by Boström et al. (2015).

**Tramadol and Its Metabolite Concentrations in Fish Brain After 23–24 Days Exposure.** None of the targeted chemicals were detected in any control (WDC and SC) samples. Tramadol brain concentrations (mean ± SD, $n = 16$) measured after the 23–24 days exposure for TG-1, TG-10, and TG-100 treatment groups were $4.6 ± 1.4 \text{ ng g}^{-1}$, $26 ± 10 \text{ ng g}^{-1}$, and $200 ± 49 \text{ ng g}^{-1}$, respectively.
T-M1 brain concentrations (mean ± SD, n = 16) measured for TG-1, TG-10, and TG-100 treatment groups were below the MDL value (0.56 ng g⁻¹), 1.4 ± 0.40 ng g⁻¹, and 11 ± 3.5 ng g⁻¹, respectively. T-M2 brain concentrations (mean ± SD, n = 16) measured for TG-1, TG-10, and TG-100 treatment groups were 0.73 ± 0.17 ng g⁻¹, 2.6 ± 0.75 ng g⁻¹, and 24 ± 7.8 ng g⁻¹, respectively. Recent results from an in vitro human blood-brain barrier model and an in vivo rodent study have shown that tramadol is actively transported from blood to brain by a proton-coupled organic cation antiporter located in the blood–brain barrier. When examining a relationship between brain and plasma individual concentrations for tramadol, T-M1, and T-M2 (Figure 2 (A)), strong positive correlations were shown for all these chemicals (r = 0.83–0.97, p < 0.001). Brain/plasma tramadol concentration ratios were consistent among treatments (Figure 2 (B)) (p = 0.86), showing a dose-independent manner. These concentration ratios (min–max: 2.2–7.5) were similar to those reported for rodents (min–max: 1.3–7.3). The similarity in brain/plasma tramadol concentration ratios between fish and rodents supports the species-extrapolation and predictive pharmacology/toxicology approaches. On the other hand, dose-dependent increases in brain/plasma concentration ratios were observed for both T-M1 (p = 0.010) and T-M2 (p = 0.0001). The reason is unclear, but metabolism of tramadol into T-M1 and T-M2 might be induced in the brain of higher dose groups due to higher tramadol concentrations in the brain. As another possible reason, the protein-unbound (free) T-M1 and T-M2 in plasma, which can penetrate the blood-brain barrier, might increase by the reduction of plasma protein binding sites available for these metabolites because of the increase in plasma tramadol concentrations. However, our fish plasma concentration data represents the total (both protein-bound and unbound) tramadol levels. Measurement of free tramadol, T-M1, and T-M2 in fish plasma will be needed to verify whether or not high dose of tramadol can increase amount of free T-M1 and T-M2. Interestingly, brain/plasma T-M1 concentration ratios (min–max: 0.59–5.9) were greater than those reported for rodents (min–max: 0.23–1.3).

**A Comparison of Fish Metabolic Data with Mammalian Data.** A comparison of...
metabolites/tramadol concentration ratios in plasma of fish with literature values reported in plasma of various species including human beings, rodents, cats, and dogs is shown in Table S5 (Supporting Information) and Figure 3 (A). T-M1/tramadol concentration ratios, which were 0.087 ± 0.028 (mean ± SD, n = 16) in plasma of fish exposed to 98 µg L⁻¹ of tramadol, were 2–6 times lower than literature values (0.17–0.52) reported in general humans⁶⁷–⁷⁰. Meanwhile, the T-M2/tramadol concentration ratio, which was 0.16 ± 0.031 (mean ± SD, n = 16) in plasma of fish exposed to 98 µg L⁻¹ of tramadol, was quite similar to literature values (0.074–0.14) reported in humans⁶⁸–⁷⁰. Interestingly, T-M1/tramadol concentration ratios measured in fish are comparable to those previously reported in a human who was classified as a CYP 2D6 poor metabolizer⁶⁷.

Although fathead minnows are able to metabolize tramadol as humans do, the apparently slower metabolism of tramadol into the active metabolite T-M1 indicates that this species of fish is less capable of metabolising tramadol into T-M1 than most humans; this might result in decreased analgesic efficacy of the drug. A recent publication⁷¹ has shown that T-M2 levels greater than T-M1 levels were present in the brain of zebrafish (Danio rerio) after administration of a single intramuscular dose of tramadol, supporting the results of our study, although that paper is not concerned with the environmental impact of pharmaceuticals, nor the relevance of drug metabolism to any potential impact. As shown in Table S5 and Figure 3 (A), the metabolites/tramadol concentration ratios are significantly variable among animal species; differences in the T-M1/tramadol concentration ratio occur not only between fish and humans, but also between different mammalian species. In rodents, high metabolic rates of tramadol into T-M1 compared with those in humans have been reported,⁶⁵ whereas in dogs, horses, and donkeys, T-M1 seemed to be a relatively minor metabolite.⁷²–⁷⁴ These animals produce less T-M1 and more T-M2, as fathead minnows do. Differences in metabolism of tramadol between different aquatic organisms as well as fish species can be a question of future interest.

A comparison of norfluoxetine (N-desmethyl fluoxetine)/fluoxetine concentration ratios in plasma of fish with literature values reported in plasma of fish, rodents, and human beings is shown...
in Table S6 (Supporting Information) and Figure 3 (B). In our previous study\textsuperscript{40}, in which fathead minnows were exposed to fluoxetine at water concentrations ranging from 0.1 to 64 µg L\textsuperscript{-1}, we found a change in the slope of the linear regression between water and fish plasma concentrations when water concentrations exceeded 16 µg L\textsuperscript{-1}. This variation in slope occurred simultaneously with the decrease of norfluoxetine/fluoxetine concentration ratios. Those results were also confirmed in the present study (Figure S1, Supporting Information), and are likely due to the saturation and/or inhibition of the enzymatic system involved in fluoxetine metabolism. Such a process has also been well documented in both humans and rodents at similar plasma concentrations.\textsuperscript{32,75–78} In clinical studies and \textit{in vivo} rodent studies, norfluoxetine/fluoxetine concentration ratios were approximately 1.0 at therapeutic plasma concentration ranges (120–500 ng mL\textsuperscript{-1})\textsuperscript{33}, showing that concentrations of circulating fluoxetine and norfluoxetine are mostly in the same range\textsuperscript{75–77,79}. On the other hand, norfluoxetine/fluoxetine concentration ratios for fathead minnows were 3.0 ± 1.1 when plasma fluoxetine levels were 390 ± 240 ng mL\textsuperscript{-1}. Nakamura and coworkers\textsuperscript{41} previously observed norfluoxetine/fluoxetine ratios between 2.2 and 8.5 for Japanese medaka (\textit{Oryzias latipes}) exposed to fluoxetine at 14–15 µg L\textsuperscript{-1} water for 30 days at water pH of 7–9. From these results, it is possible to hypothesise that fish are able to transform fluoxetine into norfluoxetine more efficiently than humans do, as suggested by the higher norfluoxetine/fluoxetine ratios.

In humans, tramadol primarily undergoes CYP 2D6-catalyzed \textit{O}-demethylation to the active metabolite T-M1, and CYP 2B6 and 3A4-catalyzed \textit{N}-demethylation to inactive metabolite T-M2. T-M1 and T-M2 are further metabolized to the following metabolites: \textit{N}, \textit{N}-didesmethyl tramadol, \textit{N}, \textit{N}, \textit{O}-tridesmethyl tramadol, and \textit{N}, \textit{O}-desmethyl tramadol. All these metabolites are finally conjugated with glucuronic acid and sulfate to be excreted by the kidneys.\textsuperscript{30} In the case of fluoxetine, CYP 2D6, 2C9, 2C19, and 3A4 are responsible for \textit{N}-demethylation of fluoxetine in humans; beside, fluoxetine undergoes direct conjugation with glucuronic acid.\textsuperscript{32,78,80} Due to an apparent deficiency of 2B, 2C, and 2D homologues of CYPs in fish, it is plausible to hypothesise
that other fish-specific CYP 2 subfamilies (e.g., CYP 2K and 2Y) are involved in the metabolism of tramadol and fluoxetine in fish. It is also important to consider that 47 CYP2 genes were identified in zebrafish, in contrast to 16 in humans. The quantitative functional properties of those isoforms remain largely unknown; nonetheless, their characterization remains an important research task for the future, as this information would dramatically increase the accuracy and predictive power of pharmacokinetic models for fish species. So far, one possible interpretation of the metabolite/parent concentration ratios obtained for tramadol in the present study is that CYP 2 subfamilies-catalyzed \( O \)-demethylation occurs slower than CYP 3 subfamilies-catalyzed \( N \)-demethylation in fish.

The comparison of metabolite/parent concentration ratios in different animals requires a note of caution because of differences in induction/inhibition/saturation dynamics of metabolic enzymes. Additionally, the results discussed here were obtained using fish exposed to the drug via water. This type of administration route results in sustained levels of the drug in the blood for the duration of the experiment, as drug uptake via the gills is continuous. This exposure scenario is to some extent different than drug administration in humans and other mammal species, which typically occur with lower intra-day frequency, resulting in plasma concentrations that display more pronounced oscillatory dynamics than in fish. At this stage, we do not know the exact quantitative implications of these different exposure dynamics on the metabolic capabilities between fish and mammal species.

Tramadol is a pro-drug that requires metabolic activation to become a pharmacologically active molecule (i.e., \( \mu \)-opioid receptor agonist). Unless fish are able to metabolise the parent tramadol into T-M1, the opioid receptor-mediated effects (e.g., sedative and analgesic effects) on fish would not be observed. Considering the fact that approximately 10% of all approved small molecular drugs on the global market are classified as pro-drugs, it is scientifically worthwhile for the environmental risk assessment to understand drug metabolism in aquatic organisms such as fish. In addition, it is meaningful to measure the concentrations of these active metabolites as well as parent inactive substances in the environment. So far, only limited data are available on the
occurrence and fate of active metabolites in the aquatic environment. There is, for example, a small amount of data on tramadol, which reported T-M1/tramadol concentration ratios ranging from 0.1 to 2.9 in WWTP effluents in Germany.\textsuperscript{21}

In conclusion, the main finding of this study is that the teleost fish fathead minnow metabolises tramadol in a similar manner to humans and other mammalian species, and that concentration ratios of T-M1 to tramadol observed in the fish were comparable to the lower range of values previously reported in humans, and much lower than the values previously found in mouse, rat, and cat, which may be highly relevant when attempting to predict the environmental risk of this compound. The presence of T-M1 in fish suggest that the opioid receptor-mediated effects (e.g., sedative and analgesic effects) of tramadol would occur in fish once internal T-M1 concentrations are high enough to produce these effects. It is therefore likely that other opioids administered as pro-drugs, such as codeine and oxycodone, will be effective in fish, because in humans both have to be activated (\textit{O}-demethylated) into their active metabolites morphine and oxymorphine, respectively.

The amount of information available so far on drug metabolism in fish, although limited, supports the contention that fish metabolise human pharmaceuticals in the same way as humans and other mammalian species do. For example, the cardiovascular drug clofibric acid is metabolised by zebrafish embryos to at least 18 metabolites,\textsuperscript{82} the calcium channel blocker diltiazem is metabolised to at least 8 metabolites,\textsuperscript{83} and the anti-epileptic carbamazepine is metabolised to two or more metabolites,\textsuperscript{10} all of which have been identified in mammals administered these drugs. We are aware of only two studies to date in which metabolism of a pro-drug by fish has been studied. Both studies\textsuperscript{84,85} showed that the glucocorticoid pro-drug beclomethasone dipropionate is readily metabolised by fish to the active moieties beclometasone 17-monopropionate and beclomethasone, just as it is in humans and other mammalian species. Thus, if no evidence of the metabolism of a pro-drug is available to utilise in an environmental risk assessment, it seems reasonable to assume that fish will metabolise the pro-drug to the same active metabolites produced in mammals, as a worst-case assumption. This realisation strengthens the arguments for utilizing the read-across
hypothesis in the environmental risk assessment of pharmaceuticals.

ACKNOWLEDGMENTS

We would like to thank members of the Ecotoxicology Research Group, Brunel University London, particularly J. Walker, N. Brodigan, and A. Ferreira for fish husbandry, and T. Thrupp, E. Lawton, and A. Baynes for fish sampling. The research at Brunel University London was internally funded by the university. This study was also supported by the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT) to a project on Joint Usage/Research Center–Leading Academia in Marine and Environment Pollution Research (LaMer), and Research Fellowships from the Japan Society for the Promotion of Science (JSPS) for Young Scientists in Japan (PD) provided to R. Tanoue (26·2800), Grants-in-Aid (KAKENHI) for Scientific Research (A) (25257403), Scientific Research (A) (16H01784). This study was also funded by the Sasakawa Scientific Research Grant from The Japan Science Society.

ASSOCIATED CONTENT

Supporting Information

Additional tables (Tables S1–S6), figure (Figure S1), and text supporting sample extraction procedures, parameters for the instrumental analysis, quality assurance and quality control. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: rumi.tanoue.lw@gmail.com; tanoue.rumi.lw@ehime-u.ac.jp; Phone: +81 89 927 8174

Notes

The authors declare no competing financial interest.
REFERENCES


(8) Xie, Z.; Lu, G.; Liu, J.; Yan, Z.; Ma, B.; Zhang, Z.; Chen, W. Occurrence, bioaccumulation, and trophic magnification of pharmaceutically active compounds in Taihu Lake, China. 


(24) Rúa-Gómez, P. C.; Püttmann, W. Degradation of lidocaine, tramadol, venlafaxine and the
metabolites O-desmethyltramadol and O-desmethylvenlafaxine in surface waters.


(29) Li, Z.; Sobek, A.; Radke, M. Flume experiments to investigate the environmental fate of pharmaceuticals and their transformation products in streams. *Environmental Science and Technology* 2015, 49, 6009–6017.


(33) Schulz, M.; Iwersen-Bergmann, S.; Andresen, H.; Schmoldt, A. Therapeutic and toxic blood
concentrations of nearly 1,000 drugs and other xenobiotics. *Critical Care* 2012, 16, R136.


of pH on fluoxetine in Japanese medaka (Oryzias latipes): acute toxicity in fish larvae and

(42) Meredith-Williams, M.; Carter, L. J.; Fussell, R.; Raffaelli, D.; Ashauer, R.; Boxall, A. B. A.
Uptake and depuration of pharmaceuticals in aquatic invertebrates. Environmental Pollution

(43) Winter, M. J.; Lillicrap, A. D.; Caunter, J. E.; Schaffner, C.; Alder, A. C.; Ramil, M.; Ternes,
T. A.; Giltrow, E.; Sumpter, J. P.; Hutchinson, T. H. Defining the chronic impacts of atenolol
on embryo-larval development and reproduction in the fathead minnow (Pimephales

(44) Nallani, G.; Venables, B.; Constantine, L.; Huggett, D. Comparison of measured and
predicted bioconcentration estimates of pharmaceuticals in fish plasma and prediction of

Sumpter, J. P. Uptake of propranolol, a cardiovascular pharmaceutical, from water into fish
plasma and its effects on growth and organ biometry. Aquatic Toxicology 2009, 93, 217–224.

Prediction of Phospholipid–Water Partition Coefficients of Ionic Organic Chemicals Using
the Mechanistic Model COSMO mic. The Journal of Physical Chemistry B 2014, 118,
14833–14842.

(47) Escher, B. I.; Schwarzenbach, R. P. Evaluation of liposome - water partitioning of organic
acids and bases . 2 . Comparison of dxperimental determination methods. Environmental

(48) Smejtek, P.; Wang, S. Distribution of hydrophobic ionizable xenobiotics between water and
lipid membranes: Pentachlorophenol and pentachlorophenate. A comparison with
octanol-water partition. Archives of Environmental Contamination and Toxicology 1993, 25,


Therapeutic levels of levonorgestrel detected in blood plasma of fish: results from screening rainbow trout exposed to treated sewage effluents. *Environmental Science & Technology* **2010**, *44*, 2661–2666.


immediate release capsules administration in dogs. *Veterinary Research Communications* 2009, 33, 875–885.

(74) Giorgi, M.; Soldani, G.; Manera, C.; Ferrarini, P. L.; Sgorbini, M.; Saccomanni, G.

Pharmacokinetics of tramadol and its metabolites M1, M2 and M5 in horses following intravenous, immediate release (fasted/fed) and sustained release single dose administration. *Journal of Equine Veterinary Science* 2007, 27, 481–488.


Figure 1. Plasma concentrations and $BCF_{\text{plasma}}$ (fish plasma/water concentration ratios) of tramadol in the fathead minnow. (A) Time-course of tramadol concentrations (mean ± SD, $n = 4$) in plasma of fish exposed to 100 µg L$^{-1}$ of tramadol at water pH 8.1 (short-term treatment A) or 8.5 (short-term treatment B). Different letters above error bars denote significant differences between time periods ($p < 0.05$, one-way ANOVA followed by a post hoc Tukey HSD test). (B) Measured (red dashed line, median, $n = 16$; black dots, individuals) and predicted (dot-dot-dashed line and dot-dashed line) plasma concentrations of tramadol after 23–24 days exposure. The grey dot-dot-dashed line and dot-dashed line were calculated by using the highest and the lowest log $K_{ow}$, respectively; the yellow dot-dashed line was calculated by using the log $D_{lipw}$; the blue dot-dot-dashed line and dot-dashed line were calculated by using the highest and the lowest log $D_{ow}$, respectively (Table S4). The grey area indicates the human therapeutic plasma concentration (HTPC) range (100–300 ng mL$^{-1}$). (C) $BCF_{\text{plasma}}$ values of tramadol at water concentrations of 1 (TG-1), 10 (TG-10), and 100 (TG-100) µg L$^{-1}$. The box plots show 5th (lower whisker), 25th (bottom edge of box), 75th (top edge of box), and 95th (upper whisker) percentiles. The horizontal line in the box represents the median value. The small dots (○) are outliers. (D) pH-dependent measured (red dashed line, median; black dots, individuals) and predicted (dot-dot-dashed line and
dot-dashed line) BCF<sub>plasma</sub> of tramadol after 23–24 days or 24–72-h exposure test. The yellow
dot-dashed line was calculated by using the log $D_{lipw}$; the blue dot-dot-dashed line and dot-dashed
line were calculated by using the highest and the lowest log $D_{ow}$, respectively (Table S4).
Figure 2. Brain concentrations and brain/plasma concentration ratios of tramadol and its metabolites in the fathead minnow. (A) The relationship (Spearman's rank correlation coefficient) between brain and plasma individual fish concentrations of tramadol, T-M1, and T-M2. Y-axis: chemical concentrations in the brain, X-axis: chemical concentrations in the plasma. The dashed line represents an exact match between chemical levels in plasma and brain. (B) Brain/plasma concentration ratios of tramadol, T-M1, and T-M2 at water concentrations of 1 (TGf1), 10 (TGf10), and 100 (TGf100) µg L\(^{-1}\). The box plots show 5th (lower whisker), 25th (bottom edge of box), 75th (top edge of box), and 95th (upper whisker) percentiles. The horizontal line in the box represents median value. The small dots (○) are outliers. The ratios of T-M1 at water concentrations of 1 µg L\(^{-1}\) (TGf1) are not shown due to non-detectable concentrations in both plasma and brain.
Figure 3. Inter-species differences in drug metabolism. (A) *O*-desmethyl tramadol/tramadol concentration ratios in plasma of mouse, rat, cat, human, dog, and fish. Fish values were calculated by dividing individual plasma concentrations of *O*-desmethyl tramadol by individual plasma concentrations.
concentrations of tramadol in the present study. Mammalian values were estimated by dividing the mean plasma concentration of \( O \)-desmethyl tramadol by the mean plasma concentration of tramadol in the literature mentioned in Table S5 (Supporting Information). (B) Norfluoxetine (\( N \)-desmethyl fluoxetine)/fluoxetine concentration ratios in plasma of fish, rat, and human. Fish values were calculated by dividing individual plasma concentrations of norfluoxetine by individual plasma concentrations of fluoxetine in the present study. Mammalian values were estimated by dividing the mean plasma concentration of norfluoxetine by the mean plasma concentration of fluoxetine in the literature mentioned in Table S6 (Supporting Information).