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# Uptake and Metabolism of Human Pharmaceuticals by Fish — A Case Study with the Opioid Analgesic Tramadol

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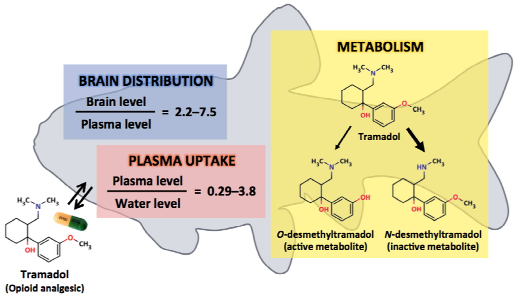
**ABSTRACT**

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.

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## 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.[1–4] Many of these pharmaceuticals can be found in aquatic organisms such as fish.[5–10] Due to the impracticality of testing all human pharmaceuticals[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,[13–17] and that the likelihood of effects is governed primarily by the presence or absence of the drug target.[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[13,19] is to be maximally useful in aiding the prediction of the effects of pharmaceuticals on non-target

100 species such as fish, drug metabolism in these species needs to be understood. This study was
101 designed to improve our knowledge of the uptake and metabolism of pharmaceuticals by fish
102 through studying how they metabolise the pro-drug tramadol.

103 Tramadol, which is an opioid analgesic, is widely used to treat moderate to severe pain, acting as
104 an agonist of μ-opioid receptors as well as being a serotonin and noradrenaline reuptake inhibitor.
105 As tramadol is only partially removed in conventional wastewater treatment plants (WWTPs),[20–23]
106 the compound has frequently been detected in surface waters around the world, at concentrations in
107 the range of ng $L^{-1}$ to low μg $L^{-1}$.[2,22,24,25] Some researchers have reported more than ten times
108 higher concentrations of tramadol in South Wales (United Kingdom) than other regions — the
109 highest concentrations were 98 μg $L^{-1}$ and 7.7 μg $L^{-1}$ in wastewater effluents[20] and surface waters[26],
110 respectively. Likewise, extremely high concentrations of tramadol ranging from 10 to 100 μg $L^{-1}$
111 were found in surface water from Cameroon.[27] In widely used biodegradability tests from the
112 OECD series, tramadol was characterized as a "not readily biodegradable" substance.[28] A
113 dissipation half-life of 49 days in degradation experiments with a bench-scale flume was previously
114 reported.[29]

115 The present study aimed at investigating the partitioning of tramadol between test water and fish
116 blood, and between blood and brain in a teleost fish, the fathead minnow (*Pimephales promelas*),
117 after 23−24 days of chronic waterborne exposure to tramadol. An additional aim was to investigate
118 tramadol metabolism in fish by measuring the active metabolite *O*-desmethyl tramadol (T-M1) and
119 the inactive metabolite *N*-desmethyl tramadol (T-M2), and to compare metabolism of tramadol in
120 fish and mammalian species. In humans, metabolism of tramadol is mainly mediated by
121 cytochrome P450 (CYP) 2D6, which transforms tramadol into the active metabolite T-M1, and by
122 CYP2B6 and CYP3A4, which transforms tramadol into the inactive metabolite T-M2.[30]
123 Considering the existing uncertainties around the evolutionary aspects and functional diversity of
124 these enzymes (especially CYP2 subfamilies) in teleost fish,[31] generating novel drug-metabolism
125 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.[32]

## 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 $L^{-1}$ (TG-1), 10 μg $L^{-1}$ (TG-10), and 100 μg $L^{-1}$ (TG-100) of tramadol, and 100 μg $L^{-1}$ of fluoxetine (FG-100), 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.[36] The original fish blood−water partition coefficient was described by the following equation[36]

$$P_{BW} = (10^{0.73\log Kow} \times 0.16) + 0.84 \quad (1)$$

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 ± 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.

$$BCF_{plasma} = (10^{0.73\log Kow} \times 0.12) + 0.88 \quad (2)$$

$$BCF_{plasma} = (10^{0.73\log D_{ow}} \times 0.12) + 0.88 \quad (3)$$

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

The pH-dependent $D_{ow}$ was calculated by

$$D_{ow} = f_{neutral} \times K_{ow\,(neutral)} + f_{ion} \times K_{ow\,(ion)} \quad (5)$$

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

$$f_{ion} = f_{neutral} \times 10^{|pH-pKa|} \quad (6)$$

It was assumed that log $K_{ow\,(ion)}$ is 3.5 log units lower than the corresponding log $K_{ow\,(neutral)}$.[52] 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 p*K*a value of 9.41 to predict $BCF_{plasma}$. Physicochemical properties of tramadol were summarized in Table S4 in the supporting information. The pH-dependent log $D_{lipw}$ was calculated by

$$D_{lipw} = f_{neutral} \times K_{lipw\,(neutral)} + f_{ion} \times K_{lipw\,(ion)} \quad (7)$$

where $f_{neutral}$ and $f_{ion}$ are the fractions of the neutral and ion species at the study pH, respectively, and $K_{lipw\,(neutral)}$ and $K_{lipw\,(ion)}$ are the respective $K_{lipw}$ values. The $K_{lipw\,(neutral)}$ was calculated with the PP-LFER equation[53] for log $K_{lipw\,(neutral,\,25^{\circ}C)}$ = 0.48 + 0.55L – 0.95S – 0.05A – 4.02B + 1.65V and chemical parameters taken from the UFZ-LSER database v 3.1[54]. The $K_{lipw\,(ion)}$ was calculated with COSMOmic extended for the description of charged organic chemicals via the implementation of the membrane bilayer potential.[37,46] Eventually, measured tramadol concentrations in plasma of fathead minnows were compared with the concentrations predicted by the $BCF_{plasma}$ 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 $BCF_{plasma}$ 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 $BCF_{plasma}$ 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 ± 3.9 μg $L^{-1}$ and 93 ± 4.4 μg $L^{-1}$ 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 $BCF_{plasma\ (24–72h)}$. Comparing $BCF_{plasma\ (24–72h)}$ 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 $BCF_{plasma\ (24–72h)}$ 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 p*K*a 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 $mL^{-1}$)[33]. 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

334 below the MDL value (0.14 ng $mL^{-1}$). Plasma T-M1 concentrations (3.8 ± 1.0 ng $mL^{-1}$) of fish
335 exposed to tramadol at 100 μg $L^{-1}$ were approximately 10 times lower than effective plasma T-M1
336 concentrations (40 ± 30 ng $mL^{-1}$) reported in humans[55]. Inactive metabolite T-M2 plasma
337 concentrations (mean ± SD, $n$ = 16) measured for TG-1, TG-10, and TG-100 treatment groups were
338 0.48 ± 0.21 ng $mL^{-1}$, 1.2 ± 0.43 ng $mL^{-1}$, and 7.2 ± 1.7 ng $mL^{-1}$, respectively.
339
340 **Measured vs. Predicted Fish Plasma Concentrations and $BCF_{plasma}$ of Tramadol.** Measured
341 plasma concentrations were compared with the concentrations predicted by the FPM (Figure 1 (B)).
342 When pH-dependent chemical lipophilicity ($D_{ow}$ or $D_{lipw}$) was used for the prediction, measured
343 median values were 2–6 times lower than predicted values. When using FPM for estimating the
344 potential risk of pharmaceuticals, an overestimated prediction would not be serious from the
345 viewpoint of precautionary principle. Nevertheless, the disagreement between measured and
346 predicted plasma tramadol concentrations in fathead minnows might be due to differences in the
347 existence form in the blood and/or hepatic clearances, between tramadol and PCBs. In fact, plasma
348 protein binding of tramadol in human was reported to be approximately 20%[56], while lipid-soluble
349 PCBs can be highly retained in the blood lipids. For the clearance of tramadol, we found its
350 metabolites T-M1 and T-M2, with the concentration ratios of tramadol: T-M1 + T-M2 = 4:1 in
351 plasma of fathead minnows. As it can be presumed that biotransformation of tramadol in fish
352 occurs much faster than PCBs, metabolism of tramadol by fathead minnow is likely involved in the
353 disagreement between measured and predicted plasma tramadol concentrations. Accounting for the
354 protein binding and metabolism, $BCF_{plasma}$ of tramadol can be predicted by

355 $$BCF_{plasma} = [(10^{0.73\log\alpha} \times 0.12 \times f_{bp}) + 0.88] \times f_{parent} \quad (8)$$

356 where α is $D_{ow}$ or $D_{lipw}$, the terms of 0.12 and 0.88 represent the organic and aqueous fractions in
357 fathead minnow whole blood, respectively, and $f_{bp}$ is the fraction bound to proteins (value
358 measured in human plasma: 0.20), $f_{parent}$ is the fraction of parent compound tramadol
359 (tramadol/tramadol + TM-1 + TM-2 concentration ratio in plasma of fathead minnow: 0.80). When

360 comparing measured tramadol concentrations in plasma of fathead minnow with the concentrations
361 predicted by the $BCF_{plasma}$ estimated using the equation (8), only 0.86−1.8 fold differences were
362 observed. In the present study, only 2 metabolites (i.e., TM-1 and TM-2) in fathead minnow were
363 measured and the $f_{parent}$ of 0.80 was applied as a provisional value. The actual value should be
364 lower than 0.80, because 23 metabolites of tramadol were previously identified in human urine.[57]
365 Additionally, several study have reported that 25–30% of an oral dose is excreted as unchanged
366 drug in the urine of human, whereas 55–60% of an oral dose is excreted as metabolites.[56]
367 Measured $BCF_{plasma}$ of tramadol for 23−24 days exposure are shown in Figure 1 (C). These
368 $BCF_{plasma}$ values, ranging from 0.29 to 1.6 for fathead minnows, were similar to or slightly lower
369 than those for rainbow trout (min–max: 2.3–3.3) exposed to treated wastewater in Sweden[58].
370 Measured $BCF_{plasma}$ values were the highest for TG-1 treatment group, followed by TG-10 and
371 TG-100 treatment groups. Combining results from preliminary short-term and 23−24 days chronic
372 experiments, it was found that $BCF_{plasma}$ values increased as the water pH increased (Figure 1 (D)).
373 The lowest (median $BCF_{plasma}$: 0.44) and the highest (median $BCF_{plasma}$: 1.8) values were found at
374 pH 7.6 and 8.5, respectively. Theoretically, 99% and 89% of tramadol are considered to be
375 positively charged at pH 7.6 and 8.5, respectively. Our result supports previous studies[5,38,42,59–61],
376 highlighting the importance of taking the water pH influence into account when $BCF_{plasma}$ of
377 ionisable chemicals are estimated. From the viewpoint of environmental risk assessment for basic
378 chemicals, using water-based threshold values from *in vivo* tests at only neutral water pH can lead
379 to underestimation of their actual risks in natural alkaline surface waters, as pointed out by Boström
380 et al. (2015)[62].
381
382 **Tramadol and Its Metabolite Concentrations in Fish Brain After 23−24 Days Exposure.** None
383 of the targeted chemicals were detected in any control (WDC and SC) samples. Tramadol brain
384 concentrations (mean ± SD, $n$ = 16) measured after the 23−24 days exposure for TG-1, TG-10, and
385 TG-100 treatment groups were 4.6 ± 1.4 ng $g^{-1}$, 26 ± 10 ng $g^{-1}$, and 200 ± 49 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.[63] 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)[64–66]. The similarity in brain/plasma tramadol concentration ratios between fish and rodents supports the species-extrapolation and predictive pharmacology/toxicology approaches[13]. 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)[64,65].

**A Comparison of Fish Metabolic Data with Mammalian Data.** A comparison of

412 metabolites/tramadol concentration ratios in plasma of fish with literature values reported in plasma
413 of various species including human beings, rodents, cats, and dogs is shown in Table S5
414 (Supporting Information) and Figure 3 (A). T-M1/tramadol concentration ratios, which were 0.087
415 ± 0.028 (mean ± SD, $n$ = 16) in plasma of fish exposed to 98 μg $L^{-1}$ of tramadol, were 2–6 times
416 lower than literature values (0.17–0.52) reported in general humans[67–70]. Meanwhile, the
417 T-M2/tramadol concentration ratio, which was 0.16 ± 0.031 (mean ± SD, $n$ = 16) in plasma of fish
418 exposed to 98 μg $L^{-1}$ of tramadol, was quite similar to literature values (0.074–0.14) reported in
419 humans[68–70]. Interestingly, T-M1/tramadol concentration ratios measured in fish are comparable to
420 those previously reported in a human who was classified as a CYP 2D6 poor metabolizer[67].
421 Although fathead minnows are able to metabolize tramadol as humans do, the apparently slower
422 metabolism of tramadol into the active metabolite T-M1 indicates that this species of fish is less
423 capable of metabolising tramadol into T-M1 than most humans; this might result in decreased
424 analgesic efficacy of the drug. A recent publication[71] has shown that T-M2 levels greater than T-M1
425 levels were present in the brain of zebrafish (*Danio rerio*) after administration of a single
426 intramuscular dose of tramadol, supporting the results of our study, although that paper is not
427 concerned with the environmental impact of pharmaceuticals, nor the relevance of drug metabolism
428 to any potential impact. As shown in Table S5 and Figure 3 (A), the metabolites/tramadol
429 concentration ratios are significantly variable among animal species; differences in the
430 T-M1/tramadol concentration ratio occur not only between fish and humans, but also between
431 different mammalian species. In rodents, high metabolic rates of tramadol into T-M1 compared
432 with those in humans have been reported,[65] whereas in dogs, horses, and donkeys, T-M1 seemed to
433 be a relatively minor metabolite.[72–74] These animals produce less T-M1 and more T-M2, as fathead
434 minnows do. Differences in metabolism of tramadol between different aquatic organisms as well as
435 fish species can be a question of future interest.

436 A comparison of norfluoxetine (*N*-desmethyl fluoxetine)/fluoxetine concentration ratios in
437 plasma of fish with literature values reported in plasma of fish, rodents, and human beings is shown

438 in Table S6 (Supporting Information) and Figure 3 (B). In our previous study[40], in which fathead
439 minnows were exposed to fluoxetine at water concentrations ranging from 0.1 to 64 μg $L^{-1}$, we
440 found a change in the slope of the linear regression between water and fish plasma concentrations
441 when water concentrations exceeded 16 μg $L^{-1}$. This variation in slope occurred simultaneously
442 with the decrease of norfluoxetine/fluoxetine concentration ratios. Those results were also
443 confirmed in the present study (Figure S1, Supporting Information), and are likely due to the
444 saturation and/or inhibition of the enzymatic system involved in fluoxetine metabolism. Such a
445 process has also been well documented in both humans and rodents at similar plasma
446 concentrations.[32,75–78] In clinical studies and *in vivo* rodent studies, norfluoxetine/fluoxetine
447 concentration ratios were approximately 1.0 at therapeutic plasma concentration ranges (120–500
448 ng $mL^{-1}$)[33], showing that concentrations of circulating fluoxetine and norfluoxetine are mostly in
449 the same range[75–77,79]. On the other hand, norfluoxetine/fluoxetine concentration ratios for fathead
450 minnows were 3.0 ± 1.1 when plasma fluoxetine levels were 390 ± 240 ng $mL^{-1}$. Nakamura and
451 coworkers[41] previously observed norfluoxetine/fluoxetine ratios between 2.2 and 8.5 for Japanese
452 medaka (*Oryzias latipes*) exposed to fluoxetine at 14–15 μg $L^{-1}$ water for 30 days at water pH of
453 7–9. From these results, it is possible to hypothesise that fish are able to transform fluoxetine into
454 norfluoxetine more efficiently than humans do, as suggested by the higher norfluoxetine/fluoxetine
455 ratios.

456 In humans, tramadol primarily undergoes CYP 2D6-catalyzed *O*-demethylation to the active
457 metabolite T-M1, and CYP 2B6 and 3A4-catalyzed *N*-demethylation to inactive metabolite T-M2.
458 T-M1 and T-M2 are further metabolized to the following metabolites: *N, N*-didesmethyl tramadol,
459 *N, N, O*-tridesmethyl tramadol, and *N, O*-desmethyl tramadol. All these metabolites are finally
460 conjugated with glucuronic acid and sulfate to be excreted by the kidneys.[30] In the case of
461 fluoxetine, CYP 2D6, 2C9, 2C19, and 3A4 are responsible for *N*-demethylation of fluoxetine in
462 humans; beside, fluoxetine undergoes direct conjugation with glucuronic acid.[32,78,80] Due to an
463 apparent deficiency of 2B, 2C, and 2D homologues of CYPs in fish, it is plausible to hypothesise

464 that other fish-specific CYP 2 subfamilies (e.g., CYP 2K and 2Y) are involved in the metabolism of
465 tramadol and fluoxetine in fish. It is also important to consider that 47 CYP2 genes were identified
466 in zebrafish, in contrast to 16 in humans.[31] The quantitative functional properties of those isoforms
467 remain largely unknown; nonetheless, their characterization remains an important research task for
468 the future, as this information would dramatically increase the accuracy and predictive power of
469 pharmacokinetic models for fish species. So far, one possible interpretation of the metabolite/parent
470 concentration ratios obtained for tramadol in the present study is that CYP 2 subfamilies-catalyzed
471 *O*-demethylation occurs slower than CYP 3 subfamilies-catalyzed *N*-demethylation in fish.
472 The comparison of metabolite/parent concentration ratios in different animals requires a note of
473 caution because of differences in induction/inhibition/saturation dynamics of metabolic enzymes.
474 Additionally, the results discussed here were obtained using fish exposed to the drug via water. This
475 type of administration route results in sustained levels of the drug in the blood for the duration of
476 the experiment, as drug uptake via the gills is continuous. This exposure scenario is to some extent
477 different than drug administration in humans and other mammal species, which typically occur with
478 lower intra-day frequency, resulting in plasma concentrations that display more pronounced
479 oscillatory dynamics than in fish. At this stage, we do not know the exact quantitative implications
480 of these different exposure dynamics on the metabolic capabilities between fish and mammal
481 species.
482 Tramadol is a pro-drug that requires metabolic activation to become a pharmacologically active
483 molecule (i.e., μ-opioid receptor agonist). Unless fish are able to metabolise the parent tramadol
484 into T-M1, the opioid receptor-mediated effects (e.g., sedative and analgesic effects) on fish would
485 not be observed. Considering the fact that approximately 10% of all approved small molecular
486 drugs on the global market are classified as pro-drugs,[81] it is scientifically worthwhile for the
487 environmental risk assessment to understand drug metabolism in aquatic organisms such as fish. In
488 addition, it is meaningful to measure the concentrations of these active metabolites as well as
489 parent inactive substances in the environment. So far, only limited data are available on the

490 occurrence and fate of active metabolites in the aquatic environment. There is, for example, a small
491 amount of data on tramadol, which reported T-M1/tramadol concentration ratios ranging from 0.1
492 to 2.9 in WWTP effluents in Germany.[21]

493 In conclusion, the main finding of this study is that the teleost fish fathead minnow metabolises
494 tramadol in a similar manner to humans and other mammalian species, and that concentration ratios
495 of T-M1 to tramadol observed in the fish were comparable to the lower range of values previously
496 reported in humans, and much lower than the values previously found in mouse, rat, and cat, which
497 may be highly relevant when attempting to predict the environmental risk of this compound. The
498 presence of T-M1 in fish suggest that the opioid receptor-mediated effects (e.g., sedative and
499 analgesic effects) of tramadol would occur in fish once internal T-M1 concentrations are high
500 enough to produce these effects. It is therefore likely that other opioids administered as pro-drugs,
501 such as codeine and oxycodone, will be effective in fish, because in humans both have to be
502 activated (*O*-demethylated) into their active metabolites morphine and oxymorphine, respectively.
503 The amount of information available so far on drug metabolism in fish, although limited, supports
504 the contention that fish metabolise human pharmaceuticals in the same way as humans and other
505 mammalian species do. For example, the cardiovascular drug clofibric acid is metabolised by
506 zebrafish embryos to at least 18 metabolites,[82] the calcium channel blocker diltiazem is metabolised
507 to at least 8 metabolites,[83] and the anti-epileptic carbamazepine is metabolised to two or more
508 metabolites,[10] all of which have been identified in mammals administered these drugs. We are
509 aware of only two studies to date in which metabolism of a pro-drug by fish has been studied. Both
510 studies[84,85] showed that the glucocorticoid pro-drug beclomethasone dipropionate is readily
511 metabolised by fish to the active moieties beclomethasone 17-monopropionate and beclomethasone,
512 just as it is in humans and other mammalian species. Thus, if no evidence of the metabolism of a
513 pro-drug is available to utilise in an environmental risk assessment, it seems reasonable to assume
514 that fish will metabolise the pro-drug to the same active metabolites produced in mammals, as a
515 worst-case assumption. This realisation strengthens the arguments for utilizing the read-across

hypothesis[13,19] in the environmental risk assessment of pharmaceuticals.

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## 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.

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### Notes

The authors declare no competing financial interest.

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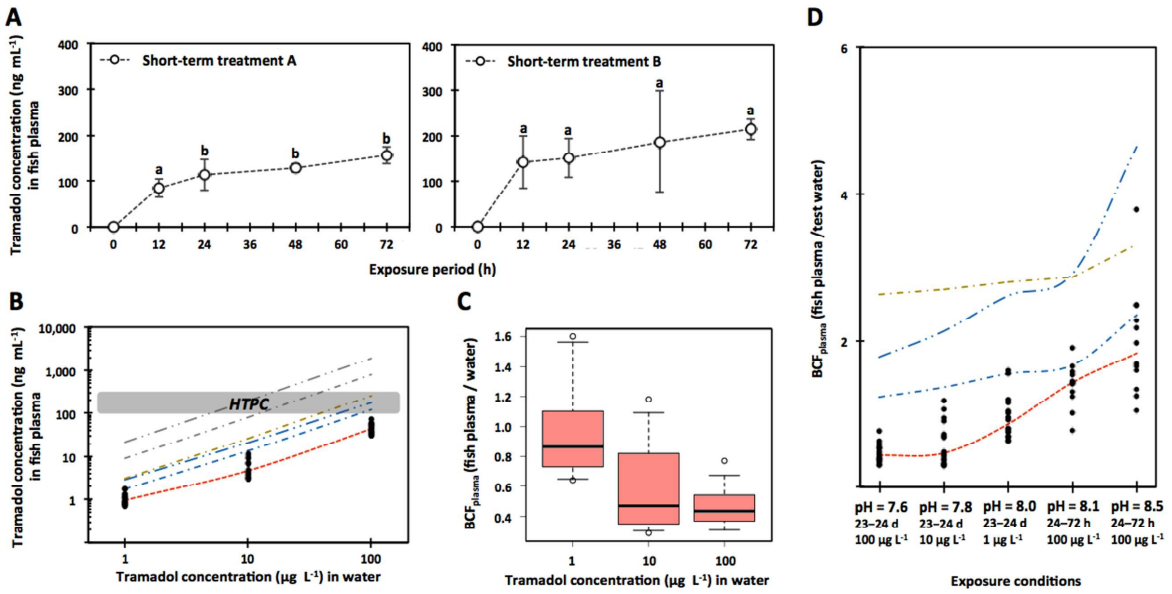
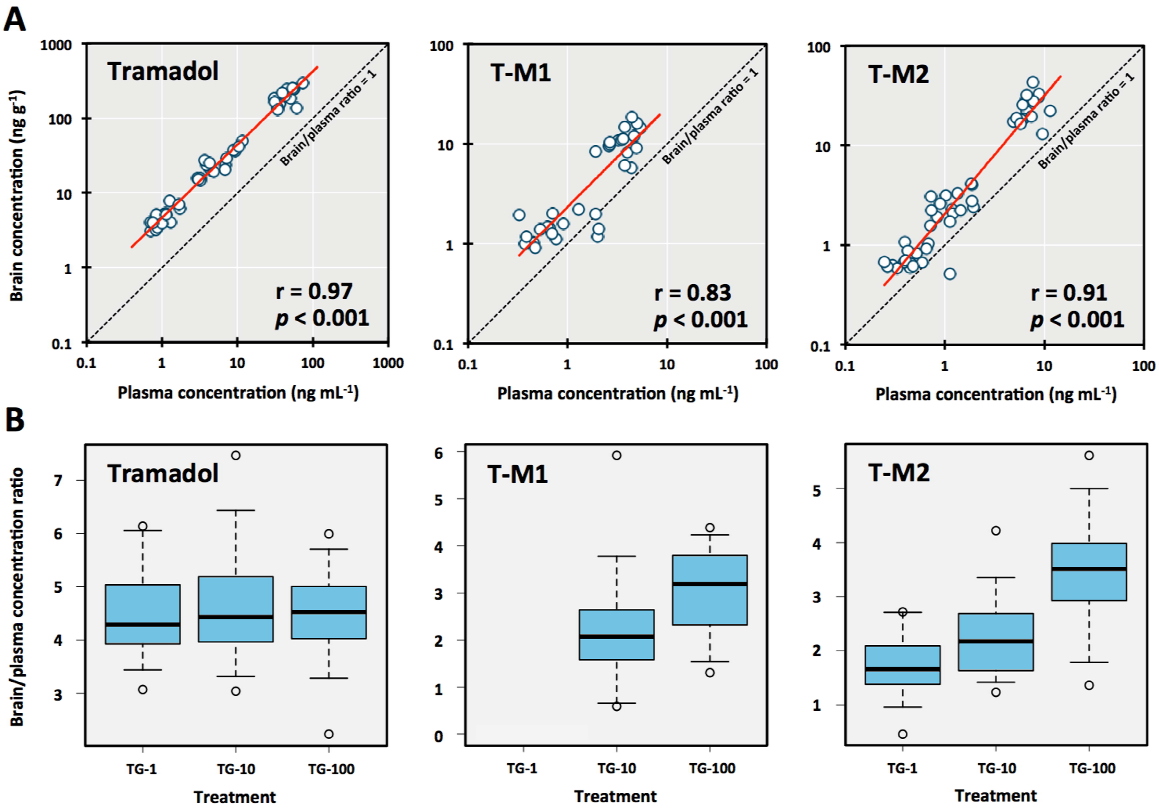



822

823 **Figure 1. Plasma concentrations and $BCF_{plasma}$ (fish plasma/water concentration ratios) of**
824 **tramadol in the fathead minnow.** (A) Time-course of tramadol concentrations (mean ± SD, $n$ = 4)
825 in plasma of fish exposed to 100 µg $L^{-1}$ of tramadol at water pH 8.1 (short-term treatment A) or 8.5
826 (short-term treatment B). Different letters above error bars denote significant differences between
827 time periods ($p < 0.05$, one-way ANOVA followed by a post hoc Tukey HSD test). (B) Measured
828 (red dashed line, median, $n$ = 16; black dots, individuals) and predicted (dot-dot-dashed line and
829 dot-dashed line) plasma concentrations of tramadol after 23−24 days exposure. The grey
830 dot-dot-dashed line and dot-dashed line were calculated by using the highest and the lowest log $K_{ow}$,
831 respectively; the yellow dot-dashed line was calculated by using the log $D_{lipw}$; the blue
832 dot-dot-dashed line and dot-dashed line were calculated by using the highest and the lowest log $D_{ow}$,
833 respectively (Table S4). The grey area indicates the human therapeutic plasma concentration
834 (HTPC) range (100–300 ng $mL^{-1}$). (C) $BCF_{plasma}$ values of tramadol at water concentrations of 1
835 (TG-1), 10 (TG-10), and 100 (TG-100) µg $L^{-1}$. The box plots show 5th (lower whisker), 25th
836 (bottom edge of box), 75th (top edge of box), and 95th (upper whisker) percentiles. The horizontal
837 line in the box represents the median value. The small dots (○) are outliers. (D) pH-dependent
838 measured (red dashed line, median; black dots, individuals) and predicted (dot-dot-dashed line and

dot-dashed line) $BCF_{plasma}$ 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).




865

866 **Figure 2. Brain concentrations and brain/plasma concentration ratios of tramadol and its**
867 **metabolites in the fathead minnow.** (A) The relationship (Spearman's rank correlation coefficient)
868 between brain and plasma individual fish concentrations of tramadol, T-M1, and T-M2. Y-axis:
869 chemical concentrations in the brain, X-axis: chemical concentrations in the plasma. The dashed
870 line represents an exact match between chemical levels in plasma and brain. (B) Brain/plasma
871 concentration ratios of tramadol, T-M1, and T-M2 at water concentrations of 1 (TG-1), 10 (TG-10),
872 and 100 (TG-100) µg $L^{-1}$. The box plots show 5th (lower whisker), 25th (bottom edge of box), 75th
873 (top edge of box), and 95th (upper whisker) percentiles. The horizontal line in the box represents
874 median value. The small dots (○) are outliers. The ratios of T-M1 at water concentrations of 1 µg
875 $L^{-1}$ (TG-1) are not shown due to non-detectable concentrations in both plasma and brain.

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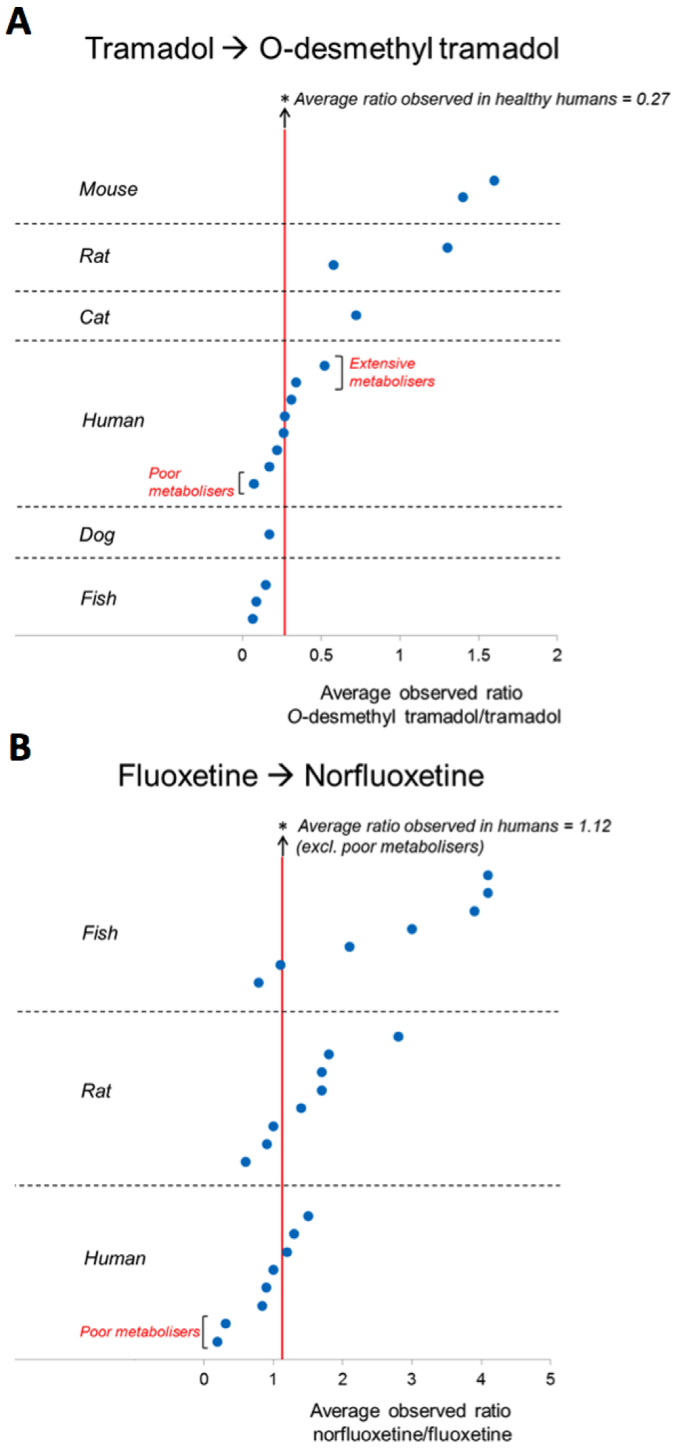


878

879 **Figure 3. Inter-species differences in drug metabolism.** (A) *O*-desmethyl tramadol/tramadol

880 concentration ratios in plasma of mouse, rat, cat, human, dog, and fish. Fish values were calculated

881 by dividing individual plasma concentrations of *O*-desmethyl tramadol by individual plasma

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).