Hazard/Risk Assessment

Trout and Human Plasma Protein Binding of Selected Pharmaceuticals Informs the Fish Plasma Model

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Abstract: Concerns are increasing that pharmaceuticals released into the environment pose a risk to nontarget organism such as fish. The fish plasma model is a read-across approach that uses human therapeutic blood plasma concentrations for estimating likely effects in fish. However, the fish plasma model neglects differences in plasma protein binding between fish and humans. Because binding data for fish plasma are scarce, the binding of 12 active pharmaceutical ingredients (APIs; acidic, basic, and neutral) to rainbow trout (Oncorhynchus mykiss) and human plasma was measured using solid-phase microextraction (SPME). The plasma/water distribution ratios ($D_{\text{plasma/w}}$) of neutral and basic APIs were similar for trout and human plasma, differing by no more than a factor of 2.7 for a given API. For the acidic APIs, the $D_{\text{plasma/w}}$ values of trout plasma were much lower than for human plasma, by up to a factor of 71 for naproxen. The lower affinity of the acidic APIs to trout plasma compared with human plasma suggests that the bioavailability of these APIs is higher in trout. Read-across approaches like the fish plasma model should account for differences in plasma protein binding to avoid over- or underestimation of effects in fish. For the acidic APIs, the effect ratio of the fish plasma model would increase by a factor of 5 to 60 if the unbound plasma concentrations were used to calculate the effect ratio. Environ Toxicol Chem 2022;41:559–568. © 2020 The Authors. Environmental Toxicology and Chemistry published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Bioaccumulation; Bioavailability; Hazard/risk assessment; Pharmaceuticals; Predictive toxicology

INTRODUCTION

Pharmaceuticals are omnipresent in the environment. They predominantly enter the aquatic environment via wastewater of households or manufacturing sites and have been found in aquatic animals (Daughton and Brooks 2011). Concerns have risen in recent years that pharmaceuticals adversely impact nontarget species (e.g., fish) in the aquatic environment, because the pharmacological targets (receptors or enzymes) may be evolutionarily conserved in nontarget organisms (Gunnarsson et al. 2008; Verbruggen et al. 2018) and what is a beneficial effect in humans might lead to an adverse effect in nontarget species. Since 2006 in the European Union, approval of new pharmaceuticals requires an environmental risk assessment (European Medicines Agency 2006). For most of the legacy drugs that were registered before 2006, a full ecotoxicity data set is missing but there remains a need to address their environmental risk. Therefore, prioritization efforts for environmental risk assessment of pharmaceuticals or active pharmaceutical ingredients (APIs) have been geared toward identifying those APIs that are likely to pose the greatest risk.

On a more general level (beyond APIs), a recent workshop has explored the integration of environmental and human health risk assessment (Rivetti et al. 2020). Rivetti et al. (2020) suggested that identifying similarities and differences between biological pathways would offer opportunities for read-across between species. The analysis should address not only the level of the target and the cellular pathway to facilitate read-across of molecular initiating events and key events from humans to environmental species but also the physiological level, that is, consider differences in absorption, distribution, metabolism, and excretion (ADME) processes.

The focus of past research has been on target conservation, with ortholog predictions well advanced, especially for pharmaceuticals (Gunnarsson et al. 2008; Verbruggen et al. 2018), but also on commonalities in the toxicity pathway, as recently
reviewed in this journal (McArdle et al. 2020). Beminger et al. (2015) proposed a so-called Mammalian Pharmacokinetic Prioritization for Aquatic Species Targeting (MaPPFAST) database to prioritize APIs for ecotoxicity testing, and Gunnarsson et al. (2019) combined the prediction of orthologs with ADME parameters to predict hazards in fish.

The fish plasma model was one of the earliest and very simple read-across approaches; the model hypothesized that fish might be adversely affected if fish plasma concentrations were similar to the human therapeutic plasma concentrations leading to pharmacological effects in humans (Huggett et al. 2003; Rand-Weaver et al. 2013). Two experimental studies that exposed fathead minnow to setraline (Valenti et al. 2012) and fluoxetine (Margiotta-Casaluci et al. 2014) gave some support to this hypothesis.

Several key assumptions and simplifications were made in the fish plasma model: 1) uptake of the API into the fish was assumed to be a passive bioconcentration process, 2) the molecular targets were assumed to be common in humans and fish, and 3) the pharmacological responses in humans were assumed to be similar to adverse responses in fish.

Although various mechanistic models also exist for bioaccumulation in fish for ionizable APIs (Erickson et al. 2006; Armitage et al. 2013, 2017), the fish plasma model took a simplified approach, assuming the same internal aqueous concentrations as external aqueous concentrations; binding to blood proteins was assumed to be mainly driven by hydrophobicity, predicted simply by the octanol/water partition constant ($K_{ow}$) for neutral chemicals (Fitzsimmons et al. 2001). At a later stage, alternative fish bioaccumulation models were applied, and the $K_{ow}$ was corrected for ionization of the APIs (Schreiber et al. 2011). All these models neglected the importance of metabolism of APIs in humans (Lienert et al. 2007) and fish (Hutchinson et al. 2014).

Another important pharmacokinetic property that has been neglected in the fish plasma model and the more sophisticated read-across approach—mainly because of the lack of experimental data—is the difference in blood plasma protein binding between humans and fish. Binding to plasma proteins limits the freely dissolved and biologically effective concentrations of APIs. Species differences in plasma protein levels complicate the understanding of interspecies pharmacodynamic and toxicological effects. This has so far not been considered for effect assessment in nontarget species or even in environmental hazard/risk assessment.

Collections of experimental binding data and also predictive models are abundant for human plasma (Kratochwil et al. 2002; Fasano et al. 2005; Zhang et al. 2012), but only a few studies have measured binding to fish plasma (Schmieder and Henry 1988; Jarboe et al. 1993; Schultz et al. 2001; Reimschuessel et al. 2005; Fitzsimmons et al. 2009; Escher et al. 2011; Nichols et al. 2013). Serum albumin determines the free fraction of the majority of drugs in human plasma (Fanali et al. 2012), but binding to lipid components and glycoproteins can also contribute to plasma binding. Albumins are also present in the plasma of several fish species, including salmonids, but their structure is very diverse (Andreeva 2010). Rainbow trout (Oncorhynchus mykiss) plasma was found to contain 2 albumin-like proteins with a structure that is similar but not identical to that of human albumin (Maillou and Nimmo 1993).

The aim of the present study was to measure and compare, in rainbow trout and humans, the plasma/water partitioning and resulting unbound fractions of pharmaceuticals with different physicochemical properties, including neutral and ionic structures. Solid-phase microextraction (SPME) is an increasingly popular measurement technique for protein binding studies (Musteata et al. 2006; Peltenburg et al. 2015). For the experiments of the present study, C18-coated SPME fibers were used. Based on the experimentally determined plasma binding data, implications for the fish plasma model are discussed.

### MATERIALS AND METHODS

#### Materials

Solid-phase microextraction (SPME) LC fiber probes from Sigma-Aldrich with C18/PAN coating were used (catalog number 57281-U, coating thickness 45 µm, coating length 1.5 cm, calculated coating volume ~520 nL). Blunt cannulas from Braun (0.8 × 22 mm) were used to transfer the fibers to the vials containing the plasma samples. Rainbow trout (O. mykiss) plasma was kindly provided by R. Brown (University of Exeter, Exeter, UK). Plasma from different individuals was pooled and stored at −80 °C until use. Human plasma was purchased from Sigma-Aldrich (catalog number P9523). For the binding experiments, the plasma was diluted using phosphate-buffered saline (PBS; 137 mM NaCl, 12 mM phosphate, pH 7.4). Bovine serum albumin (Sigma-Aldrich, catalog number A7030), triolein (Sigma-Aldrich, catalog number T-7140), 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC; Avanti Polar Lipids, catalog number 850457), and cholesterol (Sigma-Aldrich, catalog number C8667) were used as standards for the protein and lipid determination. The 12 test APIs (Table 1) were purchased from different suppliers at the highest available purity (see the Supplemental Data, Table S1 for more details). The set of test APIs included 4 organic acids that are >99% anionic at pH 7.4 (diclofenac, ibuprofen, naproxen, and warfarin), 2 organic acids that are partly charged (genistein and torasemide), 2 organic bases that are >99% cationic (metoprolol, propranolol), 2 organic bases that are partly charged (venlafaxine, and diphenhydramine), and 2 neutral APIs (lamotrigine and caffeine).

#### Experimental procedure

**Protein and lipid content determination of trout and human plasma.** The protein content of trout and human plasma was determined with the Lowry method using standard solutions of bovine serum albumin as calibration (Lowry et al. 1951). The lipid content was measured using a modified sulfo-phosphovanillin method and 3 different lipid standards as calibration (triolein, POPC, and cholesterol). The protocols of both methods are described in detail in Fischer et al. (2017).
TABLE 1: Active pharmaceutical ingredients (APIs) and their acidity constants (pKₐ) and speciation in blood

| API            | Chemical class | Acidity constant pKₐ | Speciation at pH 7.4 |
|----------------|----------------|----------------------|----------------------|
| Diclofenac     | Acid           | 4.15                 | >99% Anionic         |
| Ibufrofen      | Acid           | 4.45                 | >99% Anionic         |
| Naproxen       | Acid           | 4.15                 | >99% Anionic         |
| Warfarin       | Acid           | 4.9                 | >99% Anionic         |
| Genistein      | Base           | 7.2, 10, 13.1       | 61% Anionic, 39% Cationic |
| Torasemide     | Acid           | 6.68                 | 84% Anionic, 16% Cationic |
| Metoprolol     | Base           | 9.68                 | >99% Cationic        |
| Propranolol    | Base           | 9.42                 | >99% Cationic        |
| Venlafaxine    | Base           | 8.49                 | 91% Cationic, 9% neutral |
| Diphenhydramine| Base           | 8.98                 | 97% Cationic, 3% neutral |
| Lamotrigine    | Neutral        | 5.34                 | >99% Neutral        |
| Caffeine       | Neutral        | —                    | 100% Neutral         |

*pPhysProp database accessed via EPISuite Ver 4.1.  
+Avdeef et al. 1998.  
Ottinger and Wunderli-Allenspach 1997.  
Zielonka et al. 2003.  
Maserel 1993.  
Newton and Klaua 1978.  
Predicted using the pKₐ GALAS tool of ACD/Labs 2015 release (Build 2726).  
Shishaba et al. 2002.

Plasma/water distribution ratios. Plasma/water distribution ratios (Dₚ/ₗ) were determined at pH 7.4, which is the pH of blood and is also fairly constant for fish (Eddy et al. 1977), even if the external pH of the water in which the fish live may cover a wider range (Escher et al. 2020). The SPME method described by Peltenburg et al. (2015) was applied according to the protocol of Henneberger et al. (2020) for C18-coated SPME fibers. Before each experiment the SPME fibers were conditioned according to the manufacturer’s recommendations in methanol (2 h) and subsequently in water (20 min). To avoid spiking of solvents to the plasma samples, methanol stock solutions of the APIs were spiked to PBS at a concentration level 10 times higher than the desired plasma concentration. An aliquot of 20 µL of the spiked PBS was mixed with 180 µL of plasma (undiluted or diluted in PBS), leading to a total volume of 200 µL and a maximum plasma content of 90% in the samples. The methanol content in the final samples was always <0.1%. For all experiments with trout plasma the plasma content of the samples was 90%. For human plasma the plasma content in the samples was 90% for metoprolol, propranolol, venlafaxine, diphenhydramine, lamotrigine, and caffeine. Because of the strong binding to human albumin, the plasma concentration was only 10% for the experiments with human plasma for the acidic APIs (diclofenac, ibuprofen, naproxen, warfarin, genistein, and torasemide).

Three to 12 replicates were prepared for each API and plasma type (see also Table 2). The spiked plasma samples were placed into amber glass high-performance LC (HPLC) vials with inserts, SPME fibers were added, and the samples were equilibrated 24 h at 250 rpm on an orbital shaker (Thermo Fisher Scientific MaxQ 2000) at room temperature (20 ± 2 °C) for the trout plasma samples and on an incubated orbital shaker (Thermo Fisher Scientific MaxQ 6000) at 37 ± 0.5 °C for the human plasma samples. The fibers were removed, and the test APIs were desorbed from the fibers in a HPLC vial with an insert containing 180 µL of desorption solution (250 rpm, 2 h). The concentrations of the APIs in the desorption solutions were determined by HPLC-UV or LC/tandem mass spectrometry. The type of desorption solution used for each API is listed in the Supplemental Data, Table S1. Control samples for the SPME containing only PBS without plasma were run in parallel. For the control samples, the concentrations of the APIs were measured in the PBS phase after SPME and in the desorption solution to derive the fiber/water distribution ratios of the APIs and to calculate the mass balance.

**Instrumental analysis**

The concentrations of the APIs in the desorption solutions of the SPME fibers and the PBS phase of the control samples were quantified using either an LC system (Agilent 1260 Infinity) equipped with a diode array (Agilent 1260) and a fluorescence detector (Agilent 1100/1200) or an LC system

**TABLE 2: Experimental plasma/water distribution ratios (log Dₚ/ₗ) at pH 7.4 of all active pharmaceutical ingredients (APIs) for trout and human plasma**

| API            | Trout plasma | Human plasma |
|----------------|--------------|--------------|
|                | T [°C]       | log Dₚ/ₗ     | SD  | n  | T [°C]       | log Dₚ/ₗ     | SD  | n  |
| Diclofenac [A] | 20           | 3.25         | 0.12| 12 | 37           | 3.87         | 0.11| 4  |
| Ibufrofen [A]  | 20           | 2.46         | 0.13| 12 | 37           | 3.60         | 0.06| 4  |
| Naproxen [A]   | 20           | 2.51         | 0.14| 12 | 37           | 4.36         | 0.08| 4  |
| Warfarin [A]   | 20           | 2.13         | 0.29| 8  | 37           | 3.35         | 0.21| 8  |
| Genistein [A]  | 20           | 2.77         | 0.12| 8  | 37           | 3.27         | 0.21| 8  |
| Torasemide [A] | 20           | 2.31         | 0.18| 4  | 37           | 3.55         | 0.25| 4  |
| Metoprolol [B]| 20           | 2.00         | 0.39| 4  | 37           | 1.16         | 0.21| 3  |
| Propranolol [B]| 20           | 2.19         | 0.07| 4  | 37           | 2.35         | 0.02| 3  |
| Venlafaxine [B]| 20           | 2.27         | 0.13| 4  | 37           | 1.84         | 0.06| 3  |
| Diphenhydramine[B]| 20    | 2.02         | 0.19| 4  | 37           | 2.12         | 0.07| 3  |
| Lamotrigine [N]| 20           | 1.72         | 0.07| 4  | 37           | 1.74         | 0.05| 3  |
| Caffeine [N]   | 20           | 1.02         | 0.52| 4  | 37           | 1.42         | 0.30| 4  |

API = active pharmaceutical ingredient; SD = standard deviation; [A] = acidic API; [B] = basic API; [N] = neutral API.
PBS and in the desorption solution. Calibration solutions were prepared for the individual APIs in can be found in the Supplemental Data, Tables S2 and S3.

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The fiber/water distribution ratios ($D_{fw}$) of the APIs were derived from the control samples without plasma by dividing the fiber concentration ($C_f$) by the measured concentration in the PBS phase after SPME ($C_w$, Equation 1).

$$D_{fw} = \frac{C_f}{C_w} \quad (1)$$

The concentrations of the APIs in the SPME fibers ($C_f$) were calculated from the measured concentrations in the desorption solutions ($C_{des}$), the volume of the desorption solution ($V_{des}$), and the volume of the SPME fiber coating ($V_f = 520 \text{nL}$) by Equation 2:

$$C_f = \frac{C_{des} \times V_{des}}{V_f} \quad (2)$$

The plasma/water distribution ratios ($D_{plasma/w}$) of the APIs at pH 7.4 were derived by Equation 3 from $D_{fw}$, $V_f$ the total amount of API added to the vial ($m_{total}$), the amount of API in the SPME fiber ($n_i$), the volume of water in the plasma sample ($V_{w,plasma}$), and the total mass of proteins and lipids in the plasma sample ($m_{prot+lip}$).

$$D_{plasma/w}(\text{pH 7.4}) = \frac{L_w}{k_d \times m_{prot+lip}}$$

where $m_{prot+lip}$ is the mass of proteins and lipids in plasma.

$$= \frac{n_i \times D_{fw} \times V_f - V_w - V_f 	imes D_{fw}}{m_{prot+lip}} \quad (3)$$

The unbound fractions ($f_{unbound}$) of the APIs in plasma were calculated from the measured $D_{plasma/w}$ and the ratio of total mass of proteins and lipids in the plasma ($m_{prot+lip,plasma}$) to the volume of water in the plasma ($V_{w,plasma}$) using Equation 4.

$$f_{unbound} = 1 - f_{bound} = \left(1 + D_{plasma/w} \times \frac{m_{prot+lip,plasma}}{V_{w,plasma}}\right)^{-1} \quad (4)$$

### RESULTS AND DISCUSSION

#### Protein and lipid content of trout and human plasma

The total lipid content of both plasma types was similar, whereas the total protein content of the trout plasma was lower than that of human plasma (Table 3). The measured total protein and lipid content of trout plasma was in the range of published data. Protein contents of 41.2 g/L (Escher et al. 2011) and 35.9 g/L (Manera and Britti 2006) and lipid contents of 18.4 g/L (Escher et al. 2011) and 14 g/L (Bertelsen et al. 1998) were previously reported for rainbow trout. The determined protein content of human plasma was lower, whereas the lipid content was slightly higher than the reference composition of human plasma (protein 65–72 g/L and lipid 4.5–12.6 g/L; International Commission on Radiological Protection 1975).

The differences in lipid and protein contents of trout or human plasma may stem from true biological variability but could also be due to methodological differences. Lipid content is typically quantified gravimetrically after solvent extraction, and the recovery is determined by the type and amount of solvent, which is often not reported. Instead of gravimetry, we used the sulfo-phosphovanillin method, which reacts to double bonds in lipids using 3 different types of lipids (triolein, POPC, and cholesterol) for calibration because we had limited quantities of trout plasma available and this method is more sensitive for small masses than gravimetry. For the partition constants and the fish plasma model, it is important that the methods for trout and human plasma be the same, which facilitates direct comparisons.

### Plasma/water distribution ratios

To allow a better comparison of trout and human plasma binding, the experimentally determined $D_{plasma/w}$ (pH 7.4) values were normalized to the total amount of proteins and lipids in the respective plasma ($m_{prot+lip}$) for trout and 62.81 g/L for human plasma; Equation 3).

The measured log $D_{plasma/w}$ for the 12 tested APIs ranged from 1.02 (caffeine) to 3.25 (diclofenac) for trout plasma and from 1.16 (metoprolol) to 4.36 (naproxen) for human plasma (Table 2). Higher $D_{plasma/w}$ values were found for acidic pharmaceuticals compared with neutral and basic pharmaceuticals for both plasma types.

The comparison of $D_{plasma/w}$ between trout and human plasma revealed that the majority of the neutral (caffeine and lamotrigine) and basic pharmaceuticals (propranolol, venlaxafine, and diphenhydramine) tested bound to trout and human plasma to a similar extent, with $D_{plasma/w}$ being within a factor of 2.7 (Figure 1A). Only metoprolol showed 6.9 times higher binding to trout plasma compared with human plasma. In contrast, the acidic pharmaceuticals (diclofenac, ibuprofen, naproxen, warfarin, genistein, and torasemide) bound more strongly to human plasma compared with trout plasma. The difference was smallest for diclofenac and genistein ($D_{plasma/w}$ within a factor of 4.2). The highest difference was measured for naproxen, for which $D_{plasma/w}$ was 70.8 times higher for human plasma than for trout plasma.

Physiologically relevant temperatures were chosen for the experiments with trout (20°C) and human plasma (37°C).

| TABLE 3: Protein and lipid content of trout and human plasma |
|-------------------------------------------------------------|
| Plasma | Protein content (g/L) | Lipid content (g/L) |
|--------|-----------------------|---------------------|
| Trout  | 39.69 ± 3.35          | 12.13 ± 0.53        |
| Human  | 49.54 ± 1.24          | 13.27 ± 0.42        |

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The observed differences in $D_{\text{plasma/w}}$ between the 2 types of plasma were most likely not caused by the difference in temperature, because the distribution between condensed phases shows little temperature dependence (Opperhuizen et al. 1988; Geisler et al. 2012). The higher binding of the acidic pharmaceuticals to human plasma compared with trout plasma rather suggests that the trout plasma proteins either do not exhibit the same high-affinity binding sites that are available for organic acids on human albumin (Fasano et al. 2005) or only offer a reduced number of binding sites.

**Unbound fractions in trout and human plasma**

The unbound and bound fractions of all APIs in human and trout plasma were calculated using Equation 4 (Supplemental Data, Table S4). The unbound fractions of neutral and basic pharmaceuticals were similar in both plasma types, whereas the unbound fraction of the acidic pharmaceuticals was considerably higher in trout plasma compared with human plasma (Figure 1B). When the fractions bound/unbound of individual APIs were compared between trout and human plasma (Figure 2), they were similar for neutral and cationic APIs, with the highest ratio $f_{\text{unbound(trout)}}/f_{\text{unbound(human)}}$ of 1.8 for caffeine and the lowest ratio of 0.31 for metoprolol, thus differing by a factor of <3, which is half a log unit, within the uncertainty of the log $D_{\text{plasma/w}}$ of caffeine, and only slightly higher than the standard deviation of $D_{\text{plasma/w}}$ of the other chemicals.

In contrast, the $f_{\text{unbound}}$ values of the acidic APIs were higher in trout plasma by a factor of 3.8 for genistein to 82-fold for naproxen (Figure 2). The bound fractions in human plasma determined in the present study were also compared with data from literature and agreed for the majority of the test chemicals (Supplemental Data, Table S4). Only for the basic APIs metoprolol and venlafaxine were the bound fractions considerably higher in our study (49 and 82%, respectively) compared with literature data (8 and 27%, respectively). This discrepancy might be caused by the fact that plasma binding of basic APIs

![FIGURE 1:](image1)

**FIGURE 1:** (A) Experimental plasma/water distribution ratios ($\log D_{\text{plasma/w}}$) of all active pharmaceutical ingredients (APIs) for trout and human plasma. Error bars are sometimes hidden by the data points. (B) Comparison of unbound fractions of the APIs in trout and human plasma. The solid lines indicate the 1:1 line, and the dashed lines a deviation from the 1:1 line by a factor of ±10.

![FIGURE 2:](image2)

**FIGURE 2:** Unbound and bound fractions of the active pharmaceutical ingredients (APIs) in trout and human plasma calculated by Equation 4; [A] acidic API, [B] basic API, [N] neutral API.
in human plasma is not only determined by albumin, which has a rather constant concentration in plasma, but also by α1-acid glycoprotein, for which the concentration in plasma is more variable (Fournier et al. 2000).

**Implications for the fish plasma model**

The core equation of the fish plasma model defining an effect ratio (ER) was proposed by Huggett et al. (2003). We define the ER as the inverse of the Huggett-ER, as the ratio of steady-state fish plasma concentration (FSSPC) to the human therapeutic plasma concentrations (HPC, Equation 5). We are using the inverse equation, so that an ER > 1 indicates that the FSSPC is higher than the HPC, i.e., that there might be a risk.

\[
ER = \frac{FSSPC}{HPC} \tag{5}
\]

The HPC can be found in published literature (Schulz et al. 2012; Berninger et al. 2015), and the FSSPC values were typically estimated from the environmental water concentration (Cw) using the partition constant between the arterial blood of the fish and the surrounding water phase (Kblood/w, Equation 6).

\[
FSSPC = K_{blood/w} \times C_w \tag{6}
\]

In Equation 6 it is implicitly assumed that the external aqueous concentration (Cw) is equal to the internal aqueous concentration in blood, meaning that uptake is passive and has reached steady state and no metabolism occurs in fish.

Equations 5 and 6 can be used in 2 ways: First, if both HPC and FSSPC are experimentally available, one can calculate the effect ratio. If effect ratio > 1, adverse effects can be expected, and the API should be prioritized for ecotoxicity testing. Second, if we know the HPC and we set effect ratio = 1, from which follows HPC = FSSPC, then Equation 6 can be used to calculate the surface water concentration, which is theoretically needed to reach plasma levels in fish that are similar to the HPC (i.e., plasma levels that may be critical for the fish; Fick et al. 2010; Gunnarsson et al. 2019).

The Kblood/w value had been initially estimated from the Kow for neutral compounds using Equation 7 from Fitzsimmons et al. (2001), which was expanded to ionizable APIs by replacing the Kow by the ionization-corrected octanol/water distribution ratio at the pH of the blood (Dw; pH 7.4; Schreiber et al. 2011).

\[
\log K_{blood/w} = 0.73 \times \log D_{w} (pH \, 7.4) - 0.88 \tag{7}
\]

Note that Kblood/w is not directly comparable with Dplasma/w (pH 7.4) of the present study because Kblood/w is the ratio between the total blood concentration and the water concentration and has the unit L/w/Lblood, whereas Dplasma/w (pH 7.4) values were normalized to the total amount of proteins and lipids in plasma and have the unit L/w/kgprot+lip. For comparison, Dplasma/w (pH 7.4) in units of L/w/Lplasma (the concentration ratio between the whole plasma including the water phase and a pure water phase) were calculated with Equation 8.

\[
D_{plasma/w} (pH \, 7.4) \frac{[w/L_{plasma}]}{[w/L_{plasma}]} = \frac{D_{plasma/w} [w/L_{plasma}][g]\text{prot+lip}}{[\text{prot+lip}]} + \frac{V_{w,plasma}}{V_{plasma}} \tag{8}
\]

Experimental Dplasma/w (pH 7.4) for trout plasma and Kblood/w predicted from Dw (pH 7.4) calculated with Equation 7 showed no correlation, and Kblood/w values were lower than Dplasma/w values for the majority of the APIs by up to a factor of 151 (for naproxen, Figure 3A, blue circles and Supplemental Data, Table S5). Even though the majority of the APIs are >99% charged at pH 7.4, Kblood/w values predicted from Kow of the neutral species of the APIs agreed much better with the experimental Dplasma/w (pH 7.4) for trout plasma, within a factor of...
TABLE 4: Logarithmic fish plasma/water distribution ratios (log $D_{\text{plasma/w}}$) pH 7.4 in the appropriate units of $[L_w/L_{\text{plasma}}]$ for the fish plasma model and comparison with experimental blood/water bioconcentration factors (BCF$_{\text{blood/w}}$) in fish from the literature.

| API          | Log $D_{\text{plasma/w}}$ $[L_w/L_{\text{plasma}}]$ human | Log $D_{\text{plasma/w}}$ $[L_w/L_{\text{plasma}}]$ fish | Log BCF$_{\text{blood/w}}$ $[L_w/L_{\text{blood}}]$ fish |
|--------------|----------------------------------------------------------|------------------------------------------------------------|-------------------------------------------------------------|
| Diclofenac   | 2.67                                                     | 1.97                                                      | 0.69–0.74$^a$, 0.40–0.43$^b$, 1.33$^c$, 0.65–0.79$^d$          |
| Ibuprofen    | 2.40                                                     | 1.20                                                      | 0.52–0.62$^e$, 1.05–1.11$^f$, −0.19–0.33$^g$, 0.66–2.49$^h$    |
| Naproxen     | 3.16                                                     | 1.25                                                      | 0.13–0.14$^i$, 0.63–0.98$^j$                                  |
| Warfarin     | 2.15                                                     | 0.90                                                      |                                                             |
| Genestin     | 2.07                                                     | 1.50                                                      |                                                             |
| Torasemide   | 2.35                                                     | 1.06                                                      |                                                             |
| Metoprolol   | 0.27                                                     | 0.79                                                      |                                                             |
| Propanolol   | 1.18                                                     | 0.95                                                      | 0.46–4.36$^k$, 0.19–1.00$^l$                                  |
| Venlaxifine  | 0.72                                                     | 1.03                                                      |                                                             |
| Diphenhydramine | 0.97                                                        | 0.81                                                      | 0.74–1.17$^m$, −0.03–0.23$^n$                                 |
| Lamotrigine  | 0.64                                                     | 0.57                                                      |                                                             |
| Caffeine     | 0.42                                                     | 0.18                                                      |                                                             |

*Rainbow trout (Oncorhynchus mykiss; Lahti et al. 2011).
†Zebrafish (Danio rerio; Chen et al. 2017).
‡Fathead minnow (Pimephales promelas; Bickley et al. 2017).
§Rainbow trout (Cuklev et al. 2011).
¶Catfish (Ictalurus punctatus; Nallani et al. 2011).
‖Fathead minnow (Pate et al. 2016).
¶¶Fathead minnow (Gilto et al. 2009).
††Rainbow trout (Owen et al. 2009).
||Male fathead minnow (Nichols et al. 2015).
**Zebrafish (Chen et al. 2017).
API = active pharmaceutical ingredient.

5.8 for all APIs except caffeine (factor of 12.8, Figure 3A, yellow squares and Supplemental Data, Table S5). The $D_{\text{ow}}$ (pH 7.4) is only suitable to predict partitioning to storage lipids. It underestimates partitioning to membrane lipids and binding to proteins, especially for anionic APIs (Escher and Schwarzenbach 2002). Despite the good agreement for the data of the present study, predicting log $K_{\text{blood/w}}$ of ionizable APIs based on log $K_{\text{ow}}$ is not recommended because it has no mechanistic background and could easily lead to wrong predictions.

We also compared the experimental $D_{\text{plasma/w}}$ (pH 7.4) $[L_w/L_{\text{plasma}}]$ with experimental blood/water bioconcentration factors (BCF$_{\text{blood/w}}$) in fish from the literature (Table 4). The BCF$_{\text{blood/w}}$ values measured in rainbow trout were more than a factor of 10 lower than the $D_{\text{plasma/w}}$ (pH 7.4) values for the anionic APIs diclofenac and naproxen but also for the cationic API propanolol (Figure 3B). Some other fish species showed better agreement, but for them there remains more uncertainty regarding blood composition. This poor agreement might also mean that the external aqueous concentration is not a sufficiently good measure for the internal aqueous concentration and that uptake, excretion, and metabolism would also need to be considered. Biotransformation of the APIs in particular will significantly lower their BCFs. The fish plasma model is generally not applicable to chemicals that are biotransformed, because the equilibrium assumption is no longer fulfilled.

The difference in the unbound fractions of APIs in humans and fish (Figures 1B and 2) should be considered in the fish plasma model. For instance, the unbound fractions in rainbow trout plasma and human plasma for diclofenac were 1% and 0.2%, respectively (Supplemental Data, Table S4).

Cuklev et al. (2011) analyzed effects of diclofenac in juvenile rainbow trout that were exposed for 14 d to environmentally relevant concentrations. The average concentrations of diclofenac in blood plasma (FSSPC$_{\text{total}}$) at 14 d of exposure were approximately 6.5, approximately 46.2, and 369.5 $\mu$g/L at water concentrations of 1.6, 11.5, and 81.5 $\mu$g/L, respectively (Table 5). Lahti et al. (2012) exposed 1-yr-old rainbow trout for 10 d in cages in rivers downstream of wastewater treatment plants in Finland. On average, the fish had accumulated 18 to 30 $\mu$g/L diclofenac in their blood, and the maximum FSSPC$_{\text{total}}$
was 41 µg/L (Table 5). Ibuprofen and naproxen had lower detected concentrations and slightly lower levels (Table 5). After the total plasma concentrations were converted to the free plasma concentrations with the $f_{\text{unbound}}$ listed in the Supplemental Data, Table S4 by Equation 9, the effect ratio was calculated with Equation 5 based on total concentrations (ER$_{\text{total}}$) and based on free concentrations (ER$_{\text{free}}$). The ER$_{\text{free}}$ increased by a factor of 5 to 64 compared with the ER$_{\text{total}}$ (Table 5). While the ER$_{\text{total}}$ ranged from 0.001 to 0.74 and risk would therefore be deemed negligible, the ER$_{\text{free}}$ was much closer to 1 for diclofenac.

$$C_{\text{plasma,free}} = f_{\text{unbound}} \times C_{\text{plasma,total}} \quad (9)$$

Interestingly, in rainbow trout, diclofenac total plasma levels similar and even below the HPC affected global hepatic gene expression, which has been confirmed as diclofenac’s pharmacological action (Cuklev et al. 2011).

**CONCLUSIONS**

The present study has demonstrated that plasma/water partitioning can be measured fairly easily using SPME even for ionized APIs. So instead of continuing to improve $K_{ow}$-based models for prediction of plasma binding, it might be worthwhile to expand the experimental database of $D_{\text{plasma/w}}$, especially for ionized APIs, which may facilitate the development of mechanistic prediction models. Plasma binding is, of course, only one aspect of fish bioaccumulation with the assumption of the same external and internal aqueous concentrations, falling short for ionizable APIs (Armitage et al. 2017), and ion-trapping mechanisms might have to be invoked to model uptake into aquatic organisms (Escher et al. 2020).

Differences in binding between human and trout plasma were minor for neutral and basic APIs if $D_{\text{plasma/w}}$ (pH 7.4) values were normalized to the total protein and lipid content of the plasma but also when they were expressed in [Lw/Lplasma]. For acidic APIs, the $D_{\text{plasma/w}}$ (pH 7.4) values were 5 to 80 times higher for human plasma compared with trout plasma, probably due to specific binding sites on human albumin that may not be present in trout albumin.

Future studies should aim to expand the experimental database for fish plasma protein binding, with the ultimate goal of developing a predictive model for fish plasma protein binding. Based on these data, read-across approaches like the fish plasma model should be further refined to account for the differences in binding between human and fish plasma. A better understanding of the observed differences between human and trout plasma may be achieved by comparing the amino acid sequences and tertiary structure of trout and human albumins or by performing binding studies with purified trout albumin. Furthermore, plasma binding should be measured for other fish species as well, especially those in which albumin-like proteins are absent in plasma such as zebrafish (Noël et al. 2010) or carp (De Smet et al. 1998). For these species the differences in plasma binding might be even more pronounced than for trout.

**Supplemental Data**—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.4934.

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