C18-coated solid phase microextraction fibers for the quantification of partitioning of organic acids to proteins, lipids and cells

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KEYWORDS

SPME, mass balance modeling, protein binding, liposomes, QIVIVE

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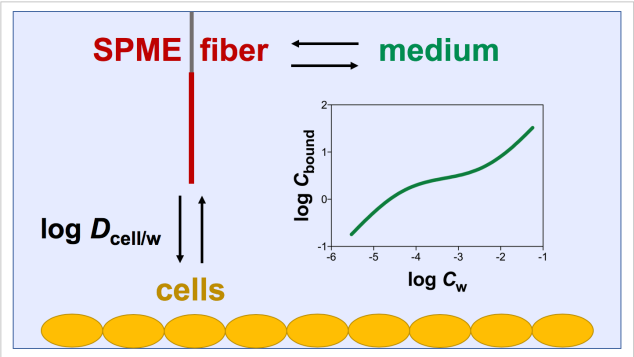

ABSTRACT

The effects measured with *in vitro* cell-based bioassays are typically reported as nominal effect concentrations (C_{nom}), but the freely dissolved concentration in the exposure medium (C_w) and the total cellular concentration (C_{cell}) are considered more quantitative dose metrics that allow extrapolation to the whole-organism level. To predict C_w and C_{cell}, the partitioning of the test chemicals to medium proteins and lipids and cells has to be known. In this study, we developed a solid phase microextraction (SPME) method based on C18-coated fibers to quantify the partitioning of diclofenac, 2,4-D, ibuprofen, naproxen, torasemide, warfarin, and genistein to bovine serum albumin (BSA), phospholipid liposomes, fetal bovine serum (FBS), and cells. For ibuprofen, 2,4-D, naproxen, and warfarin the partitioning to the SPME fibers was found to be concentration-dependent, which had to be considered for the calculation of distribution ratios to biological materials. The sorption isotherms to FBS were non-linear for diclofenac, 2,4-D, ibuprofen, naproxen, and warfarin. The FBS isotherms could be described by assuming that the total amount of chemical bound to FBS is the sum of the amount specifically bound to the binding sites of albumin and non-specifically bound to all medium proteins and lipids. The determined

cell-water distribution ratios ($D_{cell/w}$) differed considerably between four different cell lines (up to 1.83 log-units), but also between different batches of the same cell line (up to 0.48 log-units). The relative importance of protein and lipid content for $D_{cell/w}$ was evaluated with a mass balance model and different types of cellular proteins and lipids as input parameters. Existing *in vitro* mass balance models may underestimate C_w because they do not account for saturable protein binding, and overestimate C_{cell} for organic acids, if BSA is used as surrogate for cellular proteins.

1. Introduction

In vitro cell-based bioassays have great potential as an alternative test system to animal testing for chemical risk assessment. The freely dissolved concentration in the aqueous phase of the assay medium (C_w) and the total cellular concentration (C_{cell}) of the test chemicals have been proposed as suitable dose metrics for successful quantitative *in vitro* to *in vivo* extrapolation (QIVIVE).[1, 2] In QIVIVE models, C_w in the external exposure medium of the *in vitro* test system can be compared to C_w in the blood plasma of an organism, and C_{cell} can be compared to tissue concentrations. Total medium concentration and nominal concentration (C_{nom}) are less suitable because they highly depend on the composition of the medium and presence of binding phases. C_{nom}, the amount of chemical added to a defined volume of medium, can differ by orders of magnitude from C_w, which emphasizes the need for modelling and measurement techniques for C_w.[3] Mass balance models have been developed to estimate C_w and C_{cell} in various *in vitro* cell-based bioassays.[4-6] Likewise, for *in vivo* exposure assessment physiologically based toxicokinetic (PBTK) models can be used to calculate C_w of the test chemicals in blood plasma and the concentration in different organs and tissues.[7-9] These models rely on modelled or measured partitioning data to the different sorption phases as input parameters (i.e., proteins and lipids of cell culture media, cells and tissues). As biological materials are composed of numerous different

types of proteins and lipids, it is not possible to experimentally determine the partitioning to all sorption phases requiring the use of surrogates for simplification. Bovine serum albumin (BSA) is often applied as general surrogate for proteins.[10, 11] Phospholipid liposomes represent polar (membrane) lipids,[12] and octanol or triolein are suitable surrogates for non-polar (storage) lipids.[13] These surrogates usually give reasonable predictions for the partitioning of neutral organic chemicals to whole organisms and different tissues.[14]

Ionizable organic chemicals are of high environmental relevance, because many active pharmaceutical and consumer product ingredients and pesticides belong to this chemical class. They have been found in wastewater and surface water where they pose as much a risk to aquatic life as neutral chemicals.[15] The binding of ionizable organic chemicals to biological materials is generally more complex compared to neutral chemicals and cannot easily be predicted with available models. Organic acids were chosen as test chemicals for this study as they show a very distinct partitioning behavior, that is characterized by very strong and specific binding to serum albumin,[16, 17] but much lower affinities for other types of proteins[18] and for polar lipids.[19, 20] The partitioning of the ionized fraction of organic acids to non-polar lipids can probably be neglected because their partitioning into most solvents is negligible and their partition constants to octanol are usually more than two orders of magnitude lower than that of the corresponding neutral species.[21, 22]

For the experimental determination of partition constants to biological materials and C_w in *in vitro* bioassays solid phase microextraction (SPME) appears to be a suitable measurement technique as it requires only small sample volumes, reduces the use of solvents and removes biological matrices that disturb instrumental analysis.[23, 24] Previous studies have already successfully applied SPME to measure the partitioning of neutral organic chemicals to bovine

serum albumin,[25-27] structural proteins,[28] phospholipid liposomes,[29, 30] and cells and cell culture media.[4] As reviewed by Vuckovic,[31] SPME can be automated for high-throughput testing in multi-well plates. A prototype for a multifiber handling device in 96-well plate format for sample preparation prior to LC-MS analysis has already been described in the literature.[24] For the application of SPME to organic acids it is essential to identify a suitable sorption material, as ionic chemicals show very weak affinities for classical SPME coatings such as polydimethylsiloxane and polyacrylate.[30, 32, 33] Peltenburg et al. have developed a SPME method for organic bases based on "mixed-mode" C18/SCX SPME fibers that incorporate hydrophobic as well as ion exchange properties.[27, 34-36] By comparing the C18/SCX SPME fibers with commercially available C18 SPME fibers they identified hydrophobic interactions with the C18 material and adsorption to the high surface area of the C18 particles as possible sorption mechanisms.[34] Peltenburg et al. also observed strong binding of the organic acid diclofenac to the C18 SPME fibers, suggesting their use for sampling of organic acids. Because the fibers were also specifically designed for concentration measurements in biological samples, they were chosen for the experiments of this study. Other advantages of the C18 SPME fibers are that they are chemically and mechanically stable and biocompatible, which means that no fouling with proteins and lipids is expected.[37, 38]

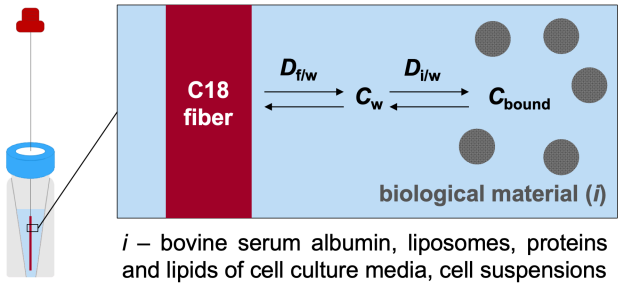

Figure 1. C18 SPME fiber experiments. C_w and C_{bound} are the freely dissolved and bound concentration of the test chemicals, $D_{f/w}$ and $D_{i/w}$ denote the distribution ratio between the fiber and water and between the tested biological material and water, respectively.

This study aimed at a better understanding of exposure of ionizable chemicals in cell-based bioassays. As the partitioning of organic acids is difficult to predict, we developed a SPME method for the measurement of partitioning of organic acids to diverse types of biological materials (Figure 1), that also has the potential for automation and direct measurements in *in vitro* cell-based bioassays. After characterizing the partitioning of seven organic acids between the C18 SPME fibers and water, the C18 SPME method was applied to quantify the partitioning of the test chemicals to BSA, phospholipid liposomes, cell culture basal media, and cell suspensions and to determine fetal bovine serum (FBS) binding isotherms. The derived binding data for cell culture medium components and cellular materials of this study were used to test the performance of a mass balance model for the prediction of *in vitro* exposure of organic acids.

2. Materials and methods

2.1 Materials

Solid phase microextraction LC fiber probes were purchased from Sigma-Aldrich (catalogue number 57281-U, all from the same batch). The fibers were made of nitinol wire (200 µm diameter)[35] and the coating consisted of C18 particles (5 µm, Discovery^{TM} C18) embedded in the biocompatible polymer polyacrylonitrile (PAN).[37, 38] The coating thickness was 45 µm, the coating length 1.5 cm, and the thickness of the nitinol wire 100 µm, leading to a calculated coating volume of approx. 520 nL. Blunt cannulas from Braun (0.8 × 22 mm) were used to safely transfer the fibers to the samples. Phosphate buffered saline (PBS, 137 mM NaCl, 12 mM phosphate) was used for the majority of the experiments. Additionally, two cell culture basal media were tested: OptiMEM (130 mM Na^+, 109 mM Cl^-, 0.76 mM phosphate, supplemented with different trace elements, fatty acids, insulin, transferrin and different vitamins) and DMEM GlutaMAX (110 mM NaCl, 0.9 mM phosphate, supplemented with amino acids, different vitamins, glucose, Phenol Red, pyruvate, and

25 mM HEPES buffer), both acquired from Gibco. To prevent changes of pH upon addition of the acidic test chemicals, 10 mM MOPS buffer (3-(N-Morpholino)propanesulfonic acid) from Roth was added to OptiMEM and DMEM GlutaMAX and the pH was adjusted to 7.4 using 10 N HCl or 2 N NaOH. BSA heat shock fraction, protease free, fatty acid free, essentially globulin free, pH 7, with a purity of ≥98 % was acquired from Sigma Aldrich (catalogue no. A7030), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) from Avanti Polar Lipids. Untreated FBS was purchased from Gibco (catalogue number 10099-141). The following cell lines were evaluated: MCF7 (with plasmid pcDNA3.1 and pGL-8xARE) obtained from Cancer Research UK, H4lle (H4L7.5c2 with AhR Luc reporter) obtained from Michael Denison, University of California, and HEK293T (GR-UAS-bla) and HEK293H (PPAR gamma-UAS-bla), both purchased from Thermo Fisher Scientific. The cell lines represent different mammalian species and common toxic endpoints. HEK293 and H4lle cells were also used in the Tox21 program.[39] Seven organic acids were chosen as test chemicals for this study that are toxicologically and environmentally relevant and cover different chemical classes, charged functional groups and speciation, including four carboxylic acids (diclofenac, 2,4-dichlorophenoxyacetic acid (2,4-D), ibuprofen, and naproxen) and the coumarin derivative warfarin, which are all >99 % charged at pH 7.4, as well as the pyridine sulfonylurea compound torasemide and the isoflavone genistein, which are 84 and 61 % ionized at pH 7.4, respectively. More information on all test chemicals (CAS-No., supplier, purity, pK_a value, speciation at pH 7.4, and chemical structure) are listed in the Supporting Information (SI, Table S1).

2.2 Experimental procedure

Fiber-water partitioning

The C18 SPME fibers were conditioned in methanol for at least two hours and at least 20 minutes in water before each experiment, following recommendations from the literature[37] and the

manufacturer of the fibers. Once conditioned, the fibers were quickly transferred to the samples to avoid drying of the coating, which decreased the sorption capacity of the fibers and increased the variability of the measured fiber concentrations in preliminary experiments (data not shown). The fibers were exposed to solutions of the test chemicals in PBS until equilibrium was reached (see Table 1 for equilibration times of the test chemicals) using an incubated orbital shaker from Thermo Fisher Scientific (MaxQ 6000), set to 250 rpm and 37°C. Subsequently, the fibers were transferred to HPLC vials with fused-in inserts containing 180 µL of desorption solution. A mixture of acetonitrile and water (90:10) was used for desorption of diclofenac and genistein, a mixture of methanol and water (50:50) for naproxen, 2,4-D, torasemide, warfarin, and ibuprofen. Complete desorption was achieved for all test chemicals by incubating the desorption vials for two hours on the orbital shaker (250 rpm and 37°C). After extraction, fibers were stored in a mixture of methanol and water (50:50) and re-used until the coating was visibly damaged.

The time to reach equilibrium between the C18 SPME fibers and water was determined for all test chemicals. Stock solutions of the chemicals were prepared in methanol and spiked to PBS (methanol content <1 %). Replicate samples of the test chemicals were prepared (volume 1 mL) and incubated for different durations between 15 min and 48 h. For each time point the fibers of two samples were extracted as described above. The concentrations of the test chemicals in the PBS samples (after SPME) and in the fiber extracts were analyzed by HPLC (see below). Control samples without fibers were always run in parallel. Inter-fiber reproducibility was checked by performing ten replicate extractions from PBS. Samples were prepared as described above and incubated until equilibrated. Chemical concentrations in the PBS samples (after SPME) and in the fiber extracts were analyzed. Similarly, for all test chemicals, sorption to the fibers was also measured for two cell culture basal media (OptiMEM and DMEM GlutaMAX, n = 3).

Partitioning to biological materials

In the experiments with the biological materials (BSA, liposomes, FBS, and cell suspensions) the samples were equilibrated 24 h to ensure equilibrium between all sorption phases. For the binding experiments with BSA, protein solutions were prepared in PBS using volumetric flasks. A BSA concentration of 10 g/L was used for all experiments. This BSA concentration was chosen to aim at a fraction bound of >20 % for all test chemicals, without saturating the protein (chemical:BSA <0.1 $mol_{chemical}/mol_{BSA}$). The BSA solutions were spiked with methanolic stock solutions of the chemicals (methanol content ≤1 %), transferred to 1.5mL-vials (volume 1 mL) and the C18 SPME fibers were inserted through the septum. After equilibrating the samples for 24 h at 250 rpm and 37°C, the fibers were extracted and the concentration in the fiber extracts was measured. POPC liposomes were prepared in PBS according to the protocol described by Kaiser et al.[40] To derive unilamellar vesicles, the liposome solutions were filtered using a syringe extruder (Avanti Polar Lipids) equipped with two 1 mL syringes and a polycarbonate membrane with a pore size of 0.1 µm. For the binding experiments liposome concentrations were used that resulted in a fraction bound of >20 % and still quantifiable concentrations in the SPME fibers: 5 g/L for diclofenac, torasemide, and genistein, 10 g/L for 2,4-D and naproxen, 15 g/L for warfarin and 10-15 g/L for ibuprofen. For each chemical three liposome suspension aliquots (volume 1 mL) were used and spiked with methanolic stocks of the chemicals at three different concentration levels. These solutions were pre-equilibrated at room temperature for 24 h using an orbital shaker from Thermo Fisher Scientific (MaxQ2000) set to 150 rpm. Subsequently, four aliquots (volume 200 µL) from each spiked liposome suspension were transferred to HPLC vials with fused-in inserts and the C18 SPME fibers were added. Two aliquots for each concentration level were equilibrated for 4 h and the other two for 24 h. The fibers were extracted as described previously and only the

fiber extracts were analyzed. For three chemicals $D_{lip/w}$ was additionally determined using equilibrium dialysis. The protocol for the dialysis experiments can be found in the SI, section S2.

Binding to FBS was measured for all chemicals at a single nominal concentration level using different FBS contents (1, 5, and 10 %) in PBS. Because the results from these experiments suggested non-linear binding to FBS for diclofenac, 2,4-D, ibuprofen, naproxen, and warfarin, binding isotherms to FBS were measured for all chemicals at a constant FBS concentration (10 %), varying the nominal concentration of the test chemicals by 1-2 orders of magnitude. All FBS samples were pre-equilibrated at room temperature for 24 h and with agitation of 150 rpm.

For the determination of cell-water distribution ratios no living cells were used to avoid experimental artefacts due to active kinetic processes like cellular uptake and biotransformation. A defined number of cells (approx. 2×10^7) was washed once with PBS, gathered in a 5 mL tube and centrifuged. The exact cell count was quantified with the automated cell counter CASY MODEL TT (Roche Innovatis, Reutlingen, Germany). The cell pellet was re-suspended in PBS and homogenized for 1 min by ultrasonic treatment (Sonoplus 2070, Bandelin, Berlin, Germany). The tubes were cooled on ice during this procedure. The cell suspensions were always used immediately for the SPME experiments. For each of the chemicals and cell lines tested, three aliquots of 180 µL of the cell suspensions were transferred to HPLC vials with fused-in insert and 20 µL of a solution of the test chemical in PBS. After pre-equilibrating the samples at room temperature for 24 h and an agitation of 150 rpm the SPME fibers were added. The samples were equilibrated again for 24 h at 37°C and the chemical concentrations were determined in the fiber extracts.

Because fiber-water distribution ratios ($D_{f/w}$) were found to be concentration-dependent for four of the seven test chemicals, control samples without biological material were prepared for each set

of experiments at fiber concentration levels similar to the samples containing biological material (BSA, liposomes, FBS, and cell suspensions). $D_{f/w}$ was determined from the control samples and used for the data evaluation (see below). The control samples were also used to check the mass balance. All experiments were performed with single chemicals and not with mixtures.

Instrumental analysis

The samples were analyzed using three different HPLC systems. For all test chemicals the majority of the samples was measured using a 1260 Infinity HPLC system from Agilent, equipped with a diode array and a fluorescence detector. For samples with low concentration levels a more sensitive measurement was required, either using a 1260 Infinity HPLC system from Agilent coupled to a mass spectrometer from ABSciex (QTrap 6500) or a Dionex HPLC UltiMate 3000 system coupled to a mass spectrometer from Thermo Scientific (LTQ Orbitrap XL). For the measurement of fiber sorption isotherms data points of UV and MS measurements were overlapping, indicating no difference between the two methods (Table S5, SI). More details on the instrumental analysis can be found in SI, section S3.

2.3 Data evaluation

Fiber-water partitioning

The measured concentrations in the fiber extracts (C_{ex}) and the volumes of desorption solution and fiber coating (V_{ex} and V_f, respectively) were used to calculate the concentration of the test chemicals in the SPME fiber (C_f):

$$C_f = \frac{C_{ex} \cdot V_{ex}}{V_f} \qquad \text{eq. 1}$$

The fiber uptake kinetics (i.e., the changes of the amount of chemical in the fiber (n_f) over time (t)) were fitted using eq. 2 for those chemicals where the aqueous concentration was negligibly depleted. Fit parameters were the amount of chemical in the fiber at equilibrium (n_f (eq)) and the apparent fiber uptake rate constant k_1.

$$n_f(t) = n_f(eq) \cdot (1 - e^{-k_1 \cdot t}) \quad \text{eq. 2}$$

The fitted n_f (eq) was also compared with n_f (t) at times where equilibrium had been attained and the values were consistent. Eq. 3 was used to fit the decrease of the amount of the chemical in the water phase (n_w (t)). Note that the rate constant in eq. 3 (k_2) is in principle the same as k_1, but not identical in this case as n_f (t) and n_w (t) were fitted separately.

$$n_w(t) = n_w(eq) + \left(n_w(t_0) - n_w(eq)\right) \cdot e^{-k_2 \cdot t} \quad \text{eq. 3}$$

The measured fiber sorption isotherms were fitted using the log-transformed Freundlich model (eq. 4) to check linearity of sorption isotherms. Freundlich coefficient and Freundlich exponent are indicated by K_{Fr} and n_{Fr}, respectively.

$$\log C_f = \log K_{Fr} + n_{Fr} \cdot \log C_w \quad \text{eq. 4}$$

The apparent fiber-water distribution ratio ($D_{f/w}$) at a constant nominal concentration in units of liter of water/liter of fiber (L_w/L_f) was calculated using eq. 5.

$$D_{f/w}(\text{pH } 7.4)[L_w/L_f] = \frac{C_f}{C_w} \quad \text{eq. 5}$$

Partitioning to biological materials

As described by Neale et al.,[41] the full mass balance (eq. 7) was used to calculate the distribution ratios between the tested biological materials and water ($D_{i/w}$, *i* refers to BSA, liposome, or the sum of proteins and lipids in the cell culture medium), because we could not assure negligible depletion conditions in all experiments. The full mass balance approach (eq. 6-8) does not require any restrains and is applicable under non-depletive and depletive conditions.

In eq. 7 C_{bound} is the concentration of the test chemical bound to the total amount of proteins and lipids in the solution, n_{tot} is the total amount of chemical added to the vial, V_w is the volume of water, and m_i is the total mass of proteins and lipids in the solution. n_{tot} is defined by eq. 6 as the

sum of the amount of the test chemical in water, in fiber, and bound to the proteins and lipids (n_{bound}).

$$n_{tot} = n_w + n_f + n_{bound} \quad \text{eq. 6}$$

$$D_{i/w}(\text{pH 7.4})\ [L_w/kg_i] = \frac{C_{bound}}{C_w} = \frac{\frac{n_{tot}}{n_f} \cdot D_{f/w} \cdot V_f - V_w - V_f \cdot D_{f/w}}{m_i} \quad \text{eq. 7}$$

The distribution ratio between the cells and water ($D_{cell/w}$) was based on the total cell volume and calculated according to eq. 8.

$$D_{cell/w}(\text{pH 7.4})\ [L_w/L_{cell}] = \frac{C_{cell}}{C_w} = \frac{\frac{n_{tot}}{n_f} \cdot D_{f/w} \cdot V_f - V_w - V_f \cdot D_{f/w}}{V_{cell}} \quad \text{eq. 8}$$

The concentration in the cells is indicated by C_{cell}. The total volume of the cells in the vial (V_{cell}) is required in eq. 8, but only the cell count of the suspensions before homogenization was known. Based on experimentally determined cellular volumes the average cell count of 1 L of cells was calculated for all cell lines of this study. This factor was used to convert the cell count of the cell suspensions to V_{cell}. For two cell lines cellular volumes were available from the literature[6] (HEK293T 1.38×10^{11} cells/L and HEK293H 3.48×10^{11} cells/L), for the other cell lines (MCF7 and H4lle) the water, protein and lipid contents were determined using the protocol described by Fischer et al.[6] to derive the cellular volume of 9.99×10^{10} and 2.83×10^{11} cells/L, respectively. The results are included in Table S6, SI.

FBS is a complex mixture of different proteins and lipids. We assumed that the amount of the test chemicals that is bound to the total amount of proteins and lipids in the FBS ($n_{bound,total}$) is the sum of the amount specifically bound to the high affinity binding sites of albumin (n_{prot}, number of binding sites is assumed to be 1)) and the amount nonspecifically bound to the total amount of proteins and lipids in the solution ($n_{nonspecific}$), eq. 9.

$$n_{bound,total} = n_{prot} + n_{nonspecific} \quad \text{eq. 9}$$

The concentrations of the chemical specifically bound to albumin (C_{prot}, in units of mmol/kg_{prot}) and nonspecifically bound ($C_{nonspecific}$, in units of mmol/$kg_{prot+lip}$) can be derived by dividing the amounts in the respective phases by the mass of proteins (m_{prot}) and the total mass of proteins and lipids in FBS ($m_{prot+lip}$), respectively (eq. 10 + 11). Protein and lipid content of untreated FBS (71.75 and 1.57 g/L, respectively) were taken from Fischer et al.[6] as FBS from the same batch was used in this study.

$$C_{\mathrm{prot}} = \frac{n_{\mathrm{prot}}}{m_{\mathrm{prot}}} \qquad \text{eq. 10}$$

$$C_{\mathrm{nonspecific}} = \frac{n_{\mathrm{nonspecific}}}{m_{\mathrm{prot+lip}}} \qquad \text{eq. 11}$$

By combining equation 9, 10, and 11, the total concentration bound to the proteins and lipids of FBS ($C_{bound,total}$) was defined as:

$$C_{\mathrm{bound,total}} = C_{\mathrm{prot}} \frac{m_{\mathrm{prot}}}{m_{\mathrm{prot+lip}}} + C_{\mathrm{nonspecific}} \qquad \text{eq. 12}$$

The nonspecific binding process was assumed to be a partitioning process with linear sorption isotherms and can be described with a single distribution ratio. Hence, $C_{nonspecific}$ was calculated by simply multiplying the distribution ratio between the total amount of proteins and lipids in FBS and water ($D_{prot+lip/w}$) with C_w (eq. 13). The protein is included in the calculation of non-specific binding again, because one protein molecule can bind more than one molecule of the test chemical.

$$C_{\mathrm{nonspecific}} = D_{\mathrm{prot+lip/w}} \cdot C_{\mathrm{w}} \qquad \text{eq. 13}$$

$D_{prot+lip/w}$ was derived by linear regression of the upper points of the FBS isotherm and subsequently C_{prot} was calculated by eq. 14.

$$C_{\mathrm{prot}} = \left(C_{\mathrm{bound,total}} - C_{\mathrm{nonspecific}}\right) \frac{m_{\mathrm{prot}}}{m_{\mathrm{prot+lip}}} \qquad \text{eq. 14}$$

If the FBS isotherm was lacking a sufficient number of data points at high concentration levels, which was the case for 2,4-D and warfarin, $D_{lip/w}$ was used as a proxy for $D_{prot+lip/w}$. The specific

binding to albumin is saturable, because the number of binding sites is limited, and is defined by eq. 15.

$$C_{\mathrm{prot}} = \frac{C_{\mathrm{prot,max}} \cdot C_{\mathrm{w}}}{K_{\mathrm{d}} + C_{\mathrm{w}}} \qquad \text{eq. 15}$$

The maximum concentration bound to albumin and the dissociation constant of the albumin-chemical complex, i.e. the inverse of the specific binding constant, are indicated by $C_{\mathrm{prot,max}}$ and K_{d}, respectively, and were derived by non-linear regression of the dataset for C_{prot}. Finally, $C_{\mathrm{bound,total}}$ can be calculated by eq. 16.

$$C_{\mathrm{bound,total}} = D_{\mathrm{prot+lip/w}} \cdot C_{\mathrm{w}} + \frac{C_{\mathrm{prot,max}} \cdot C_{\mathrm{w}}}{K_{\mathrm{d}} + C_{\mathrm{w}}} \cdot \frac{\mathrm{m_{prot}}}{\mathrm{m_{prot+lip}}} \qquad \text{eq. 16}$$

For chemicals that showed linear sorption to FBS (torasemide and genistein), the isotherms were fitted by linear regression.

For all experiments, the mass balance was calculated for the samples containing no biological material. Only experiments with a mass balance of 85-115 % were considered for the data evaluation.

3. Results and discussion

3.1 Kinetics of fiber-water partitioning

Equilibrium between the C18 SPME fibers and water was established within six hours for all test chemicals (Fig. 2 for diclofenac, Fig. S1, SI for all other test chemicals and Table 1). The short equilibration times are beneficial for direct measurements in *in vitro* cell-based bioassays, but the tolerance of living cells towards the applied shaking speed of this study (250 rpm) has to be evaluated in future studies.

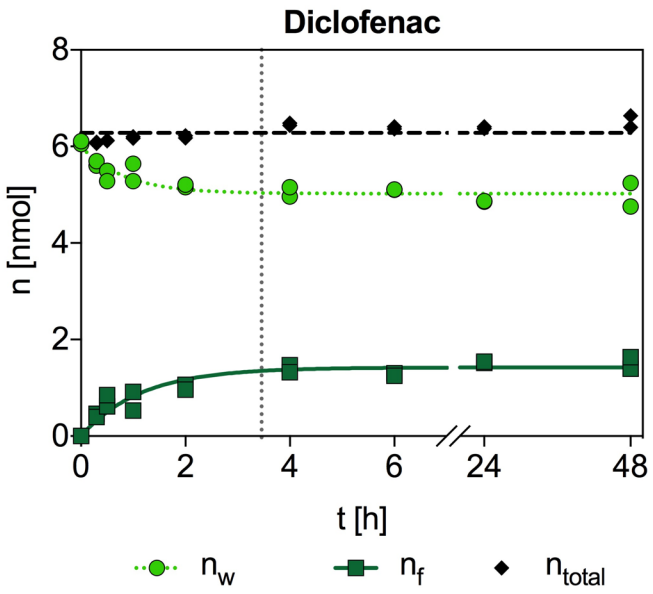

Figure 2. Fiber uptake kinetics of diclofenac. The dotted grey vertical line indicates the time until 95 % equilibrium was reached ($t_{95\%}$). The black diamonds are the sum of the amount of the chemical in the fiber (n_f) and in water (n_w) and can be compared with the dashed black horizontal line, which is the total amount of chemical added to the vial (nominal amount). The solid line is the fit with eq. 2, which is only applicable for constant aqueous concentrations, the dotted line is the fit with eq. 3.

3.2 Inter-fiber reproducibility and fiber sorption isotherms

The results from the inter-fiber reproducibility tests and the fitted parameters for the measured fiber sorption isotherms for the C18 SPME fibers can be found in Table 1. The fiber sorption isotherms of diclofenac and ibuprofen are shown in Fig. 3, the isotherms of the other test chemicals can be found in the SI, Figure S2.

Table 1. Equilibration times ($t_{95\%}$), logarithmic fiber-water distribution ratios (log $D_{f/w}$) calculated from the inter-fiber reproducibility experiments (n = 10) at a constant nominal concentration (C_{nom}) and fitting parameters of fiber sorption isotherms of the test chemicals determined in PBS at pH 7.4 and 37°C. Log $D_{f/w}$ are the arithmetic means of 10 experiments and SD is the standard

deviation. Fiber sorption isotherms were fitted using the log-transformed Freundlich model (eq. 4) and compared to linear sorption (n_{Fr} = 1) using the extra sum-of-squares F test. Significant deviation from linear sorption is indicated by a P value <0.05.

Chemical	$t_{95\%}$ [h]	log $D_{f/w}$ (pH 7.4) [L_w/L_f]	SD	C_{nom} [mmol/L]	Isotherm	P value	n_{Fr} [-]	log K_{Fr} [(mmol/L_f)·(mmol/L_w)$^{-nFr}$]
diclofenac	3.5	2.47	0.04	0.016	linear	0.1	0.96	2.53
2,4-D	0.3	1.50	0.06	0.009	non-linear	<0.0001	0.79	1.10
ibuprofen	0.8	2.52	0.13	0.009	non-linear	<0.0001	0.74	1.90
naproxen	1.5	2.23	0.06	0.009	non-linear	0.02	0.95	2.16
torasemide	2.4	3.16	0.06	0.006	linear	0.67	1.01	3.16
warfarin	6.0	1.96	0.05	0.006	non-linear	<0.0001	0.91	1.73
genistein	3.3	2.62	0.03	0.007	linear	0.05	0.95	2.51

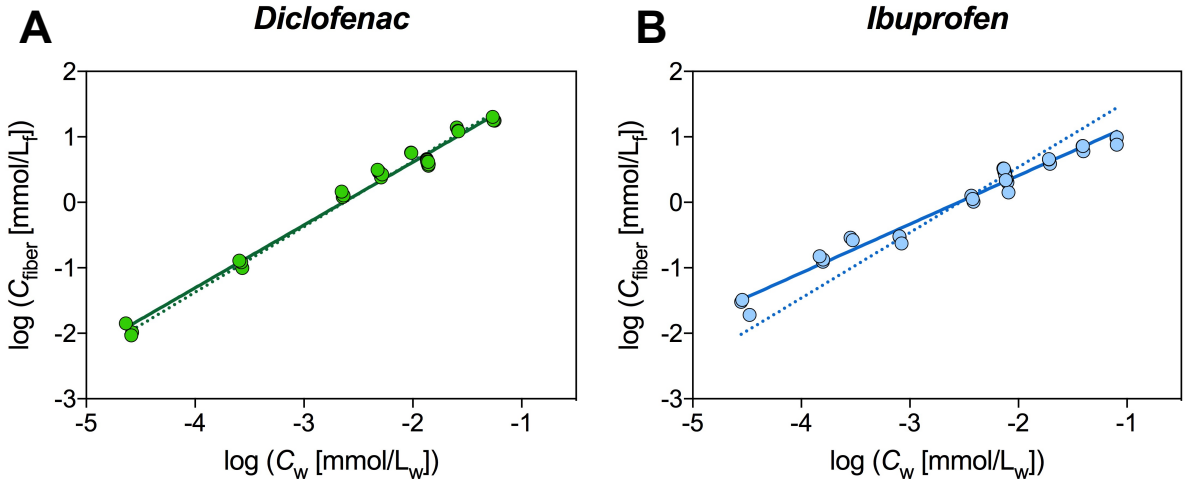

Figure 3. Fiber sorption isotherms of diclofenac and ibuprofen. The solid lines indicate the fitted Freundlich isotherms (eq. 4), the dotted lines show the fit with a fixed slope of 1 (eq. 4 with n_{Fr}=1).

The partitioning to the fibers was reproducible, if tested on a single concentration level, with standard deviations for $D_{f/w}$ ≤0.1 log-units except for ibuprofen (SD = 0.13 log-units) (Table 1). However, a significant decrease of $D_{f/w}$ with increasing concentration levels was measured for four test chemicals (2,4-D, ibuprofen, naproxen, and warfarin). Non-linear binding to C18-based SPME

fibers was already described by Peltenburg et al.[36] for the sorption of 19 organic bases to C18/SCX fibers. Musteata et al.[38] found a linear range of only two orders of magnitude for extraction of warfarin from PBS using C18 SPME fibers. The fiber sorption isotherms were therefore fitted using the log-transformed Freundlich model (eq. 4). The slope of the regression line is equal to the Freundlich exponent (n_{Fr}). Linear (i.e., concentration independent) sorption is indicated by $n_{Fr} = 1$. The extra sum-of-squares F test was performed to determine whether the slope of the measured fiber sorption isotherms was significantly different from one, indicated by a P value <0.05. For diclofenac (Fig. 3A), torasemide, and genistein, (both Fig. S2, SI) the slope of the regression line was close to one and linear sorption can be assumed for the tested concentration range. For ibuprofen (Fig. 3B), 2,4-D, naproxen, and warfarin (Fig. S2, SI) the isotherms were non-linear. Hence, $D_{f/w}$ is not only a function of pH, but also of the chemical concentration, which has to be considered for the evaluation of the binding data for biological materials. Consequently, the value for $D_{f/w}$ used for the calculation of $D_{i/w}$ and $D_{cell/w}$ (eq. 7 and 8, respectively), had to be derived at fiber concentration levels similar to the fiber concentrations in the samples with biological materials to account for the concentration dependence of $D_{f/w}$. Hence, $D_{f/w}$ was always measured in parallel at similar C_w in the following experiments.

3.3 Partitioning to bovine serum albumin and phospholipid liposomes

Distribution ratios to BSA were successfully determined for all test chemicals with standard deviations ≤0.11 log-unit. All data for $D_{BSA/w}$ of this study were determined at low chemical concentrations (chemical:BSA was always below 0.1 $mol_{chemical}/mol_{BSA}$), well below saturation of the specific binding sites of BSA. The measured values for $D_{BSA/w}$ and corresponding binding data from the literature are listed in Table 2. Although literature data were not available for diclofenac, torasemide, and genistein and data for human serum albumin had to be used for the comparison,

the derived data for binding to BSA of this study generally agreed well with the values from the literature, with differences of ≤0.21 log-units.

Table 2. Experimental logarithmic BSA-water (log $D_{BSA/w}$ exp.) and liposome-water distribution ratios (log $D_{lip/w}$ exp.) for the test chemicals of this study determined in PBS at pH 7.4 and 37°C and comparison with available literature data for serum albumin and liposome partitioning ($D_{SA/w}$ literature and $D_{lip/w}$ literature, respectively).

Chemical	log $D_{BSA/w}$ SPME [L_w/L_{BSA}]	SD	log $D_{SA/w}$ literature [L_w/L_{SA}]	Ref	log $D_{lip/w}$ SPME [L_w/L_{lip}]	SD	log $D_{lip/w}$ literature [L_w/L_{lip}]	Ref
diclofenac	4.40	0.08	4.20 [a]	42	3.26	0.24	2.64	20
2,4-D	3.56	0.02	3.41 [b]	43	2.02	0.16	-	-
ibuprofen	4.02	0.09	4.04 [b]	44	2.77	0.65	1.81	20
naproxen	5.21	0.11	5.36 [a]	45	2.17	0.08	-	-
torasemide	3.81	0.07	3.61 [c]	46	3.21	0.37	2.05±0.05[d]	-
warfarin	3.46	0.08	3.67 [a]	47	1.62	0.30	1.40	48
genistein	2.83	0.03	2.68 [a]	49	3.32	0.10	-	-

[a] calculated from binding constant

[b] converted from [L_w/kg_{SA}] to [L_w/L_{SA}] using a protein density of 1.36 kg/L

[c] calculated from serum protein binding in %

[d] determined using equilibrium dialysis

Liposome-water partitioning has linear isotherms for organic ions up to concentration ranges where electrostatic interactions come into play.[50] In this study, $D_{lip/w}$ was determined at three different concentration levels. No difference between the different concentration levels was found (for details see Table S7, SI) and all data were combined for the calculation of $D_{lip/w}$ (Table 2). Unfortunately, $D_{lip/w}$ could be obtained only for four test chemicals (2,4-D, naproxen, warfarin, and genistein) with the C18 SPME method. The standard deviations are higher compared to the results for BSA (up to 0.3 log-units). For 2,4-D, naproxen, and warfarin the variability of the samples equilibrated for only four hours was considerably higher compared to the samples

equilibrated for 24 h, suggesting that equilibrium with the liposomes was not reached within four hours and only the data for 24 h were used for the calculation of $D_{lip/w}$ in Table 2. For diclofenac, ibuprofen, and torasemide, the results showed very high variabilities of up to 0.7 log-units that were independent of the chemical concentration and equilibration time. The determined $D_{lip/w}$ values for diclofenac and ibuprofen were also up to one order of magnitude higher than the literature data. This means, that the measured fiber concentrations and consequently the calculated C_w were lower than expected. The low fiber concentrations can be explained by reduced affinity of the chemicals to the C18 SPME fibers. Possibly, POPC monomers can sorb to the surface of the C18 SPME fibers blocking the binding sites for organic acids (fiber fouling). Fiber fouling would also explain the higher variability for the other acidic chemicals. Musteata et al.[38] studied the matrix effect of human plasma for the C18 SPME fibers used in this study by measuring the amount of nitrogen and sulfur on the fiber surface with X-ray photoelectron spectroscopy after exposure to plasma samples. They found no significant increase in the N and S content after exposure to plasma and concluded no matrix effect. Hence, we do not expect matrix effects for the BSA and FBS samples (comparable to plasma), but cannot exclude them for liposomes and cell suspensions.

Another explanation for the high variability could be that the method used for liposome preparation did produce multilamellar instead of unilamellar vesicles. To test this hypothesis $D_{lip/w}$ of diclofenac, ibuprofen, and torasemide were additionally determined by equilibrium dialysis as described in SI section 2. For diclofenac and ibuprofen, the determined log $D_{lip/w}$ of 2.8 and 1.7 are similar to the literature values, indicating that the liposomes were prepared correctly. Because reliable data for $D_{lip/w}$ are required for the mass balance modelling of the binding to cells and media, the literature values were used for diclofenac and ibuprofen and $D_{lip/w}$ determined in this

study by equilibrium dialysis for torasemide. At this stage, we do not recommend to determine $D_{lip/w}$ with the C18 SPME method, because one cannot exclude experimental artefacts due to fiber fouling by POPC. An improved cleaning of the surface prior to extraction might improve the reliability of the measurements with liposomes.

3.4 Partitioning to cell culture medium components

The complete composition of commercial cell culture basal media is not reported by the suppliers, but they are generally known to be supplemented with different trace elements, vitamins, amino acids, and fatty acids. Since the C18 SPME method is intended to be used for the direct measurement of C_w in *in vitro* bioassays, we evaluated if the basal media without FBS supplement used in such assays would influence the partitioning to the C18 SPME fibers.

For the majority of the test chemicals the values for $D_{f/w}$ determined in PBS, OptiMEM and DMEM agreed within 0.5 log-units, except for torasemide and genistein for which differences were up to 0.7 log-units (Fig. 4 and Table S8, SI). For all chemicals except diclofenac the sorption to the fibers was weaker in OptiMEM and DMEM compared to PBS.

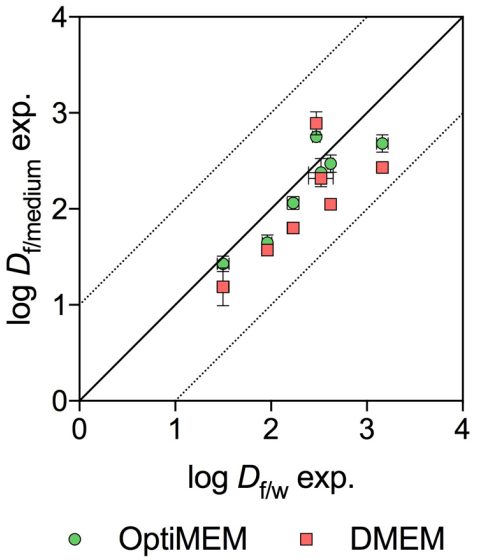

Figure 4. Comparison of measured fiber-water distribution ratios ($D_{f/w}$) in PBS and fiber-medium distribution ratios ($D_{f/medium}$) determined using two cell culture basal media: OptiMEM (green circles), DMEM (red squares) for all test chemicals, the solid line indicates the 1:1 line, the dotted lines a difference of 1 log-unit.

OptiMEM and DMEM have been reported to contain small but detectable amounts of proteins and lipids.[6] If the test chemicals bind to these proteins and lipids, the distribution ratio between the fiber and the basal media ($D_{f/medium}$) will be lower than $D_{f/w}$. The observed differences between $D_{f/w}$ and $D_{f/medium}$ were much smaller than expected from the measured protein and lipid contents.[6] However, the methods used for lipid and protein quantifications are operationally defined and could give false-positive results. The lipid content determined with the sulfo-phosphovanillin method is based on a reaction with the double bonds of unsaturated fatty acids. Other hydrophobic constituents of the medium that are extractable with the used solvent and contain double bonds may lead to detectable signals. The proteins detected in the basal media using the Lowry assay could also be small peptides that are lacking suitable binding sites for organic acids.

For organic acids the contribution of the basal media to the sorption capacity of the medium is negligible compared to the large sorption capacity of FBS. Given the small differences between PBS and the basal media, PBS was used for all experiments with FBS. The binding to FBS was measured at different concentration levels of FBS using a constant concentration of the chemicals and at a single FBS concentration varying the nominal concentration of the chemicals by a factor of 10-120 to derive isotherms (Fig. 5 and S3, SI). As there was no linear correlation between C_{nom} and C_{free} for several test chemicals, the corresponding C_{free} of these chemicals varied by a factor of 85-5700.

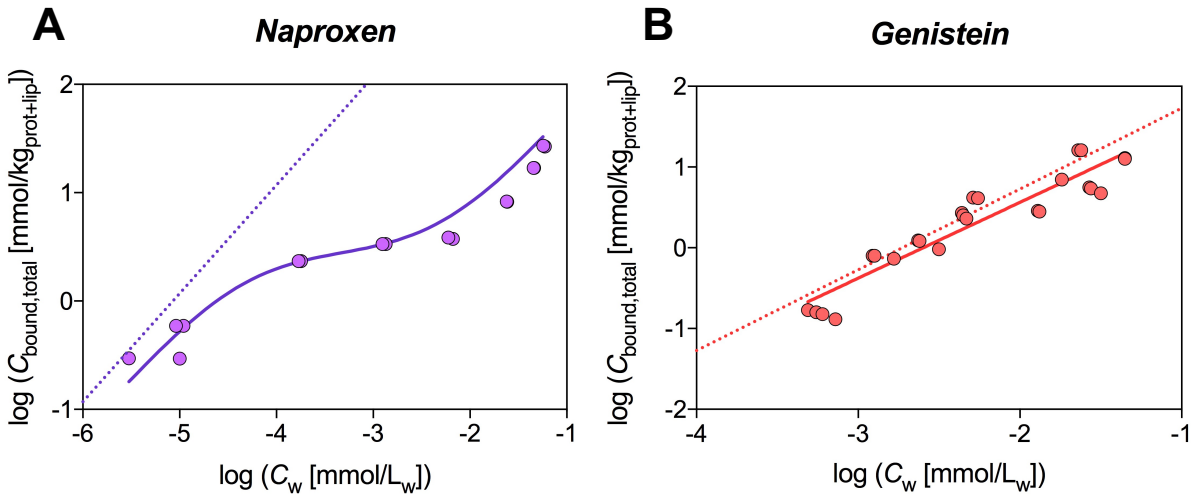

Figure 5. Relationships between freely dissolved (C_w) and bound concentrations ($C_{bound,total}$) in FBS determined for naproxen and genistein. The solid curve of naproxen was fitted using the model described in the method section (eq. 16), the solid line of genistein was derived by linear regression. The dotted lines indicate the binding predicted using the mass balance model for $D_{FBS/w}$ (eq. 17).

For the two chemicals that are partly present as their neutral species, genistein (Fig. 5B) and torasemide (Fig. S3, SI), the concentration bound to the sum of proteins and lipids of FBS (C_{bound}) was increasing linearly with C_w. For the interpretation of the experimentally determined non-linear sorption isotherms we compared them with predicted distribution ratio between the sum of proteins and lipids in FBS and water ($D_{FBS/w}$) calculated with a mass balance model (eq. 17).

$$D_{FBS/w}(\text{pH } 7.4)\left[L_w/kg_{prot+lip}\right] = VF_{prot,medium} \cdot D_{BSA/w} + VF_{lip,medium} \cdot D_{lip/w} \qquad \text{eq. 17}$$

This mass balance equation uses experimentally determined protein and lipid contents of the medium reported by Fischer et al.[6] ($VF_{prot,medium}$, $VF_{lip,medium}$). To simplify the composition of FBS, bovine serum albumin was used as surrogate for all medium proteins and phospholipid liposomes as model for all lipids. The predicted $D_{FBS/w}$ are provided in the SI, Table S9.

The experimentally determined C_{bound} agree well with the prediction from the mass balance model for genistein and torasemide (indicated in Fig. 5 and S3 with a dotted line). For all other test chemicals, all of which are >99 % anionic at pH 7.4 (diclofenac, 2,4-D, ibuprofen, naproxen, and warfarin), a large deviation from the linear isotherm was observed. As described previously, the sorption of these chemicals to FBS can be divided into two different binding types. At low chemical concentration levels, the concentration bound to proteins and lipids increased linearly with C_w and according to the predictions from the model (eq. 17). At higher concentration levels, it can be assumed that the limited number of high affinity bindings sites on the medium proteins become saturated with the test chemical. With even further increase in the concentration, linear binding was observed again for diclofenac, ibuprofen, and naproxen. This part of the isotherm can be explained by nonspecific (moderate to low) binding to the proteins and lipids in the medium. Based on the assumption that the total amount of chemical bound to FBS is the sum of the amount bound specifically and nonspecifically, the isotherms of diclofenac, 2,4-D, ibuprofen, naproxen, and warfarin were fitted successfully by eq. 16 (indicated in Fig. 5A and S3 by the solid lines, for fit parameters of the FBS isotherm see Table S10, SI).

3.5 Partitioning to cells

The partitioning of diclofenac, 2,4-D, and genistein was similar for all evaluated cell lines (Table 3 and Fig. S4) with difference in $D_{cell/w}$ <0.5 log-units. In contrast, considerable differences were measured for ibuprofen (up to 0.77 log-units), naproxen (1.33 log-units), warfarin (1.33 log-units), and torasemide (1.83 log-units) (Table 3 and Fig. S4, SI). However, there are no obvious trends in the partitioning and the differences in the measured distribution ratios are probably caused by variability in the composition of the cells. This hypothesis is strengthened by the fact that the partitioning did not only vary between different cell lines, but also between different batches/passages of cells, e.g., for ibuprofen for H4lle cells (0.47 log-units) and for torasemide for

MCF7 cells (0.48 log-units), although the macroscopic composition, i.e., the total protein and lipid content, of the cells is very similar (Fischer et al.[6] and SI, Table S6).

Table 3. Experimental logarithmic cell-water (log $D_{cell/w}$ [L_w/L_{cell}]) distribution ratios for the test chemicals of this study determined in PBS at pH 7.4 and 37°C.

Chemical	log $D_{cell/w}$ (MCF7)	log $D_{cell/w}$ (HEK293H)	log $D_{cell/w}$ (HEK293T)	log $D_{cell/w}$ (H4lle)
diclofenac	1.47 ± 0.02	1.62 ± 0.05	1.19 ± 0.09	1.47 ± 0.42
2,4-D	0.99 ± 0.03	1.19 ± 0.14	0.97 ± 0.21	1.45 ± 0.24
ibuprofen	0.77 ± 0.24	1.46 ± 0.11	1.45 ± 0.09	1.54 ± 0.47
naproxen	1.25 ± 0.13	2.20 ± 0.12	0.87 ± 0.20	1.88 ± 0.24
torasemide	0.87 ± 0.48	2.70 ± 0.05	1.51 ± 0.27	1.76 ± 0.22
warfarin	0.35 ± 0.16	1.68 ± 0.05	0.83 ± 0.14	1.60 ± 0.29
genistein	1.06 ± 0.11	1.17 ± 0.13	0.98 ± 0.07	1.09 ± 0.26

A mass balance model was also used to predict $D_{cell/w}$. The partitioning to a whole cell can be approximated by multiplying the volume fractions of storage lipids ($VF_{SL,\,cell}$), membrane lipids ($VF_{lip,\,cell}$), functional proteins ($VF_{FP,cell}$), structural proteins ($VF_{SP,cell}$), and water (VF_w) and with the respective distribution ratios between storage lipid and water ($D_{SL/w}$), liposomes and water ($D_{lip/w}$), BSA and water ($D_{BSA/w}$) and structural proteins and water ($D_{SP/w}$) (eq.18).

$$D_{\mathrm{cell/w}}(\mathrm{pH\ 7.4})[\mathrm{L_w/L_{cell}}] = VF_{\mathrm{SL,cell}} \cdot D_{\mathrm{SL/w}} + VF_{\mathrm{lip,cell}} \cdot D_{\mathrm{lip/w}} + VF_{\mathrm{FP,cell}} \cdot D_{\mathrm{BSA/w}} + VF_{\mathrm{SP,cell}} \cdot D_{\mathrm{SP/w}} + VF_{\mathrm{w,cell}} \quad \text{eq. 18}$$

Assuming that only the neutral fraction of the organic acids partitions to storage lipids, the required values for $D_{SL/w}$ can be derived by multiplying the octanol-water partition constant of the neutral species (K_{ow}) with the fraction of the neutral species at pH 7.4 (f_{neutal}, eq. 19 and Table S11).

$$D_{\mathrm{SL/w}}(\mathrm{pH\ 7.4})[\mathrm{L_w/L_{SL}}] = K_{\mathrm{ow}} \cdot f_{neutral} \quad \text{eq. 19}$$

We cannot differentiate between the volume fractions of storage and membrane lipids because both respond similarly to the sulfo-phosphovanillin method of lipid determination after total lipid

extraction. Likewise, a differentiation between different protein types is not possible using the total protein content determined with the Lowry assay. We analyzed the relative importance of different lipid and protein types by modelling four different scenarios (Fig. 6 for HEK293H cells, and Fig. S5 for the other three cell lines). Diclofenac, ibuprofen, and naproxen were chosen for the simulation of cell-water distribution, because $D_{SP/W}$ are available from the literature for these chemicals (Table S11).[18] If BSA was used as surrogate for cellular proteins, the protein fraction was completely dominating the partitioning with minimal contribution of lipids, while the lipid and water phase significantly contributed to the partitioning when using structural proteins for the calculation. If the lipids were modelled as membrane lipid, the lipid fraction was predicted to dominate the partitioning of naproxen to HEK293H cells (Fig. 6, chart on the lower right).

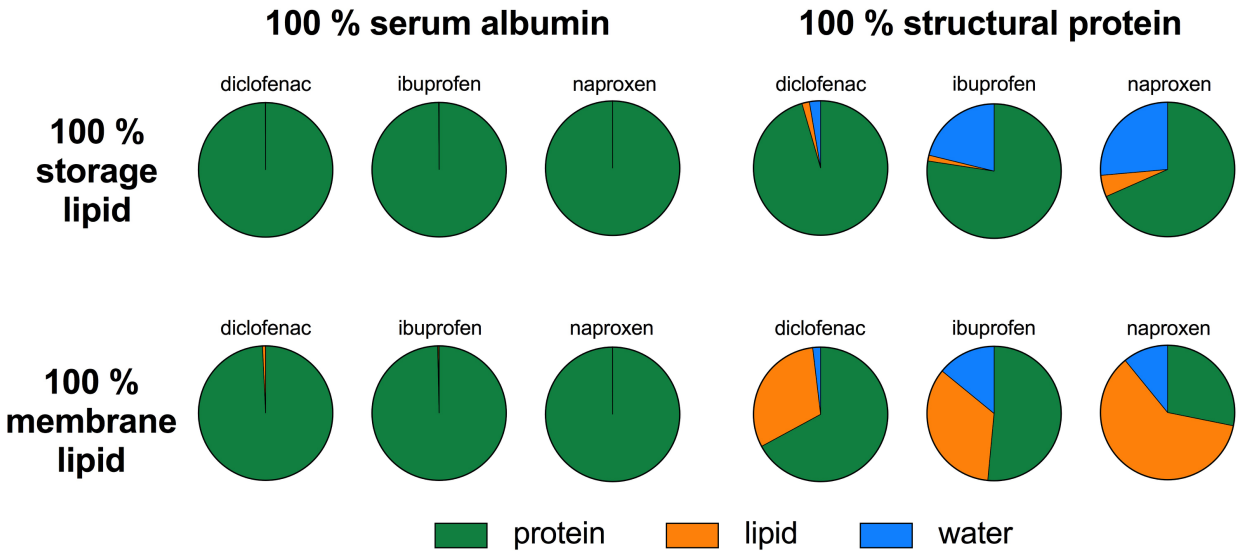

Figure 6. Chemical fractions in the protein, lipid, and water phases of HEK293H cells, using either bovine serum albumin or structural proteins as surrogate for cellular proteins and storage lipids or membrane lipids as surrogate for lipids.

For better comparison of experimental and modelled $D_{\text{cell/w}}$ we simplified eq. 18 by assuming that all cellular lipids are membrane lipids (eq. 20) and tested how the prediction changes by using different volume fractions of functional and structural proteins.

$$D_{\text{cell/w}}(\text{pH 7.4})[\text{L}_\text{w}/\text{L}_\text{cell}] = VF_{\text{lip,cell}} \cdot D_{\text{lip/w}} + VF_{\text{FP,cell}} \cdot D_{\text{BSA/w}} + VF_{\text{SP,cell}} \cdot D_{\text{SP/w}} + VF_{\text{w,cell}} \quad \text{eq. 20}$$

Previous studies have simply assumed that all cellular proteins are similar to BSA with regards to chemical binding.[5, 6] For all cell lines the model (eq. 20) overestimated $D_{\text{cell/w}}$ of the evaluated anions, often by more than one order of magnitude, if only BSA was used as surrogate for cellular proteins (Fig. 7 upper points and Table S12). Structural proteins of the cytoskeleton are presumably most abundant in mammalian cell lines. Previous studies demonstrated that organic acids show much weaker binding to structural proteins than to BSA by up to 3.5 orders of magnitude.[18] The ranges between modelled and predicted $D_{\text{cell/w}}$ using different protein surrogates are probably highest for organic acids compared to neutral and basic chemicals, as they show the largest differences in partitioning between BSA and structural proteins.[18] Fig. 7 (lower points) also shows the predicted values for $D_{\text{cell/w}}$ using only structural proteins instead of BSA as surrogate for cellular proteins. For these three chemicals the prediction seemed to improve, as the difference is smaller than 1 order of magnitude for the majority of the data points, but the predicted values often underestimated $D_{\text{cell/w}}$ (see also Table S13). Assuming that the cells are composed of functional proteins (represented by BSA) and structural proteins in a ratio of 1:100 leads to reasonable predictions of $D_{\text{cell/w}}$ (Fig. 7 points in the middle and Table S14).

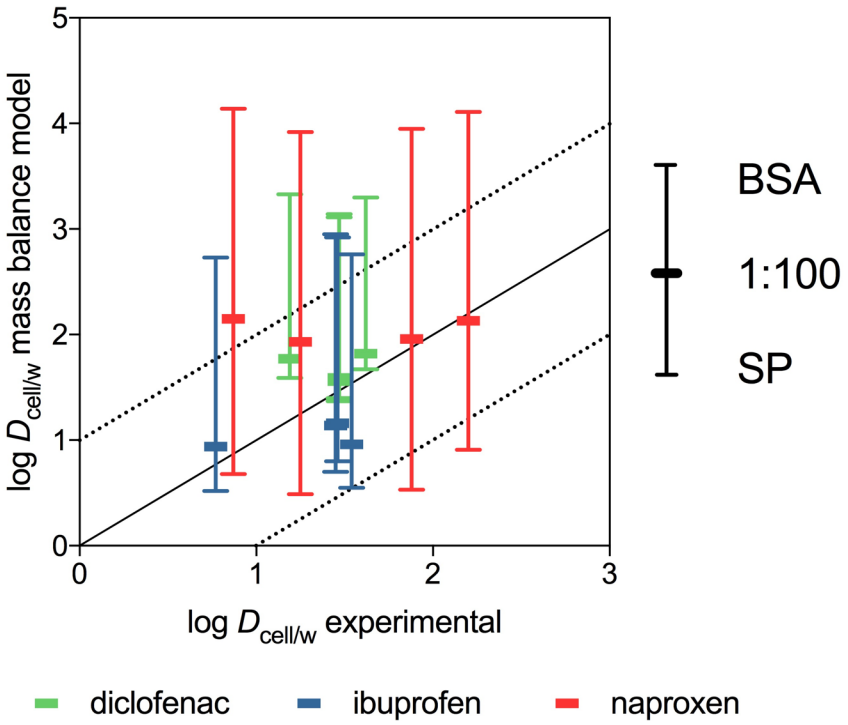

Figure 7. Comparison of experimentally determined cell-water distribution ratios ($D_{cell/w}$) with mass balance model prediction (eq. 20) for diclofenac (green), ibuprofen (blue), and naproxen (red) using either only BSA (upper points), only structural protein (SP, lower points) or a mixture of BSA and SP (ratio 1:100) as surrogate for cellular proteins. The four different data points for each chemical are the four different cells lines tested. The solid line indicates the 1:1 line, the dotted lines a difference of 1 log-unit.

More data for cell-water partitioning will be required to assess, whether the prediction of $D_{cell/w}$ is significantly improved by including structural proteins and different lipid types into *in vitro* mass balance models. Only few literature data are available for binding to structural protein and including it in the mass balance model for the calculation of $D_{cell/w}$ would involve significant experimental work. The generation of such data is also difficult, as larger amounts of structural proteins are not commercially available and have to be extracted from tissue for the binding

experiments.[18, 28] Alternatively, one could also use a "generic" cell as a surrogate for cells, which could be a preparation of a mixture of typical mammalian cell lines, given that there are no systematic differences in $D_{cell/w}$ for different cell lines. This might be experimentally simpler than isolating large quantities of structural proteins.

3.6 Implications for *in vitro* exposure assessment of organic acids

Based on the generated binding data for cell culture medium components and cellular material, we can draw some conclusions regarding the *in vitro* partitioning behavior of organic acids. As stated above, organic acids show very strong binding to the medium proteins, resulting in considerably lower C_w compared to C_{nom} added to the bioassay. The experimental data generated in this study also indicate a strong binding to isolated serum proteins (Table 2) and to FBS at low concentration levels. Saturation of FBS binding was observed at elevated concentrations for five of the seven organic acids tested. As a consequence, the concentration-effect curves obtained for these chemicals in *in vitro* cell-based bioassays will not only be shifted to lower concentration levels when plotted using C_w instead of C_{nom}, but the overall shape of the concentration-effect curves will be changed. The non-linear relationship between C_w and C_{nom} will have to be considered for *in vitro* exposure assessment of organic acids, as the use of simple mass balance models assuming linear (i.e., unsaturable) binding to all sorption phases can lead to an underestimation of C_w at high concentration levels. The calculation of C_w of organic acids could be simplified by testing these chemicals only at low concentrations *in vitro* bioassays, at which linear sorption to FBS can be expected. However, effects (e.g., cytotoxicity) are often observed at higher concentrations and it will likely be necessary to dose organic acids at a concentration range that leads to non-linear sorption isotherms to obtain full concentration-effect relationships. The saturation binding to FBS also emphasizes the need for measured freely dissolved effect concentrations. The C18 SPME method presented is this study has the potential to be used for

direct experimental exposure assessment in *in vitro* cell-based bioassays. Because non-linear fiber sorption isotherms were measured for several chemicals, it should be evaluated in future research projects, if the C18 SPME method is applicable to mixtures of chemicals as well or if this technique is limited to the measurement of C_w for single chemicals. Furthermore, it should be evaluated, if the non-linear FBS sorption isotherms measured in this study are similar for different batches of the same FBS type and among different types of FBS, if the isotherms are normalized to an experimentally determined protein and lipid content.

We also found that the partitioning of organic acids to cells is predicted more accurately when considering different types of cellular proteins in *in vitro* mass balance models, whereas the current assumption that all cellular proteins are BSA[5, 6] would lead to an overestimation of C_{cell} for the evaluated chemicals. To improve the prediction of C_{cell} by existing models, a larger and more diverse dataset in terms of chemical properties that includes also neutral and basic chemicals, will be required to identify suitable surrogates for cellular proteins and lipids.

ASSOCIATED CONTENT

Supporting Information.

The following files are available free of charge.

Additional information on the test chemicals, details on the equilibrium dialysis experiments and instrumental analysis, water, protein and lipid contents of different cell lines, additional figures showing the fiber uptake kinetics, fiber sorption isotherms and FBS sorption isotherms, results from the mass balance model and a graphical presentation of the determined cell-water distribution ratios are provided in a PDF document.

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