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Equilibrium Sorption of Structurally Diverse Organic Ions to Bovine Serum Albumin

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ABSTRACT

Reliable partitioning data are essential for assessing the bioaccumulation potential and the toxicity of chemicals. In contrast to neutral organic chemicals, the partitioning behavior of ionogenic organic chemicals (IOCs) is still a black box for environmental scientists. Partitioning to serum albumin, the major protein in blood plasma, strongly influences the freely dissolved concentration of many chemicals (including IOCs), which affects their transport and distribution in the body. Because consistent datasets for partitioning of IOCs are rarely available, bovine serum albumin-water partition coefficients ($K_{BSA/w}$) were measured in this study for 45 anionic

and 4 cationic organic chemicals, including various substituted benzoic and naphthoic acids, sulfonates and several pesticides and pharmaceuticals. The results of this study suggest that binding to BSA is substantially influenced by the three dimensional structure of the chemicals and the position of substitutions on the sorbing molecules. For example, we found a difference of > 1.5 log units between isomeric chemicals such as 3,4-dichlorobenzoic acid and 2,6-dichlorobenzoic acid, 1-naphthoic acid and 2-naphthoic acid, and 2,4,6-trimethylbenzoic acid and 2,4,6-trimethylbenzenesulfonate. Conventional modeling approaches (e.g. based on octanol-water partition coefficients) poorly predict log $K_{BSA/w}$ of organic ions ($R^2 \leq 0.5$), partially because they do not capture the observed steric effects. Hence, alternative modeling strategies will be required for accurate prediction of serum albumin-water partition coefficients of organic ions.

Introduction

Partitioning of ionogenic organic chemicals (IOCs) into biological tissues and their constituents such as various lipids and proteins is of great importance, as it has direct implications for bioconcentration, bioaccumulation, and toxicity of IOCs. However, established models to predict relevant partition coefficients are almost exclusively focused on neutral organic chemicals including the neutral species of ionizable chemicals. For example, a simple regression with log K_{ow} is widely used as a screening model, which sometimes gives a surprisingly good approximation (e.g., for membrane-water partition coefficients).[1] Polyparameter linear free energy relationships (PP-LFERs) can give more accurate predictions for broader ranges of chemicals and partition phases.[2, 3] These empirical models are convenient but applicable only for neutral chemicals. To our knowledge, there is only one model that was

41 developed specifically for the prediction of biopartitioning of IOCs in general.[4] One reason for
42 the absence of models may be the lack of experimental data that are measured under consistent
43 conditions for partitioning of charged chemicals.

44 Bioaccumulation models used for risk assessment often assume that the dominant sorption
45 phase in organisms is the total lipid fraction. For IOCs storage lipids are expected to play a minor
46 role while phospholipid membranes and the protein fraction of an organism are supposed to
47 contribute significantly to the overall partitioning process.[5] Serum albumin, the most abundant
48 protein in blood plasma, is likely an important sorption phase for IOCs, because research on
49 pharmaceuticals and endogenous chemicals has long shown that serum albumin binds a broad
50 spectrum of chemicals, especially hydrophobic anions of medium size.[6-8] Binding to serum
51 albumin has major impact on transport and distribution of a target chemical in the body, because
52 it increases the sorption capacity of the blood and decreases the free, unbound concentration of
53 the chemical.[9, 10] Moreover, fetal bovine serum is widely used for cell culture assays, where
54 serum albumin binding often determines the bound and the unbound fractions of the test
55 chemical in the medium.[11] Furthermore, serum albumin is often considered a generic protein to
56 represent various properties of the bulk protein fraction of organisms, including sorption
57 properties,[12] although this assumption may not always be valid and thus needs careful
58 evaluation.[13]

59 In the literature, binding of a target chemical to protein, e.g., bovine serum albumin (BSA), is
60 typically reported as association constant K_a [M^{-1}]. For 1:1 binding K_a is defined as:

61 $$K_a = \frac{C_{bound}}{C_{free}[BSA]} \qquad (1)$$

62 where C_{free} [mol/L$_{water}$] is the freely dissolved molar concentration of the chemical, C_{bound} the
63 molar concentration of the chemical bound to BSA, and [BSA] the free molar concentration of
64 BSA in the solution. The extent of binding to BSA can also be expressed as BSA-water partition
65 coefficient ($K_{BSA/w}$ [L$_{water}$/kg$_{BSA}$]), which is preferably used by environmental scientists and is
66 defined as the ratio of the concentration of the target chemical in BSA (C_{BSA} [mol/kg$_{BSA}$]) to the
67 freely dissolved concentration of the chemical:

68 $$K_{BSA/w} = C_{BSA}/C_{free} \quad (2)$$

69 In case the chemical concentration is so low that BSA binding is far below saturation, K_a and
70 $K_{BSA/w}$ are convertible with a constant factor (i.e., $K_a = 10^{1.83}\ K_{BSA/w}$).[14]

71 Large amounts of data are available in the literature for binding of pharmaceuticals (including
72 also IOCs) to human serum albumin. However, reliability and comparability of these data are
73 difficult to evaluate. As reviewed by Nilsson et al.,[15] data for protein binding are often published
74 without carefully controlling all the factors that can influence the partitioning process, e.g.,
75 temperature; the pH value; concentrations of the test chemical, salt, and the protein; non-specific
76 adsorption to the used equipment; equilibrium disturbances; and leaking of dialysis membrane.
77 Moreover, pharmaceuticals are often multifunctional molecules with complex structure. A
78 collection of data for such diverse chemicals is difficult to interpret in terms of structural
79 influences on the binding constant. Systematically measured data for a series of chemicals with
80 incremental changes in the substructure units (e.g., the number of Cl on the benzene ring) or data
81 for a pair of chemicals that differ by only one structural feature may shed additional light on the
82 binding mechanisms of IOCs to serum albumin.

83 This study aims to elucidate how the molecular structure of organic ions (e.g., different basic
84 structures, substitutions and charged functional groups) influences their partitioning to serum

albumin and which mechanisms underlie the sorption process. To this end, we investigated the partitioning of systematically selected organic anions and cations to BSA, starting from simple compounds onwards to more complex structures. Particularly, many varyingly substituted benzenes and naphthalenes with an anionic functional group were included to study the influences of molecule structures on serum albumin binding. All measurement conditions were thoroughly controlled. Additionally, influence of pH value, dependence on the concentration of inorganic ions, and reversibility of BSA binding were determined experimentally. Finally, the data obtained were correlated with various descriptors to gain an insight into the requirements of successful modeling for serum albumin binding.

Materials and methods

Materials

Bovine serum albumin (essentially fatty acid free) was purchased from Sigma Aldrich (Product No. A3803) and used without further purification. BSA was chosen because of its good availability and comparability to the previous publication for neutral compounds.[14] Water purified with a Milli-Q Gradient A10 system from Millipore was used. Methanol (Suprasolv) was obtained from Merck and acetonitrile (gradient grade) from Sigma Aldrich. Unless otherwise noted below, all sorption experiments were performed using Hanks' balanced salt solution (HBSS, without phenol red and sodium bicarbonate, Sigma Aldrich) buffered with 10 mM tris(hydroxymethyl)aminomethane (Tris) from Carl Roth. After addition of sodium bicarbonate and Tris, the pH value of HBSS was adjusted to 7.4 using 1 N HCl or NaOH solution from Merck. The exact salt concentrations of HBSS and a comparison with human plasma are shown in Table S3, SI. Ammonium acetate, formic acid, orthophosphoric acid, bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (Bis-Tris), sodium bicarbonate, sodium

108 chloride, sodium sulfate and sodium azide were purchased from Sigma Aldrich, Carl Roth or
109 Merck.

110 The chemicals used for the binding experiments were different organic acids and bases, or salts
111 of them, all of which have only one ionizable functional group and are more than 99 % ionized at
112 pH 7.4. To investigate the influence of different substitutions on the binding constant, a series of
113 benzoic acids, naphthoic acids and sulfonates were chosen for the dataset. More complex
114 compounds (i.e., pesticides and pharmaceuticals) were included, because of their environmental
115 relevance. All test chemicals are listed in Table 1 and had purity of at least 98%. More details on
116 the test chemicals (e.g., CAS number, provider, analytical method used for quantification,
117 chemical structure, pK_a values and recovery from control experiments) can be found in the
118 Supporting Information Table S1. In this work, weak acids and bases are always denoted with
119 the chemical names of their neutral species (e.g., 4-chlorobenzoic acid) because they are more
120 common than the names for the ionic species (e.g., 4-chlorobenzoate), despite the fact that weak
121 acids and bases in our test solutions are always predominantly in their ionic form and that $K_{BSA/w}$
122 reported in this work is thus for the ionic species.

123 Dialysis experiments

124 Our experimental procedure for the dialysis experiments has been described previously in
125 detail.[16] In short: a custom-made dialysis cell that consists of two half-cells (total volume approx.
126 10 mL) and dialysis membranes from Spectrum Laboratories (type Spectra/Por 4 RC with a
127 molecular weight cutoff of 12-14 kD) was used. One half-cell of the dialysis unit received 5 mL
128 HBSS buffer and the other half-cell 4.9 mL BSA solution (1-50 g/L). All samples were spiked
129 with 100 µL of a dilution of the test chemical in HBSS, which was prepared from a concentrated
130 stock solution in methanol (final concentration of methanol in the system ≤0.5 vol%), and the

131 dialysis cells were equilibrated at 37°C. Three to four replicates were prepared. Aliquots of 100
132 µL were taken from the buffer side of the BSA samples after two and three days (with no
133 significant difference observed between the two time points). The concentration of the test
134 chemicals (C_{free}) was quantified in all samples as described in the instrumental analysis section,
135 C_{BSA} was obtained from the mass balance calculation, and log $K_{BSA/w}$ was calculated using eq 2.
136 Test chemicals for which the fraction bound to BSA was less than 20 % were excluded from the
137 dataset. At high concentrations BSA possibly causes a colloid osmotic pressure that leads to a
138 volume shift in the dialysis cell. However, this was not observed in our experiments. In
139 preliminary experiments we also determined the amount of proteins that passes the dialysis
140 membrane using the Bradford assay. Only 0.01 % of the total protein were found to diffuse
141 through the membrane, which is not expected to influence the determination of $K_{BSA/w}$ in our
142 experiments. Control samples without BSA were also prepared and measured in parallel. If the
143 recovery for a test chemical from the control was consistently below 95 % or above 105 %, the
144 concentration in the BSA samples was corrected according to the recovery. This correction is
145 justified, because a consistent deviation from 100 % recovery was found to result from the first
146 dilution step of the methanolic stock solution in HBSS, which should cause exactly the same
147 error in the dose amounts for BSA and control samples. Test chemicals with recoveries below 90
148 and above 120 % were excluded from the dataset. The average recovery from the control
149 samples for the remaining test chemicals was 91-117 %. All sorption experiments were
150 performed for individual chemicals and not with mixtures. Additionally, in all experiments the
151 amount of the bound test chemicals was kept well below the amount of BSA (i.e., $\leq$ 0.1 mol/mol
152 at equilibrium, see also Table S2, SI), to avoid saturation of the binding sites of BSA. More
153 details on the dialysis experiments are listed in Table S2, SI (e.g., concentration of stock

154 solutions in methanol, initial water phase and measured equilibrium water phase concentrations

155 of all test chemicals, concentration of BSA solution used for the dialysis experiments).

156 **Reversibility of BSA binding**

157 To test the reversibility of binding and the mass conservation, the following experiment was

158 performed for a subset of the test chemicals. Dialysis cells with BSA were prepared as described

159 above, but additionally with 300 mg/L sodium azide to prevent microbial activity. This was

160 necessary to extend the experimental time without causing precipitation of BSA. Additional

161 experiments showed no significant influence of sodium azide on the partitioning of benzoic acid,

162 2-phenoxyacetic acid and 2-methoxy-1-naphthoic acid to BSA (data not shown). After

163 equilibrium was established (three days) the buffer-containing half-cell was emptied completely

164 and 5 mL of fresh buffer were added. Additional three days were given for re-equilibration and

165 the buffer was sampled again. With both equilibrium buffer concentrations after three and six

166 days, log $K_{BSA/w}$ was calculated, assuming the mass conservation. For samples taken after six

167 days, the removal of test chemical due to clearance of buffer after three days was considered in

168 the mass balance calculation. If binding to BSA was fully reversible and no mass loss occurred,

169 the determined $K_{BSA/w}$ after three and six days should be the same. If either (or both) of the

170 conditions is not fulfilled, $K_{BSA/w}$ calculated for the six days sample should become larger than

171 that for the three days sample.

172 **Dependence of BSA binding on pH value and salt concentration**

173 The partitioning of IOCs to proteins may be influenced by pH value and salt concentration. In

174 this study sorption of 2,6-dichlorobenzoic acid to BSA was measured at pH 6, 7 and 8 at a

175 constant concentration of Cl^- (150 mM). To control the pH value in the experiments, 10 mM Bis-

176 Tris (pK_a = 6.5) were added for solutions at pH 6 and 10 mM Tris (pK_a = 8.06) for pH 7 and 8

177 (for more details see SI). Salt concentration dependence was investigated by measuring the BSA-
178 water partition coefficient of 2,6-dichlorobenzoic acid at Cl^- concentrations of 10, 50, 300 and
179 500 mM (adjusted with NaCl) and at a SO_4^{2-} concentration of 163 mM (added as Na_2SO_4; the
180 same ionic strength as 500 mM Cl^-). These salt solutions contained 10 mM Tris and 10 mM HCl
181 and pH was adjusted to 7 with 0.1 N NaOH solution.

182 Instrumental analysis

183 For the majority of the test chemicals an HPLC system from JASCO was used, equipped with
184 either a UV detector (UV-970 M, JASCO) or a fluorescence detector (RF-10AXL, Shimadzu).
185 For chemicals that needed a sensitive quantification method, LC-MS/MS measurements were
186 performed with two different instruments: an Acquity UPLC system from Waters with a Xevo
187 TQ mass spectrometer and an UPLC system from Agilent Technologies (1290 Infinity Series)
188 equipped with a 6400 Triple Quad mass spectrometer. Details on the instrumental analysis are
189 presented in the Supporting information.

190 Results and discussion

191 Reversibility tests

192 The results from the reversibility tests with BSA performed for eight test chemicals are
193 presented in Table S6, SI. No significant difference between the partition coefficients determined
194 after three and six days was found (difference between the mean values was <0.03 log units for
195 all chemicals), indicating that binding to BSA is a fully reversible process and that there was no
196 significant mass loss for the chemicals tested. For all other test chemicals for which we report the
197 partition coefficients in this study, we, therefore, assume that the interaction with BSA is non-
198 covalent and, in principle, reversible. It is reasonable to think that usual sorption of organic

199 chemicals to BSA is reversible, because, otherwise, serum albumin could not transport the
200 chemical from one place to the other within the body.

201 **Dependence of BSA binding on pH value and salt concentration**

202 Changes in pH can influence the partitioning of IOCs to serum albumin in different ways. First,
203 the speciation of IOCs can change by changing pH, and ionic and neutral species of a chemical
204 possibly have different affinities for the protein. Second, the speciation of ionizable functional
205 groups of the protein is also pH dependent, which alters the overall charge of the protein and can
206 influence the interactions with IOCs. Third, serum albumin changes its conformation depending
207 on the pH of the solution,[9] possibly changing the binding site structure. Salt type and
208 concentration of the medium is another crucial factor that has to be considered, because chloride,
209 for example, is reported to compete with warfarin[17] and fatty acids[18] (both are anionic chemicals)
210 for the high affinity binding sites of serum albumin.

211 For 2,6-dichlorobenzoic acid the observed pH dependence was relatively small; log $K_{BSA/w}$ is
212 1.86, 1.82 and 1.72 for pH 6, 7, and 8, respectively. A former study found an increasing, a
213 decreasing, or no clear trend in pH dependence of BSA binding for perfluoroalkyl acids of
214 different chain lengths.[7] These results indicate that changes in pH have different effects on the
215 sorption behavior, depending on the chemicals. In contrast to the relatively small pH dependence
216 observed here, the results for the measurements at different salt concentrations for 2,6-
217 dichlorobenzoic acid show a clear competition effect (Figure S2, SI). An increase of the Cl^-
218 concentration by a factor of 50 decreases $K_{BSA/w}$ by a factor of 17 (i.e., 1.2 log units). Moreover,
219 $K_{BSA/w}$ of 2,6-dichlorobenzoic acid was determined to be 1.8 times higher at 163 mM SO_4^{2-} than
220 at 500 mM Cl^- (i.e., at the same ionic strength), which indicates that the type of competing ion
221 also has an influence on $K_{BSA/w}$.

222 While further research is clearly needed to fully understand pH and salt dependence of $K_{BSA/w}$
223 for IOCs, the results obtained do support the initial statement that pH and salt concentration
224 should be controlled to assure comparability of data. As a consequence, all data discussed in the
225 following sections were measured with a buffer of the same composition, as described in the
226 method section.

227 BSA-water partition coefficients

228 BSA-water partition coefficients ($K_{BSA/w}$) were successfully measured for 45 anionic and 4
229 cationic organic chemicals. The determined log $K_{BSA/w}$ range from 0.97 to 5.27 (Table 1).

230 **Table 1.** Determined logarithmic BSA-water partition coefficients (log $K_{BSA/w}$) at 37°C (pH 7.4
231 in HBSS with 10 mM Tris). Data are all for ionic species.

Test chemical	log $K_{BSA/w}$ [L/kg]	SD
Benzoic acids		
benzoic acid	2.23	0.04
2-chlorobenzoic acid	1.84	0.02
3-chlorobenzoic acid	3.22	0.02
4-chlorobenzoic acid	3.21	0.02
3,4-dichlorobenzoic acid	4.06	0.02
2,6-dichlorobenzoic acid	1.65	0.02
4-fluorobenzoic acid	2.84	0.03
4-nitrobenzoic acid	2.66	0.02
4-bromobenzoic acid	3.48	0.02
4-methylbenzoic acid	2.67	0.02
2-methylbenzoic acid	1.99	0.03
4-ethylbenzoic acid	3.03	0.04
4-butylbenzoic acid	3.73	0.02
4-hexylbenzoic acid	4.23	0.02
2,4,6-trimethylbenzoic acid	2.26	0.03
2-cyclohexylbenzoic acid	3.55	0.04
Naphthoic acids		
2-naphthoic acid	4.36	0.07
2-naphthaleneacetic acid	4.77	0.05
1-naphthoic acid	2.81	0.02

1-naphthaleneacetic acid	3.43	0.03
4-fluoro-1-naphthoic acid	3.46	0.01
1-bromo-2-naphthoic acid	4.02	0.03
2-methoxy-1-naphthoic acid	2.16	0.01
2-ethoxy-1-naphthoic acid	2.66	0.02
3-methoxy-2-naphthoic acid	2.86	0.01
Phenoxy acids		
2-phenoxyacetic acid	2.53	0.07
2,4-dichlorophenoxyacetic acid	3.28[a]	0.10
4-(2,4-dichlorophenoxy)butyric acid	4.12	0.03
2,4,5-trichlorophenoxyacetic acid	3.83	0.02
mecoprop[b]	3.74	0.06
Arylpropionic acids		
ketoprofen[b]	3.31	0.01
ibuprofen[b]	3.91	0.02
fenoprofen[b]	3.92	0.02
α,4-dimethylphenylacetic acid[b]	3.00	0.03
Phenols		
pentachlorophenol	5.27	0.10
bromoxynil	5.18	0.06
Coumarines		
coumachlor	3.41	0.02
coumafuryl	2.84	0.02
Others		
mefenamic acid	4.36	0.02
sulcotrione	1.72	0.06
Sulfonates		
4-ethylbenzenesulfonate	3.17[a]	0.03
4-n-octylbenzenesulfonate	4.84	0.02
2,4,6-trimethylbenzenesulfonate	4.23	0.05
4-bromobenzenesulfonate	3.32	0.04
naphthalene-2-sulfonate	4.71	0.04
Cations		
(S)-(-)-propranolol	1.43	0.05
alprenolol[b]	1.02	0.07
imipramine	1.58	0.09
verapamil[b]	0.97[a]	0.13

[a] log $K_{BSA/w}$ are taken from our previous work[16]

[b] Chiral compounds. Because no information on the

enantiomeric composition was available, we assumed the racemic mixture.

232 The organic anions measured in this study tended to show high affinities for BSA. Given that
233 serum albumin is present with a fraction of 2.9 vol%[19] and is the dominant sorption phase in
234 blood plasma, the majority of the anions tested (38 out of 45) are expected to be more than 90 %
235 bound to serum albumin in plasma. Even the smallest anion in the dataset (benzoic acid) binds
236 strong enough to perform a binding experiment. In contrast, binding of the tested cations to BSA
237 was often too weak to be measureable (i.e., fraction bound <20 % in our dialysis experiments)
238 and log $K_{BSA/w}$ was finally only determined for four cationic chemicals. Organic cations that
239 could not be measured because of too weak sorption include serotonin, (S)-(-)-nicotine,
240 dibenzylamine and different quaternary ammonium compounds (e.g., difenzoquat and
241 benzyldimethyloctylammonium). The finding that anions tend to be much stronger bound to
242 serum albumin than cations is in accordance with previous studies,[20] although this relationship
243 appears not to be a strict rule, as some cationic drugs have been reported to show similar strong
244 binding as anions to serum albumin.[21] Because only four cationic chemicals were successfully
245 measured in this study, the discussions below focus on the partitioning data for anions.

246 Influences of molecular structure on log $K_{BSA/w}$

247 From the dataset presented in Table 1, it can be seen that addition of a CH_2 group or a longer
248 chain to the molecule increases log $K_{BSA/w}$ by, on average, 0.28-0.62 log units per CH_2 unit
249 (Table S7, SI). For example, a linear increase of log $K_{BSA/w}$ was measured for a homologous
250 series of 4-alkylbenzoic acids with a slope of 0.31 log units/CH_2 (Figure S1, SI). Similarly, log
251 $K_{BSA/w}$ increases from 4.36 for 2-naphthoic acid to 4.77 for 2-naphthaleneacetic acid, from 2.81
252 for 1-naphthoic acid to 3.43 for 1-naphthaleneacetic acid and from 2.16 for 2-methoxy-1-
253 naphthoic acid to 2.66 2-ethoxy-1-naphthoic acid (for more examples, see Table S7, SI). A

254 similar increase of log $K_{BSA/w}$ with CH_2 was observed for neutral compounds before (0.35-0.50
255 log units/CH_2).[14] A consistent increase of a partition coefficient with the addition of CH_2 unit is
256 common for neutral compounds in many systems including solvent-water partition systems in
257 general.[22] Thus, in this regard, the observed sorption behavior of IOCs to BSA is generally in
258 line with what we see with neutral compounds (although the observed range of CH_2 influences
259 on log $K_{BSA/w}$ for IOCs is somewhat broader than usual for neutral compounds).

260 An interesting outcome of this work is that, for a series of anionic substituted benzenes and
261 naphthalenes measured in this study, a remarkably high influence of the substitution pattern of
262 the molecules on BSA binding was found. As presented in Figure 1A, data for benzoic acids
263 show that substitution in direct vicinity to the charged carboxylate group lowers the partition
264 coefficient substantially. For example, chlorinated benzoic acids show the following trends: 3-
265 chlorobenzoic acid and 4-chlorobenzoic acid have similar partition coefficients (log $K_{BSA/w}$ is
266 3.22 and 3.21, respectively), while log $K_{BSA/w}$ for 2-chlorobenzoic acid is only 1.84 (Figure 1A).
267 For the two constitutional isomers, 3,4-dichlorobenzoic acid and 2,6-dichlorobenzoic acid, a log
268 $K_{BSA/w}$ of 4.06 and 1.65 was determined, respectively, which means a difference of more than
269 two orders of magnitude. A similar trend was found for methylated benzoic acids: log $K_{BSA/w}$ is
270 2.67 for 4-methylbenzoic acid, but only 1.99 for 2-methylbenzoic acid. It is also interesting that
271 log $K_{BSA/w}$ of 2,4,6-trimethylbenzoic acid is just 2.26, although it has two carbon atoms more
272 than 4-methylbenzoic acid (2.67). These consistent decreases of $K_{BSA/w}$ upon ortho-substitutions
273 on benzoic acid suggest steric effects on the BSA binding, e.g., reduced accessibility to binding
274 sites. For the chlorinated benzoic acids, the observed effects of substitution position could
275 partially be non-steric, because strongly electron-withdrawing Cl has various influences on
276 molecular properties of aromatic acids, which are reflected by, e.g., solvent-water partition

coefficients[23] and pK_a of their neutral species. For methylated benzoic acids the effect is probably only steric. To obtain an additional insight, 3D structures of the substituted benzoic acid molecules were optimized using the quantum chemical software Turbomole (Figures S3-S18, SI). From these structures it can be seen that ortho-substituted benzoic acids (i.e. 2-chlorobenzoic acid, 2,6-dichlorobenzoic acid, 2-methylbenzoic acid and 2,4,6-trimethylbenzoic acid) show a twisted carboxylate group, i.e., the two oxygen atoms of the carboxylate are aligned perpendicular to the benzene ring, whereas the other benzoic acids show the parallel conformation. This structural feature could be related to the lower affinities of the vicinal substituted benzoic acids for BSA. Note that the steric effect discussed here was indicated for benzoic acids but not for sulfonates (for further discussion see the last paragraph of this section).

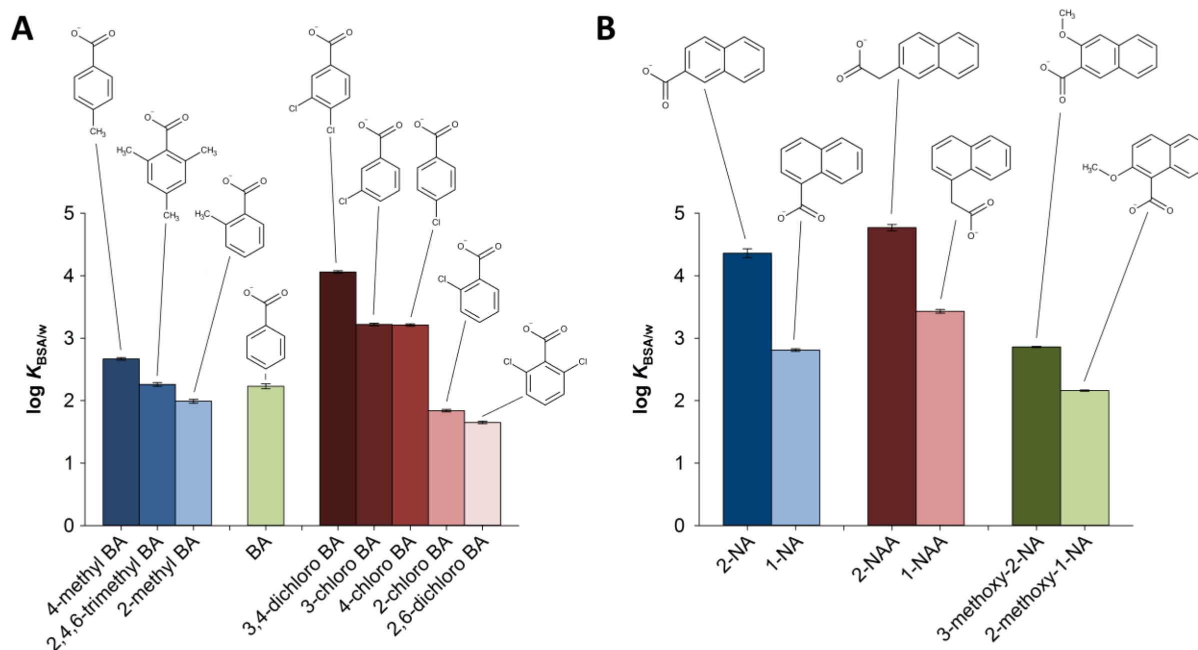


Figure 1. Influence of substitution position on log $K_{BSA/w}$ and observed steric effects; BA - benzoic acid, NA - naphthoic acid, NAA - naphthaleneacetic acid.

290 Another notable finding is that 1-naphthoic acids consistently have much lower BSA-water
291 partition coefficients than corresponding 2-naphthoic acids (Figure 1B). The determined log
292 $K_{BSA/w}$ of 1-naphthoic acid is 2.81, while it is 4.36 for 2-naphthoic acid; 1-naphthaleneacetic acid
293 and 2-naphthaleneacetic acid have log $K_{BSA/w}$ of 3.43 and 4.77, respectively and 2-methoxy-1-
294 naphthoic acid and 3-methoxy-2-naphthoic acid of 2.16 and 2.86, respectively. The difference
295 might be explained by the 3D shape of these chemicals, because 2-naphthoic acids are more
296 linear, whereas the 1-naphthoic acids have a more bulky structure.

297 Stereoselectivity is often reported for serum albumin in the literature.[24-26] For chiral drugs
298 binding of the different enantiomers to human serum albumin normally differs only up to a factor
299 of 1.5.[24, 27] Larger differences in serum albumin binding were found for specific isomers, e.g., the
300 amino acid tryptophan, for which the L chiral form is reported to bind 10 - 100 times more to
301 serum albumin than the D form.[25, 28, 29] By comparison, the high influences of the 3D structure on
302 the partitioning of benzoic and naphthoic acids to BSA that were found in this study are
303 remarkable, considering the generally broad specificity of the albumin binding. In general,
304 organic anions are believed to bind to a hydrophobic pocket of serum albumin with additional
305 electrostatic interactions between the negative charge of the chemical and the positive charge of
306 a lysyl- or arginyl residue.[9] Possibly, the accessibility of such a binding site is hampered for
307 some of the chemicals tested in this study due to their 3D shape.

308 In the dataset, we included four pairs of chemicals that have the same non-ionic substructure
309 but different charged functional groups (i.e., sulfonate vs carboxylate): 4-ethylbenzenesulfonate
310 and 4-ethylbenzoic acid, naphthalene-2-sulfonate and 2-naphthoic acid, 4-
311 bromobenzenesulfonate and 4-bromobenzoic acid, and 2,4,6-trimethylbenzenesulfonate and
312 2,4,6-trimethylbenzoic acid. For the first three pairs, the type of the charged functional group has

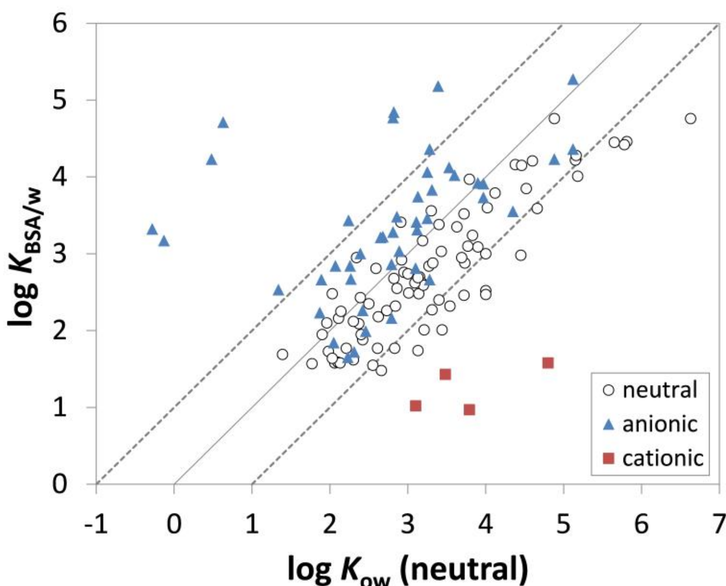
313 only a minor influence on log $K_{BSA/w}$ (0.14-0.35 log units). However, a large difference (1.97 log
314 units) was found between 2,4,6-trimethylbenzenesulfonate and 2,4,6-trimethylbenzoic acid,
315 which cannot easily be explained. As already mentioned above, 2,4,6-trimethylbenzoic acid has
316 two methyl substitutions in direct vicinity of the charged group and its $K_{BSA/w}$ value is even
317 lower than that of benzoic acid most likely due to a steric effect. In contrast, 2,4,6-
318 trimethylbenzenesulfonate appears not to experience such a steric effect, as is indicated by the
319 $K_{BSA/w}$ value being greater than that of 4-ethylbenzenesulfonate. Corroborating this interpretation,
320 the sulfonate group of 2,4,6-trimethylbenzenesulfonate optimized by Turbomole shows no
321 structural difference compared to 4-ethylbenzenesulfonate (Figure S19, SI).

322 Comparison with other partition coefficients

323 As a first step towards developing a predictive model for partitioning of organic ions to serum
324 albumin, we plotted log $K_{BSA/w}$ determined in this study against various other partition
325 coefficients that can be derived more easily than log $K_{BSA/w}$ itself (Figures 2 and 3).

326 First, the data are compared with logarithmic octanol-water partition coefficients of the neutral
327 species of the test chemicals (log K_{ow} (neutral)), although we are not hypothesizing that there is a
328 mechanistic relationship between log $K_{BSA/w}$ and log K_{ow} (neutral). Log K_{ow} (neutral) data are
329 readily available from the literature and many environmental models are based on correlations
330 with log K_{ow} (neutral), although it has been shown that such models are purely on an empirical
331 basis and can be inaccurate even for neutral chemicals.[2] All log K_{ow} values shown in Figure 2 are
332 taken from the EPI-Suite data base (version 4.1) provided by the U.S. Environmental Protection
333 Agency. If no experimental log K_{ow} (neutral) was available, the value calculated from EPISuite
334 was used instead (Table S8, SI). For comparison, BSA binding data for 83 neutral chemicals
335 from Endo et al.[14] are included in Figure 2. For the neutral chemicals, there is a relatively weak

336 but clear positive trend between log $K_{BSA/w}$ and log K_{ow} (R^2 = 0.75).[14] In contrast, for the organic
337 anions and cations of this study, there is no overall trend (R^2 = 0.13 for all anions). The high
338 scatter appears to result from several reasons. First, according to EPI-Suite, sulfonates have
339 extremely small log K_{ow} (neutral), except for naphthalene-2-sulfonate, which is the only
340 sulfonate for which an experimental log K_{ow} (neutral) was available. The reliability of the log
341 K_{ow} (neutral) calculated by EPI-Suite for sulfonates is unclear, because experimental log K_{ow} for
342 neutral species of sulfonates are difficult to measure. Second, for the cationic test chemicals, the
343 relationship between log $K_{BSA/w}$ (ion) and log K_{ow} (neutral) appears to be substantially different
344 from that of anions. Third, while log K_{ow} (neutral) captures the increase of log $K_{BSA/w}$ by addition
345 of CH_2 increments, the other trends that were found in the dataset for benzoic and naphthoic
346 acids of this study (e.g., influence of substitution position and differences between 1- and 2-
347 naphthoic acids) cannot be depicted with log K_{ow} (neutral) (see also Figure S20A, SI).



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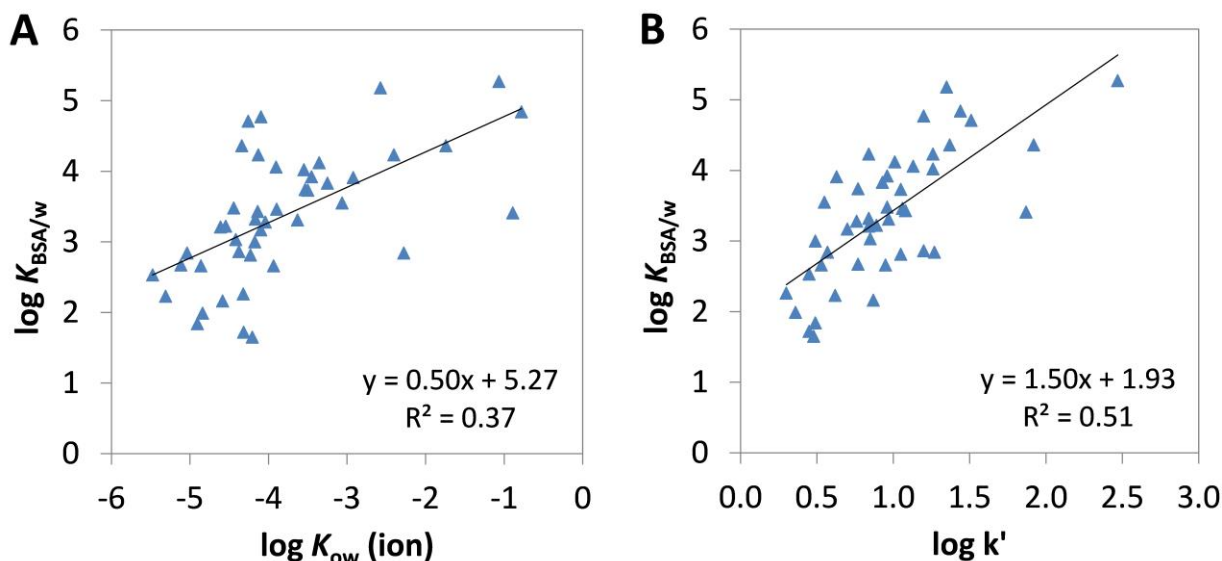
349 **Figure 2.** Comparison of determined log $K_{BSA/w}$ (ion) with octanol-water partition coefficients of
350 the neutral species (log K_{ow} (neutral)).

351 We also plotted the experimental log $K_{BSA/w}$ values of this study against other partition
352 coefficients of the ionic species (Figure 3), expecting a better correlation compared to log K_{ow}
353 (neutral), because these partition coefficients directly reflect physicochemical properties of the
354 ionic species of the test chemicals. For the 45 anions measured in this study, theoretical log K_{ow}
355 of the ionic species (log K_{ow} (ion)) were calculated using the quantum chemically based software
356 COSMOtherm.[30] For ionic chemicals, COSMOtherm calculates single-ion partition coefficients
357 at infinite dilution that are inaccessible by experiment (because counter-ions also partition in real
358 systems). While a positive trend does exist, the correlation of measured log $K_{BSA/w}$ with
359 calculated log K_{ow} (ion) is rather weak (R^2 = 0.37, Figure 3A). We also tried other solvents than
360 octanol such as methanol, acetone, and hexadecane, but none of them resulted in a better
361 correlation. Similar to log K_{ow} (neutral), log K_{ow} (ion) only accounts for the increase of log
362 $K_{BSA/w}$ with the number of CH_2 groups, but not at all for the observed differences between 1- and
363 2-naphthoic acids and the influence of substitution position (Figure S20B in SI). According to
364 COSMOtherm calculations, log K_{ow} (ion) for the structural isomers are rather similar (difference
365 $\leq$ 0.3 log units). The fact that sulfonates and benzoic acids with the same non-ionic substructure
366 show very similar log $K_{BSA/w}$ (ion) is captured correctly by log K_{ow} (ion) (predicted difference
367 between benzoic acids and sulfonates 0.08-0.32 log units). However, the large difference
368 between 2,4,6-trimethylbenzenesulfonate and 2,4,6-trimethylbenzoic acid is not predicted by log
369 K_{ow} (ion) (measured difference 1.97 log units, predicted difference only 0.19 log units).



370

371 **Figure 3.** Correlation of determined log $K_{BSA/w}$ for anions with (A) calculated octanol-water
372 partition coefficients of the ionic species (log K_{ow} (ion)) and (B) measured retention factors on a
373 weak anion exchange column (log k').

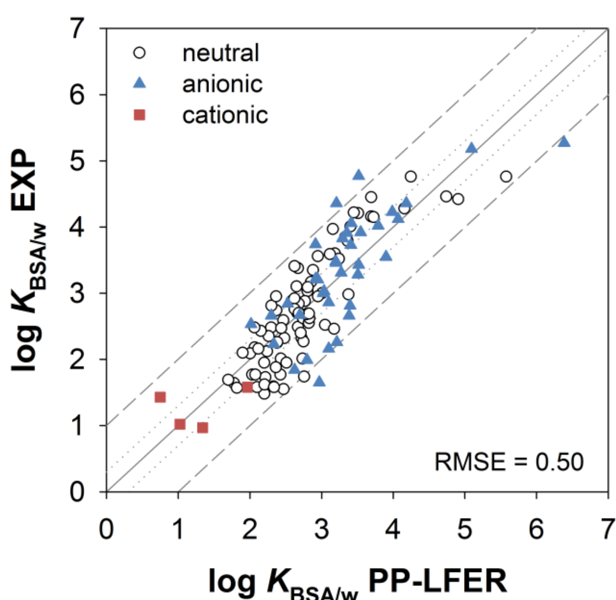
374 Additionally, in Figure 3B the data for anions are compared with measured logarithmic
375 retention factors on a weak anion exchange column (log k', Luna NH2 column, Phenomenex,
376 unpublished in-house data, see Table S8 in SI). The increase of log $K_{BSA/w}$ by addition of CH_2
377 increments is correctly depicted by log k' and also the observed steric effects are captured better
378 by log k' (Figure S20C in SI) compared to log K_{ow} (both, neutral and ion). Nevertheless, the
379 overall correlation (R^2 = 0.51) may still be too unsatisfying to be considered as a predictive
380 model.

381 Modeling considerations

382 The findings of this study suggest that conventional modeling approaches like correlation with
383 log K_{ow} would fail to accurately predict the partitioning of IOCs to serum albumin. Descriptors
384 that are supposed to directly reflect the properties of the ionic species like calculated log K_{ow} of

385 ionic species and measured retention factors on an ion exchange column do not show a good
386 correlation with measured log $K_{BSA/w}$ either.

387 Polyparameter linear free energy relationship (PP-LFER) models as described by Endo et al.[14]
388 are only applicable for neutral chemicals. However, Abraham et al. have proposed a PP-LFER
389 approach that can be used for ions as well.[31, 32] This PP-LFER model was fitted to the data of this
390 study, including 82 neutral chemicals from Endo et al[14] (a detailed description of the PP-LFER
391 modeling can be found in the SI). The derived PP-LFER equation gives a better correlation for
392 log $K_{BSA/w}$ than the other approaches discussed above (R^2 = 0.70, RMSE = 0.58 for all ionic
393 chemicals, Figure 4), but again important trends in the dataset such as the influence of
394 substitution position and differences between 1- and 2-naphthoic acids are not captured correctly
395 (Figure S20D, SI). This deficiency of the model is not surprising, because the solute descriptors
396 used for PP-LFER models incorporate only the volume of the solute, but not the specific 3D
397 structure.[2] Because there may well be unknown structural effects that cause even larger errors,
398 we do not recommend a general use of the PP-LFER equation derived within this study to predict
399 sorption of organic ions to serum albumin.



400

401 **Figure 4.** Comparison of experimentally determined BSA-water partition coefficients (log
402 $K_{BSA/w}$ EXP) with PP-LFER fitted BSA-water partition coefficients (log $K_{BSA/w}$ PP-LFER); solid
403 line denotes the 1:1 line, dotted and dashed lines indicate a deviation of 0.3 and 1 log unit,
404 respectively.

405 In conclusion, an important reason why the conventional descriptors tested above are
406 inappropriate for modeling serum albumin binding appears to be that they cannot account for the
407 observed steric effects and the influence of the 3D structure. Thus, for a better prediction, a
408 modeling approach that correctly captures the 3D structure effects needs to be applied. There is
409 an ongoing effort in our research group to use 3D quantitative structure activity relationships
410 (3D-QSARs) for modeling serum albumin binding constants, which will be reported soon.

411 ASSOCIATED CONTENT

412 **Supporting Information**. Further information on the test chemicals, instrumental analysis and
413 PP-LFER modeling; 21 additional figures (including 3D structures for selected chemicals) and 9
414 additional tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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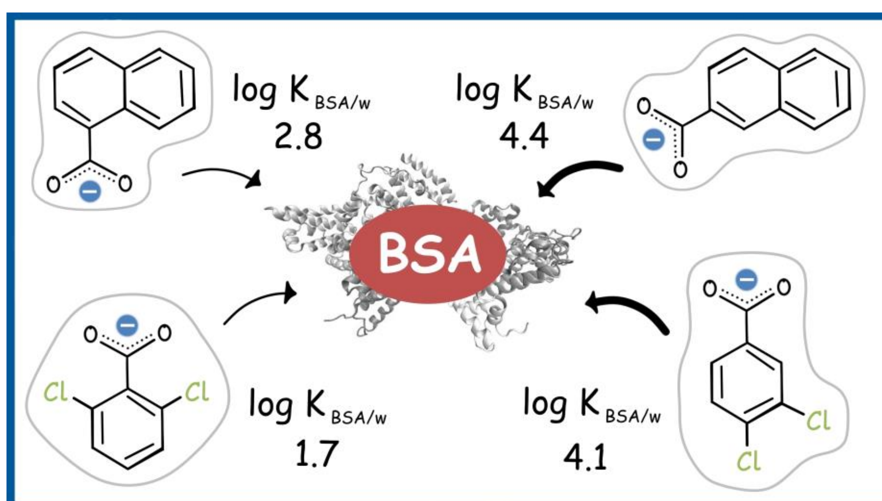
512 Table of Contents Graphic



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