The prediction of unbound fractions for *in vitro-in vivo* extrapolation of biotransformation data

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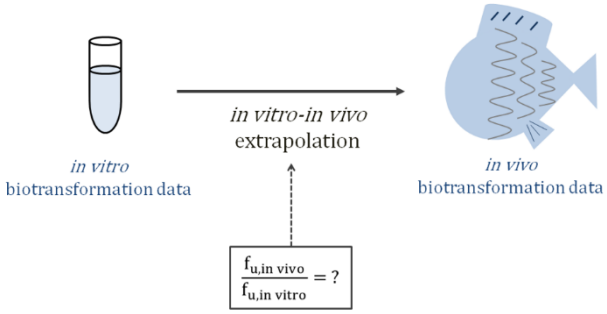

ABSTRACT

For *in vitro-in vivo* extrapolation of biotransformation data the different sorptive environments *in vitro* and *in vivo* need to be considered. The most common approach for doing so is using the ratio of unbound fractions *in vitro* and *in vivo*. In the literature, several algorithms for prediction of these unbound fractions are available. In this study, we present a theoretical evaluation of the most commonly used algorithms for prediction of unbound fractions in S9-assays and blood and compare prediction results with empirical values from the literature.

The results of this analysis prove a good performance of 'composition-based' algorithms, i.e. algorithms that represent the inhomogeneous composition of *in vitro* assay and *in vivo* system and describe sorption to the individual components (lipids, proteins, water) in the same way. For strongly sorbing chemicals, these algorithms yield constant values for the ratio of unbound fractions *in vitro* and *in vivo*. This is mechanistically plausible, because in these cases the chemicals are mostly bound and the ratio of unbound fractions is determined by the volume ratio of sorbing components in both phases.

INTRODUCTION

Methods for prediction of the bioaccumulation behavior of chemicals are in demand because they may offer an alternative to *in vivo* bioaccumulation tests [1]. The performance of bioaccumulation models was shown to increase significantly when biotransformation of the chemical in the organism is considered [2-4]. One frequently used approach for measuring the required biotransformation involves substrate depletion assays with trout liver S9 fractions or hepatocytes [5, 6] followed by *in vitro-in vivo* extrapolation [7].

For extrapolation from a biotransformation assay to an organism certain differences need to be accounted for: the different amounts of metabolically active components and the different amounts of freely dissolved chemical in both systems that are directly available for biotransformation. As shown in a recent paper [8], both the ratio of unbound fractions in both systems or the corresponding partition coefficients can be used to consider the different sorption capacities of the two systems. Both approaches are mathematically equivalent and yield the same results as long as the relevant input parameters are consistent, but the extrapolation using unbound fractions is the more commonly used variant. The so generated extrapolation results, however, often do not match the corresponding *in vivo* data well and an overestimation of *in vivo* BCFs is often observed [2, 9]. Among other possible explanations, it is suggested that errors in the sorption correction, i.e. in the ratio of unbound fractions (often called 'f_u' term), might be responsible for the observed discrepancies [9, 10]. In the literature, different approaches for calculation of f_u exist causing some uncertainty which algorithms to use. In the following, we present an evaluation of the existing algorithms for prediction of f_u for neutral chemicals and comparisons with available experimental f_u values to assess the reliability of the different existing algorithms.

THEORY

For *in vitro-in vivo* extrapolation using the unbound fractions *in vivo* and *in vitro* for sorption correction, the f_u term is calculated as

$$f_u = \frac{f_{u,blood}}{f_{u,in\ vitro\ assay}} \quad (1)$$

For estimation of the unbound fractions in assays and blood, different algorithms in the literature exist. In this study, we compared three different algorithms to estimate the unbound fraction *in vivo* (i.e. in blood/plasma; $f_{u,blood}$ or $f_{u,plasma}$) and four different algorithms to estimate the fraction unbound in the *in vitro* assay ($f_{u,in\ vitro\ assay}$) for calculation of f_u according to eq. (1). Despite there being different types of *in vitro* biotransformation assays (S9, hepatocyte or microsome assays), we here focus on rainbow trout liver S9-assays because for this type of assay the most experimentally determined f_u values are available for a comparison with the different predicted f_u values. For binding *in vivo*, experimental data showed that binding in

plasma and in whole blood is approximately equal [11] enabling direct comparisons of unbound fractions measured in plasma with unbound fractions predicted for whole blood.

1) Algorithms for estimation of fraction unbound *in vivo* ($f_{u,blood}$ or $f_{u,plasma}$)

$f_{u,blood}$ - Fitzsimmons et al. 2001 [12]

Fitzsimmons et al. derived an empirical correlation for prediction of blood-water partition coefficients $K_{blood/W}$ (called P_{BW} in the Fitzsimmons publication) using the octanol-water partition coefficient K_{OW}:

$$K_{blood/W} = 10^{0.73 \log K_{OW}} * 0.16 + 0.84 \quad (2)$$

This algorithm was derived from a dataset of experimental values for 11 chemicals (measured by Fitzsimmons et al. [12] and Bertelsen et al. [13]) and the assumption that blood consists of 16 % non-aqueous constituents (by weight).

The unbound fraction in blood $f_{u,blood}$ is then calculated using the water content of blood w_{blood} according to

$$f_{u,blood} = \frac{w_{blood}}{10^{0.73 \log K_{OW}} * 0.16 + 0.84} \quad (3)$$

$f_{u,plasma}$ - Saunders et al. 2020 [14]

Saunders et al. published an algorithm for calculation of unbound fractions in plasma. This algorithm is mechanistic (in contrast to the empirically derived one from Fitzsimmons) in that it considers the tissue composition in an additive approach:

$$f_{u,plasma} = \frac{F_{w,plasma}}{F_{L,plasma} * K_{OW} + F_{P,plasma} * 0.05 * K_{OW} + F_{w,plasma}} \quad (4)$$

In this equation $F_{L,plasma}$, $F_{P,plasma}$ and $F_{W,plasma}$ are the fractions of lipid, protein and water in plasma.

2) Algorithms for estimation of fraction unbound in S9 *in vitro* assays ($f_{u,S9}$)

$f_{u,S9}$ - Han et al. 2009 [15]

Han et al. derived an empirical relationship to predict the unbound fraction in rainbow trout S9-assays

$$f_{u,S9} = \frac{1}{C_{S9} * 10^{0.694 \log K_{OW} - 2.158} + 1} \quad (5)$$

Here, C_{S9} is the S9 concentration used in the *in vitro* assay (mg_{S9}/mL). The algorithm is based on an empirical dataset that Austin et al. [16] originally measured for 37 compounds with rat microsomes. For derivation of eq. (5), Han et al. excluded compounds with a log K_{OW} < 1.5 and refitted the data of the remaining 30 compounds. The Han algorithm for S9 assays in

combination with the Fitzsimmons algorithm for blood is the most commonly used algorithm combination to predict the f_u ratio[7].

$f_{u,S9}$ - Nichols et al. 2018 [17]

Nichols derived another empirical relationship to predict the unbound fraction in rainbow trout S9-assays

$$f_{u,S9} = \frac{1}{C_{S9} * 10^{1.33 \log K_{OW} - 4.6} + 1} \quad (6)$$

This algorithm is derived from experimental results for three PAHs (polycyclic aromatic hydrocarbons) measured with rainbow trout S9 fraction.

$f_{u,S9}$ - Lee et al. 2017 [18]

Lee et al. published an algorithm for calculation of unbound fractions in S9-assays. Like the Saunders approach for plasma, this algorithm is mechanistic (in contrast to the empirically derived ones from Han and Nichols) in that it considers the tissue composition in an additive approach:

$$f_{u,S9} = \frac{F_{w,S9}}{F_{L,S9} * K_{OW} + F_{P,S9} * 0.05 * K_{OW} + F_{w,S9}} \quad (7)$$

In this equation $F_{L,S9}$, $F_{P,S9}$ and $F_{W,S9}$ are the fractions of lipid, protein and water in S9-assay.

3) pp-LFER approaches for S9 and blood [19]

Like the Lee approach for S9 and the Saunders approach for plasma, the pp-LFER (polyparameter linear free energy relationship) approach is also a mechanistic one. The difference is that pp-LFER based partition coefficients are used for the different tissue components instead of K_{OW} correlations. The following tissue components are distinguished: membrane lipids, storage lipids, albumin, structural proteins and water. The unbound fractions in assay and blood are then calculated from a combination of the sorption data with the relative amounts of the protein, lipid and aqueous components in blood and assay (also implemented in the LSER database [20]).

RESULTS AND DISCUSSION

Generic comparison

For a first generic comparison, the unbound fractions in assay and in blood are calculated with the above equations over a range of log K_{OW}. The pp-LFER approach, however, could not be included in this generic comparison, because it does not rely on a log K_{OW} correlation (and by this it could not be plotted against log K_{OW}). The calculated $f_{u,blood}$ and $f_{u,S9}$ were plotted against the range of log K_{OW} (Figure 1):

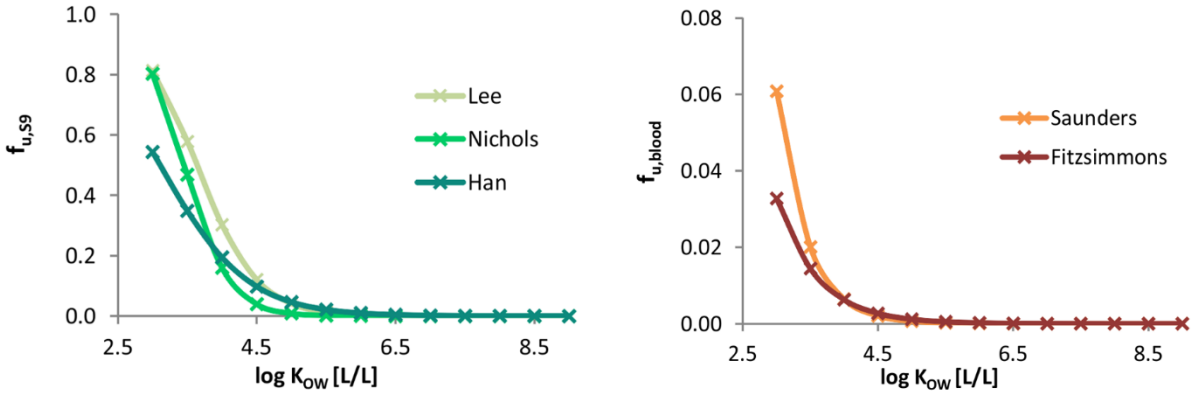

Figure 1: Predicted unbound fractions in blood or S9 assay for a range of log K_{OW}.

The two figures show rather small differences between the algorithms. For *in vitro-in vivo* extrapolation, however, the ratio of fraction unbound in blood and fraction unbound in assay $f_{u,blood}/f_{u,S9}$ is required.

Accordingly, we applied the most commonly used algorithm combinations and calculated the ratio of unbound fractions in assay and blood.

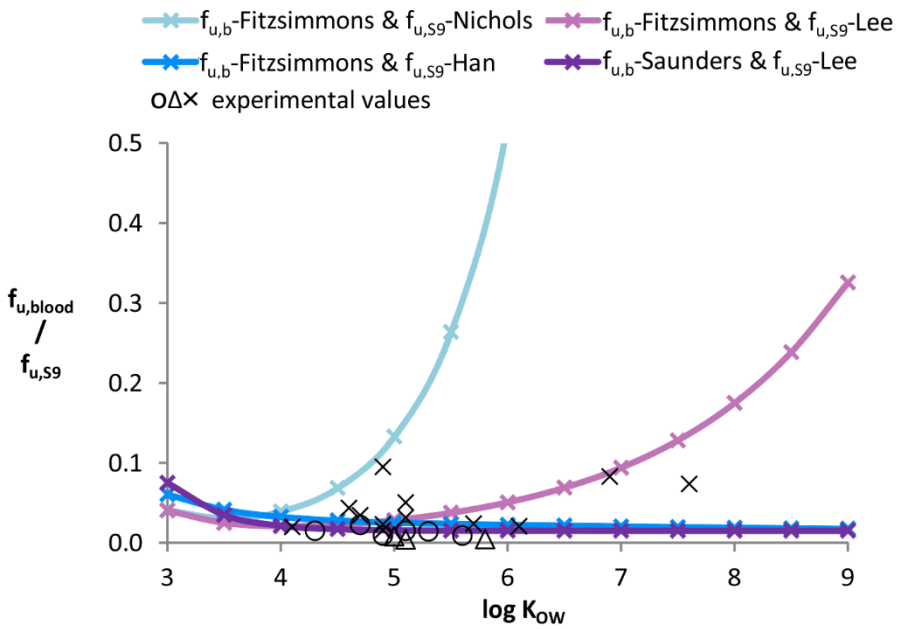

Figure 2: Predicted ratios of unbound fractions in blood and assay over a range of log K_{OW} using the different algorithm combinations. For comparison, experimentally determined values for $f_{u,blood}/f_{u,S9}$ from three studies are collected and included in the plot (× - data from Saunders et al.[14], Δ - data from Escher et al.[21], o – data from Laue et al.[22]).

Figure 2 shows that all algorithms yield similar results for log K_{OW} ranging from 3 to 4. With increasing log K_{OW}, however, the differences in the predictions increase dramatically. The Fitzsimmons/Nichols and Fitzsimmons/Lee combinations yield exponentially increasing $f_{u,blood}/f_{u,S9}$ for increasing log K_{OW} while the Fitzsimmons/Han combination yields decreasing

$f_{u,blood}/f_{u,S9}$ for increasing log K_{OW}. The Saunders/Lee combination yields a plateau for increasing log K_{OW}. Numerically, the Saunders/Lee and the Fitzsimmons/Han combinations yield similar values for the here shown range of log K_{OW}, while the Fitzsimmons/Nichols and Fitzsimmons/Lee combinations yield notably different values for $f_{u,blood}/f_{u,S9}$.

The question is why the different combinations of prediction algorithms perform so differently. The generic explanation for this has to do with the fact that ratios of two calculated parameters (i.e. the unbound fractions) are compared: when two calculated parameters are divided by each other, rather small inaccuracies in the estimates of the single parameters can lead to significant errors in the resulting ratio. However, this principal mathematical problem can be avoided: Blood as well as assay consist of the same sorbing components (lipids, proteins, water) just in different composition, blood and assay could thus be called two heterogeneous mixtures of the same components. Hence, the overall sorption coefficients to both assay and blood should be based on the same specific sorption coefficients ($K_{lipid/water}$, $K_{protein/water}$). If this is done then errors in the quotient of $f_{u,assay}$ and $f_{u,blood}$ will tend to cancel out rather than to fortify each other. Among the presented algorithms, only the ppLFER algorithms and the Saunders/Lee algorithms fulfill this requirement.

Comparison with experimental data

For further evaluation, we collected experimental data from the literature where both unbound fractions ($f_{u,blood}$ and $f_{u,S9}$) were measured within one study[14, 21, 22]. These experimental values are included in Figure 2 and summarized together with the corresponding prediction results in Table 1.

Table 1: Experimental values for $\mathbf{f_{u,blood}/f_{u,S9}}$ from the literature [14, 21, 22] and predicted $f_{u,blood}/f_{u,S9}$ using the different algorithm combinations

chemical	log K_{ow}	$f_{u,blood}/f_{u,S9}$					
		exp.	Fitzsimm. /Nichols	Fitzsimm. /Lee	Fitzsimm. /Han	Saunders /Lee	ppLFER /ppLFER
musk xylene	4.1	0.020[14]	0.043	0.021	0.031	0.020	0.018
polysantol	4.3	0.015[22]	0.054	0.021	0.029	0.018	0.013
1,2,4,5-tetrachlorobenzene	4.6	0.043[14]	0.078	0.023	0.027	0.017	0.022
cyclohexyl 2-hydroxybenzoate	4.7	0.022[22]	0.089	0.024	0.027	0.016	0.008
pentachlorobenzene	4.7	0.034[14]	0.089	0.024	0.027	0.016	0.008
pyrene	4.9	0.009[22]; 0.023[21]; 0.016[14]	0.116	0.027	0.026	0.016	0.015
4-methylbenzylidene camphor	4.9	0.095[14]	0.116	0.027	0.026	0.016	0.015
chlorpyrifos	5	0.008[21]	0.133	0.028	0.025	0.016	0.012
ambrofix	5.1	0.015[22]	0.152	0.030	0.025	0.015	0.015
methoxychlor	5.1	0.004[21]; 0.031[14]	0.152	0.030	0.025	0.015	0.010
9-methylanthracene	5.1	0.050[14]	0.152	0.030	0.025	0.015	0.005
galaxolide	5.3	0.014[22]	0.200	0.033	0.024	0.015	0.017
karanal	5.6	0.009[22]	0.302	0.040	0.023	0.015	0.009
hexachlorobenzene	5.7	0.024[14]	0.347	0.042	0.023	0.015	0.017
nonylphenol	5.8	0.004[21]	0.398	0.045	0.023	0.015	0.006
PCB 52	6.1	0.021[14]	0.603	0.054	0.022	0.015	0.006
octocrylene	6.9	0.083[14]	1.820	0.088	0.021	0.015	0.005
PCB 155	7.6	0.074[14]	4.788	0.136	0.019	0.015	0.028

Table 1 shows that all experimental values of this collection are in a range of 0.004 to 0.095. This is of relevance because several studies reported that assuming a value of 1 for f_u, i.e. assuming identical binding *in vitro* and *in vivo*, improves the agreement between extrapolation results and *in vivo* BCFs [2, 9, 21]. This procedure, however, is mechanistically not plausible and, as the above data show, cannot be justified empirically either.

Furthermore, Table 1 shows that most of the available experimental data belong to chemicals in the log K_{OW} range from 4 to 6. For this range, the Saunders/Lee, the Fitzsimmons/Han as well as the ppLFER algorithm combinations yield predictions that are close to the measured values for most of the cases (discrepancy less than a factor of 3 between prediction and measurement for the majority of the values). The Fitzsimmons/Nichols algorithm combination, in contrast, tends to overestimate the ratio of unbound fractions for the most of the cases. The

Fitzsimmons/Lee algorithm combination in turn also yields values close to the measured values. Similar findings have also been described in three recent studies [10, 14, 22]. Interestingly, the two chemicals with the highest log K_{OW} (octocrylene and PCB 155) have two of the highest experimental f_u ratios of the experimental data set. Both values were determined by Saunders et al. in a recent study using a novel method for measurement of binding terms [14]. Only the prediction results of the Fitzsimmons/Lee algorithm combination come close to these two values. It is therefore difficult to identify the most reliable algorithm just by comparison with experimental data. The otherwise well-performing Saunders/Lee, Fitzsimmons/Han and ppLFER algorithm combinations notably underestimate these two experimental f_u ratios. As mentioned above, the Fitzsimmons/Han combination results in decreasing values, whereas the Saunders/Lee combination results in a plateau. The ppLFER combination also yields more or less the same ratio of unbound fractions for all evaluated chemicals in Table 1. The reason for this is that sorption to one of the components dominates, e.g. the compound will mostly reside in the lipid parts of blood and assay. In this case, the value of $f_{u,blood}/f_{u,S9}$ is determined by the ratio of the sorbing contents of both phases. We therefore suggest that the good performance of the ppLFER algorithm combination and Lee/Saunders algorithm combination for the most chemicals is systematic and not by accident. Hence, for strongly sorbing compounds $f_{u,blood}/f_{u,S9}$ should become insensitive to errors in the partition coefficients. Instead, knowledge on the composition of assay and blood becomes important, because the relative contents of sorbing components dictate the value of the plateau for strongly sorbing compounds. Following this consideration, it is surprising that the available experimental data (Table 1) yields two of the highest $f_{u,blood}/f_{u,S9}$ ratios for the two chemicals with the highest log K_{OW}. The reason for this observation is unclear. Determination of very high log K_{OW} values of chemicals is experimentally challenging as are all other partition measurements for highly sorbing chemicals. Additional experimental $f_{u,blood}/f_{u,S9}$ ratios for more chemicals in the high log K_{OW} range, as will be determined in the recently launched Cefic LRI 47 project, could help to further elucidate this issue.

CONCLUSION

As shown above, quite a number of algorithms for estimation of unbound fractions do exist. Given the above presented theoretical considerations and the available experimental data, it is clear that assuming a value of 1 for the f_u ratio cannot be justified. Furthermore, we believe that it is possible to clearly identify which algorithms yield the most reliable prediction results with lowest error sensitivity: these are the algorithms that are ‘mechanistic’ (pp-LFER and Saunders/Lee algorithms) - meaning that they account for the inhomogeneous composition of

assay and blood and describe sorption to the individual components in the same way. The advantage of these composition-based algorithms is that they truly represent the differences in composition causing the different sorption capacities of assay and blood. We thus conclude that the composition-based algorithms should be preferred whenever unbound fractions *in vitro* and *in vivo* are predicted. Such predictions are not only required for *in vitro-in vivo*-extrapolation for bioaccumulation assessment but also for *in vitro-in vivo*-extrapolation of toxicity data. With these composition-based algorithms, the uncertainty of $f_{u,in\ vivo}/f_{u,in\ vitro}$ will be small, especially for strongly sorbing compounds.

SUPPORTING INFORMATION

Tables of $f_{u,S9}$ and $f_{u,blood}$ values predicted for the above chemicals with the different algorithms.

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BIOGRAPHIES

Kai-Uwe Goss is professor for environmental chemistry at the University of Halle-Wittenberg and head of the department for Analytical Environmental Chemistry at the Helmholtz Centre for Environmental Research in Leipzig. He received his PhD from the University of Bayreuth and did a postdoc at the University of Minnesota followed by a research stay at ETH Zürich. His early work was centered at equilibrium partitioning of organic chemicals in various environmental systems. In recent years, his research focus has shifted to bioaccumulation and toxicokinetics of organic chemicals. He has published more than 170 papers in peer reviewed journals (h-index: 44).

Sophia Krause received her PhD in Chemistry from the University of Halle-Wittenberg and is currently working as a postdoctoral researcher in Kai-Uwe Goss' group at the Helmholtz Centre for Environmental Research in Leipzig. Her work focuses on *in vitro-in vivo* extrapolation of biotransformation data and the use of PBTK models for bioaccumulation assessment.

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