In vitro – in vivo extrapolation of hepatic metabolism for different scenarios – a toolbox

Sophia Krause[a], Kai-Uwe Goss[a,b,*]

[a] Helmholtz Centre for Environmental Research UFZ, Department of Analytical Environmental Chemistry, Permoserstr. 15, 04318 Leipzig, Germany

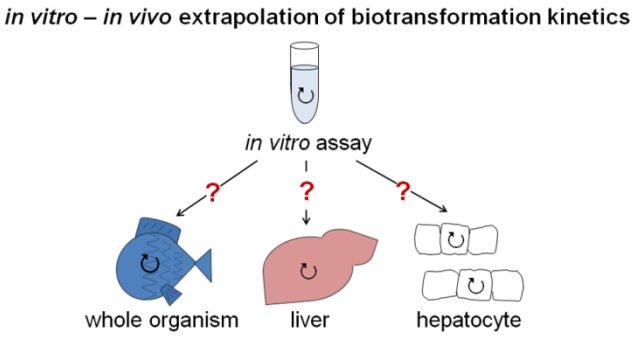
[b] University of Halle-Wittenberg, Institute of Chemistry, Kurt-Mothes-Str. 2, 06120 Halle, Germany

* Address correspondence to Kai-Uwe Goss, kai-uwe.goss@ufz.de

ABSTRACT

The extrapolation of metabolism data from *in vitro* experiments to *in vivo* clearances can provide useful information in the fields of pharmacokinetics and toxicokinetics. Depending on the purpose, different toxicokinetic models are used and these different models require the *in vivo* metabolic information in different forms. In this study, a comprehensive toolbox for *in vitro – in vivo* extrapolation (IVIVE) of hepatic metabolism is presented addressing a variety of different extrapolation goals: extrapolation to hepatic blood clearance, extrapolation to organ clearance, extrapolation to whole-body clearance and extrapolation to clearance at the level of hepatocytes. The use of the extrapolated clearances for calculation of extraction efficiencies and the use in physiologically based pharmacokinetic models are discussed. Furthermore, a sensitivity analysis demonstrates which parameters affect the accuracy of the extrapolation results the most and the presented extrapolation procedure is evaluated by comparison to experimental data from perfused liver experiments.

TOC graphic (?)



INTRODUCTION

There is a broad interest in pharmacology and toxicology on kinetic information about hepatic metabolism of organic chemicals in organisms. In the context of the Replace, Reduce and Refine targets (3Rs) for the use of animals in scientific research this information should preferably come from *in vitro* assays. An extrapolation step is needed in order to use this kinetic *in vitro* information in any kind of toxicokinetic model. This *in vitro – in vivo* extrapolation (IVIVE) has to account for the different amounts of metabolically active components (hepatocytes, microsomal protein or S9 material) *in vitro* and *in vivo* and it has to account for the different environment in both cases. This extrapolation can also contain information on additional kinetic effects on the hepatic metabolism such as blood flow limitation in the liver.

The first equations offered for this extrapolation challenge have been presented in the 1970s in the pharmacology literature [1]. However, a systematic discrepancy between predicted and observed *in vivo* metabolic clearance has frequently been found [2-5]. This and the fact that most published extrapolation schemes focus on one specific scenario [4, 6-8] while other relevant IVIVE scenarios are not covered led us to revisit and explore the mathematics that are required for a consistent and comprehensive IVIVE scheme.

This work is structured as follows: we first state the commonly accepted assumptions and boundary conditions that are needed for a stringent problem definition. In a next section, we summarize the various relevant scenarios of IVIVE of metabolic information and their applications in the context of pharmaco- and toxicokinetics. We then try to give a short literature overview concerning IVIVE schemes that have already been suggested for single specific scenarios. Afterwards we present the mathematics that solves the various extrapolation tasks introduced in the first section. This is followed by a sensitivity analysis for the impact that various variables have on the extrapolation result. Finally we apply our

extrapolation scheme to published data from a perfused liver experiment and discuss the results.

Defining the assumptions for IVIVE

A clear definition of the assumptions and boundary conditions forming the basis of the IVIVE is needed as a starting point. For the *in vitro* experiments this can be summarized as follows: The *in vitro* assay is assumed to be well-stirred with instantaneous sorption equilibrium between all its parts at all times. Any sorption equilibria (e.g. between hepatocytes and water) can be described by known equilibrium partition coefficients:

$$K_{phase1/phase2} = \frac{C_{phase\ 1}}{C_{phase\ 2}}$$

where C_{phase} (g/mL$_{phase}$) refers to the equilibrium concentration in the corresponding total phase and the unit of $K_{phase1/phase2}$ is mL$_{phase2}$/mL$_{phase1}$. The metabolically active components (hepatocytes, microsomal protein or S9 material) in the *in vitro* assay are assumed to behave the same as those *in vivo*. Based on measured concentration-time curves, the *in vitro* clearance in the assay can be determined. By definition, the reported clearance value refers to the assay volume that is cleared of the chemical per unit time [9]. Accordingly, the unit of the *in vitro* clearance in the assay (CL_{assay}) is ml of total assay volume per unit time (mL$_{assay}$/min).

Overview of the manifold extrapolation goals

The goals of the IVIVE process using the measured clearance in the assay, CL_{assay} (in mL$_{assay}$/min), can be manifold. Figure 1 gives an overview of the different extrapolation goals that will be discussed in this text:

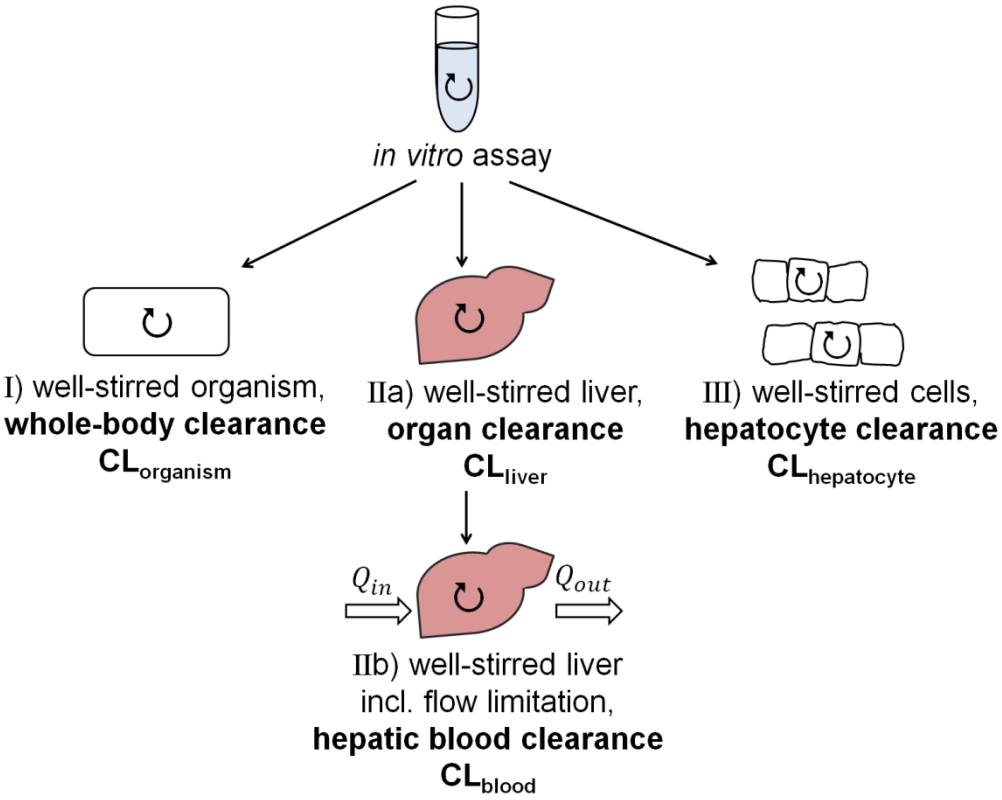


Figure 1: Overview of various extrapolation goals.

I. Extrapolation to a whole organism, $CL_{organism}$ (in $mL_{organism}$/min), that is assumed to be well-stirred with an instantaneous sorption equilibrium.

II. Extrapolation to the liver as a well-stirred organ with instantaneous sorption equilibrium. Here, a subdivision is needed:

IIa) Extrapolation to the actual organ clearance, CL_{liver} (mL_{liver}/min). This CL_{liver} serves as an intermediate step for extrapolation to IIb).

IIb) Extrapolation to a clearance of blood that passes through a well-stirred liver with instantaneous sorption equilibrium within the organ. This hepatic blood clearance additionally accounts for potential blood flow limitation of liver metabolism. It is noteworthy that this is the only case where clearance does not refer to the volume of the well-stirred compartment in which the metabolic transformation takes place but to another, external volume; here blood. For better discrimination, we introduce the

variable CL_{blood} (mL_{blood}/min) for this blood clearance. Note, though, that this hepatic blood clearance is often called 'hepatic clearance' (CL_{hep} or CL_H) in the literature.

III Extrapolation to hepatocytes as well–stirred cells with instantaneous sorption equilibrium within the cells. This hepatocyte clearance, $CL_{hepatocytes}$ ($mL_{hepatocyte}$/min), reflects the capacity of hepatocytes to transform the compound that they contain.

Applications of the extrapolated clearances

I. A whole-body clearance, $CL_{organism}$, based on a one-compartment organism model is useful information as such. In environmental sciences, the whole-body clearance is used as an input parameter for prediction of bioconcentration factors.

IIa) Liver clearance, CL_{liver}, is a required input parameter for multi-compartment toxicokinetic models that eventually allow to calculate whole-body clearance but also other information such as peak concentration in blood and area under the curve (AUC) or explicit concentration time curves in all considered compartments/organs (see Stadnicka-Michalak et al. [10] as an example). Blood circulation is represented explicitly by such models so that blood flow limitation of hepatic metabolism is implicitly covered.

IIb) Hepatic blood clearance can be used to calculate the extraction efficiency of the liver under steady-state conditions [1, 9]. In contrast to liver clearance, this extraction efficiency E is a measure of how efficient blood is cleared from a chemical when passing the liver. E considers potential limitations by blood flow and is defined as the concentration difference between inflowing and outflowing blood relative to the concentration in inflowing blood.

III. Hepatocyte clearance is a required input information for more complex toxicokinetic models in which the liver is not treated as a single compartment, but divided up into liver blood, hepatocytes and other liver tissue. Such a detailed

approach allows to cover additional kinetic processes with potential impact on the final metabolism rate such as: active uptake or excretion in/out of hepatocytes by transporter proteins, permeability limitations between blood and the hepatocytes and slow desorption kinetics of the chemical from albumin in the blood [11]. Hepatocyte clearance is also needed if one wants to calculate an extraction efficiency for the liver that accounts for these additional kinetic processes. In [12] we have shown how all these additional limitations can be built into a formula for the extraction efficiency under steady-state conditions.

IVIVE procedures in the literature

There is a vast amount of literature on IVIVE and a complete review is not possible here. We therefore focus this overview on a few exemplary papers that we found to be the most influential or that seem to represent the state of the art in the field.

First attempts to predict *in vivo* hepatic metabolism from *in vitro* experiments were made more than 40 years ago [1]. Their goal was the prediction of the hepatic extraction efficiency via extrapolation to the hepatic blood clearance (case IIb) above). In the 1990s, this extrapolation procedure was refined regarding the implementation of scaling factors and the consideration of nonspecific binding by introducing binding corrections [6, 13]. Recently, novel methods for binding corrections have been published considering ionization of the metabolized compound and protein-facilitated uptake [14, 15]. The common goal of all these IVIVE-methods remained the extrapolation to the hepatic clearance of blood including blood flow limitation (i.e. case IIb) above). Extrapolation schemes for the other scenarios from our list above are not explicitly covered in the literature. In the following, we derive a mathematic formalism addressing all the scenarios introduced above.

METHODS

Extrapolation to clearance in well-stirred organism, liver or hepatocytes

The general idea of the extrapolation procedure is as follows: There is an intrinsic metabolizing capacity of the hepatocytes, microsomes or S9 material for the studied chemical. The assay and all other systems to which we want to extrapolate (scenarios I to III) have this intrinsic capacity or clearance in common. An extrapolation scheme has to extract this information on intrinsic clearance out of the assay experiments and can then apply it to any new scenario or system. This intrinsic clearance must be normalized to the available amount of viable hepatocytes, S9 material or microsomes.

The following step-by-step derivation follows this general idea: Based on the determined *in vitro* clearance in the assay CL_{assay} (mL_{assay}/min), the decline of total mass ($M_{in\ assay}^{total}$) of a chemical in the assay can be described as a function of time by the following differential equation:

$$\frac{dM_{in\ assay}^{total}}{dt} = \frac{dC_{in\ assay}^{total} V_{assay}^{total}}{dt} = CL_{assay}\ C_{in\ assay}^{total} \qquad (1)$$

with $C_{in\ assay}^{total}$ (in g/mL_{assay}) as the total chemical concentration in the assay. This is the starting point of any IVIVE procedure. It is obvious that the clearance measured in this way must be a function of the amount of metabolic active component (hepatocytes, S9 material or microsomal protein) present in the assay, or, in other words, the assay clearance actually follows a pseudo-first order kinetics. The first step towards extrapolation must therefore be to normalize clearance to the amount of metabolic active component because the amount of metabolic active component at the endpoint of our extrapolation (e.g. in the liver) will differ from the one in the assay. This normalization is done by expanding the equation by the amount of metabolically active component $N_{in\ assay}^{hep/mp}$ (number of hepatocytes or mg of S9 material or microsomal protein in the assay):

$$\frac{dM_{in\ assay}^{total}}{dt} = \frac{CL_{assay}}{N_{in\ assay}^{hep/mp}} * C_{in\ assay}^{total} * N_{in\ assay}^{hep/mp} \quad (2)$$

This new expression is still specific for the assay because the clearance refers to the total assay concentration. Note that the term $\frac{CL_{assay}}{N_{in\ assay}^{hep/mp}}$ is equivalent to what is often called *in vitro* intrinsic clearance in the literature [5, 14]. In the next step we now have to change this specific reference into one that is universal and can be used in all systems. It is generally accepted that only the unbound concentration in the aqueous parts of any system is directly available to enzymatic transformation. Consequently, a universal clearance should refer to the water volume that is cleared from the unbound chemical by the available hepatocytes, S9 material or microsomes. This is achieved by transforming the equation such that it now refers to the unbound concentration in the water of the assay ($C_{assay}^{unbound}$, in mg/mL$_{water}$). For doing so, we replace $C_{in\ assay}^{total}$ by $\frac{C_{assay}^{unbound} V_{assay}^{water}}{f_{assay}^{unbound} V_{assay}^{total}}$:

$$\frac{dM_{in\ assay}^{total}}{dt} = \frac{CL_{assay}}{N_{in\ assay}^{hep/mp}} * \frac{V_{assay}^{water}}{f_{assay}^{unbound} V_{assay}^{total}} C_{assay}^{unbound} * N_{in\ assay}^{hep/mp} \quad (3)$$

Here, $f_{assay}^{unbound}$ refers to the unbound fraction of the compound in the assay, V_{assay}^{water} to the volume of water in the assay (mL$_{water}$) and V_{assay}^{total} to the total volume of the assay (mL$_{assay}$). The term $\frac{V_{assay}^{water}}{V_{assay}^{total}}$ is the water content of the assay expressed as volume fraction, we thus introduce the variable w_{assay} (mL$_{water}$/mL$_{assay}$) for this term. For simplicity we can introduce a new variable, CL_{water}, that quantifies the volume of water in the assay that is cleared from the freely dissolved compound either per hepatocyte and unit time or per mg S9 material or microsomal protein and unit time ($\frac{\mathrm{ml_{water}}}{\mathrm{min} * \mathrm{N^{hep/mp}}}$), defined as

$$CL_{water} = \frac{CL_{assay}}{N_{in\ assay}^{hep/mp}} * \frac{V_{assay}^{water}}{f_{assay}^{unbound} V_{assay}^{total}} \quad (4)$$

So that eq. 3 can be written as

$$\frac{dM_{in\ assay}^{total}}{dt} = CL_{water} * C_{assay}^{unbound} * N_{in\ assay}^{hep/mp} \quad (5)$$

In summary, CL_{water} was obtained by normalizing the experimentally derived CL_{assay} to the amount of metabolically active component and by accounting for the sorption effects in the assay such that the new CL_{water} directly applies to the unbound concentration of the chemical in the aqueous parts of assay.

The same mathematical procedure can be applied to describe clearance in any well-stirred system, e.g. the liver or the whole organism, as follows: We start with the following equation describing the change of total compound mass in the liver ($M_{in\ liver}^{total}$) using the clearance in the liver (CL_{liver}, mL_{liver}/min) and the total compound concentration in the liver ($C_{in\ liver}^{total}$, mg/mL_{liver}):

$$\frac{dM_{in\ liver}^{total}}{dt} = \frac{d\ C_{in\ liver}^{total} V_{liver}^{total}}{dt} = CL_{liver}\ C_{in\ liver}^{total} \quad (6)$$

which is analogue to eq. (1) and develop it further (as shown above for the assay) to eventually receive

$$\frac{dM_{in\ liver}^{total}}{dt} = \frac{CL_{liver}}{N_{in\ liver}^{hep/mp}} * \frac{V_{liver}^{water}}{f_{liver}^{unbound}\ V_{liver}^{total}} C_{liver}^{unbound} * N_{in\ liver}^{hep/mp} \quad (7)$$

Here, $N_{in\ liver}^{hep/mp}$is the amount of metabolically active component in the liver, V_{liver}^{water} is the volume of water in the liver (mL_{water}), $f_{liver}^{unbound}$ is the unbound compound fraction in the liver V_{liver}^{total} (mL_{liver}) is the total volume of the liver and $C_{liver}^{unbound}$ is the unbound concentration in the aqueous parts of the liver (mg/mL_{water}).

Analogously, for a whole well-stirred organism the following equation results:

$$\frac{dM_{in\ organism}^{total}}{dt} = \frac{CL_{organism}}{N_{in\ organism}^{hep/mp}} * \frac{V_{organism}^{water}}{f_{organism}^{unbound}\ V_{organism}^{total}} C_{organism}^{unbound} * N_{in\ organism}^{hep/mp} \quad (8)$$

Here, $CL_{organism}$($mL_{organism}$/min) is the clearance in the organism, $N_{in\ organism}^{hep/mp}$ is the amount of metabolically active component in the organism, $V_{organism}^{water}$ (mL_{water}) is the volume of water

in the organism, $f_{organism}^{unbound}$ is the unbound compound fraction in the organism, $V_{organism}^{total}$ ($mL_{organism}$) is the total volume of the liver and $C_{organism}^{unbound}$(mg/mL_{water}) is the unbound compound concentration in the aqueous parts of the organism.

Again, we can extract an intrinsic clearance for water CL_{water} ($\frac{\mathrm{ml_{water}}}{\mathrm{min} * \mathrm{N}^{\mathrm{hep/mp}}}$) from these expressions according to

$$CL_{water} = \frac{CL_{liver}}{N_{in\ liver}^{hep/mp}} * \frac{V_{liver}^{water}}{f_{liver}^{unbound}\ V_{liver}^{total}} \tag{9}$$

for the liver, or

$$CL_{water} = \frac{CL_{organism}}{N_{in\ organism}^{hep/mp}} * \frac{V_{organism}^{water}}{f_{organism}^{unbound}\ V_{organism}^{total}} \tag{10}$$

for a whole organism. In these equations, the terms $\frac{V_{liver}^{water}}{V_{liver}^{total}}$ and $\frac{V_{organism}^{water}}{V_{organism}^{total}}$ are the water contents of liver and organism expressed as volume fractions. For simplicity, we replace these terms by the variables w_{liver} (in mL_{water}/mL_{liver}) and $w_{organism}$ (in $mL_{water}/mL_{organism}$).

In all three systems (the assay, the liver and the whole organism) the water clearance normalized to the amount of hepatocytes, microsomes or S9 material must be the same, if the hepatocytes, microsomes or S9 material are of the same type. Hence, by equating eq. (4) and eq. (9) we get the extrapolation scheme for extrapolation from the assay to the well-stirred liver and by equating eq. (4) and eq. (10) we get the extrapolation scheme for extrapolation to the well-stirred organism. Using the extrapolation to the well-stirred liver as an example, i.e. equating eq. (4) and eq. (9), yields:

$$\frac{CL_{liver}}{N_{in\ liver}^{hep/mp}} * \frac{w_{liver}}{f_{liver}^{unbound}} = \frac{CL_{assay}}{N_{in\ assay}^{hep/mp}} * \frac{w_{assay}}{f_{assay}^{unbound}} \tag{11}$$

This can be rearranged to

$$CL_{liver} = \frac{f_{liver}^{unbound}}{f_{assay}^{unbound}} * CL_{assay} * \frac{N_{in\ liver}^{hep/mp}}{N_{in\ assay}^{hep/mp}} * \frac{w_{assay}}{w_{liver}} \quad (12)$$

Analogously, the expression for extrapolation from assay to well-stirred organism is:

$$CL_{organism} = \frac{f_{organism}^{unbound}}{f_{assay}^{unbound}} * CL_{assay} * \frac{N_{in\ organism}^{hep/mp}}{N_{in\ assay}^{hep/mp}} * \frac{w_{assay}}{w_{organism}} \quad (13)$$

And for extrapolation to well-stirred hepatocytes:

$$CL_{hepatocyte} = \frac{f_{hepatocyte}^{unbound}}{f_{assay}^{unbound}} * CL_{assay} * \frac{N_{in\ hepatocyte}^{hep/mp}}{N_{in\ assay}^{hep/mp}} * \frac{w_{assay}}{w_{hepatocyte}} \quad (14)$$

In fact, eq. (12), (13) and (14) now represent the solutions for extrapolation to the goals I), IIa) and III) described above.

However, the unbound fractions required in these equations can be difficult to measure. If linear equilibrium partitioning is assumed, one can proceed by rearranging the terms containing unbound fractions so that they contain only volumes and partition coefficients. The volume information is easy to achieve and the required partition coefficients can be predicted from combining the contributing sorption to proteins, lipids and water of the phase of interest (i.e. liver, organism or hepatocyte) to the overall sorption to this phase [16]. Accordingly, the term $\frac{f_{liver}^{unbound}}{f_{assay}^{unbound}}$ can be substituted by $\frac{K_{assay/water}}{K_{liver/water}} * \frac{w_{liver}}{w_{assay}}$ (see SI section 3 for details of this step). Here, $K_{assay/water}$ (mL_{water}/mL_{assay}) and $K_{liver/water}$ (mL_{water}/mL_{liver}) are the equilibrium partition coefficients between assay and water or liver and water, w_{liver} (mL_{water}/mL_{liver}) and w_{assay} (mL_{water}/mL_{assay}) are the water contents of liver and assay.

In case one prefers alternative methods for the binding correction, e.g. methods additionally considering ionization of the metabolized compound and protein-facilitated uptake [14, 15], this step can be adapted accordingly. Here we focus on the case of linear equilibrium partitioning and substitute $\frac{f_{liver}^{unbound}}{f_{assay}^{unbound}}$ so that eq. (12) simplifies to:

$$CL_{liver} = CL_{assay} * \frac{K_{assay/water}}{K_{liver/water}} * \frac{N_{in\ liver}^{hep/mp}}{N_{in\ assay}^{hep/mp}} \quad (15)$$

Analogously, the equation for extrapolation from assay to well-stirred organism simplifies to:

$$CL_{organism} = CL_{assay} * \frac{K_{assay/water}}{K_{organism/water}} * \frac{N_{in\ organism}^{hep/mp}}{N_{in\ assay}^{hep/mp}} \quad (16)$$

and for extrapolation to the well-stirred hepatocytes:

$$CL_{hepatocyte} = CL_{assay} * \frac{K_{assay/water}}{K_{hep/water}} * \frac{N_{in\ hep}^{hep/mp}}{N_{in\ assay}^{hep/mp}} \quad (17)$$

By this, equations (15), (16) and (17) also represent solutions for the extrapolation goals I), IIa) and III). Of course, they are mathematically equivalent to the equations (12), (13) and (14), the only difference is that they use partition coefficients instead of unbound fractions to consider the sorption effects that differ between assay and liver, organism or hepatocytes.

Eq. (12) - (17) are either based on fractions unbound or on partition constants but not on a mixture of both. However, practitioners may often be in the situation where they know fraction unbound in the assay but only the partition constant for the system to which the extrapolation is aiming (e.g. the liver, whole organism, hepatocytes). For these cases, one can derive alternative forms of the IVIVE equations using 'mixed' partition information. These equations can be found in SI section 6.

Further Extrapolation to hepatic blood clearance with flow limitation

The above derivation has demonstrated that extrapolation from one well-stirred system (e.g. the assay) to any other well-stirred system (liver, organism, hepatocytes) always follows the same scheme. For our extrapolation scenario IIb) however, the goal is different: in IIb) we want to arrive at the clearance of blood that passes through a well-stirred liver. Accordingly, this clearance has to account for the effects of blood flow limitation; i.e. the situation where the actual clearance is smaller than expected based on CL_{liver} because the transport capacity of

the blood for the chemical is not sufficiently high. The solution of this case can be derived by combining the previously extrapolated liver clearance with the liver blood flow in a well-stirred liver model as introduced by Rowland et al. [9] ($k_m V_{liver}$ used by Rowland is equivalent to CL_{liver}):

$$CL_{blood} = \frac{Q_H * CL_{liver} K_{liver/blood}}{Q_H + CL_{liver} K_{liver/blood}} \quad (18)$$

As mentioned above, this clearance is commonly called hepatic clearance CL_{hep} in the literature, but we prefer the variable CL_{blood} for better discrimination from CL_{liver}. In this equation, Q_H is the liver blood flow in mL of blood per unit time (mL_{blood}/min) and $K_{liver/blood}$ is the liver-blood partition coefficient for the compound. Substituting CL_{liver} by the expression presented in eq. (12) allows direct calculation of the hepatic clearance of blood, CL_{blood}, from the measured CL_{assay}:

$$CL_{blood} = \frac{Q_H * \frac{f_{liver}^{unbound}}{f_{assay}^{unbound}} * CL_{assay} * \frac{N_{in\ liver}^{hep/mp}}{N_{in\ assay}^{hep/mp}} * \frac{w_{assay}}{w_{liver}} * K_{liver/blood}}{Q_H + \frac{f_{liver}^{unbound}}{f_{assay}^{unbound}} * CL_{assay} * \frac{N_{in\ liver}^{hep/mp}}{N_{in\ assay}^{hep/mp}} * \frac{w_{assay}}{w_{liver}} * K_{liver/blood}} \quad (19)$$

After suitable rearrangement (see SI section 4 for details) this equation simplifies to:

$$CL_{blood} = \frac{Q_H * \frac{f_{blood}^{unbound}}{f_{assay}^{unbound}} * CL_{assay} * \frac{N_{in\ liver}^{hep/mp}}{N_{in\ assay}^{hep/mp}} * \frac{w_{assay}}{w_{blood}}}{Q_H + \frac{f_{blood}^{unbound}}{f_{assay}^{unbound}} * CL_{assay} * \frac{N_{in\ liver}^{hep/mp}}{N_{in\ assay}^{hep/mp}} * \frac{w_{assay}}{w_{blood}}} \quad (20)$$

Here, w_{assay} of course still refers to the water content of the assay (mL_{water}/mL_{assay}) and $f_{assay}^{unbound}$ to the unbound compound fraction in the assay and, analogously, w_{blood} refers to the water content of blood (mL_{water}/mL_{blood}) and $f_{blood}^{unbound}$ to the unbound compound fraction in blood. Eq. (20) yields the hepatic blood clearance CL_{blood} including potential blood flow limitation in the unit mL_{blood}/min. If one prefers the blood clearance expressed in a unit that is normalized to kg of bodyweight, one has to use blood flow normalized to kg bodyweight

($Q_{H,bodyweight}$, in $mL_{blood}/min/kg_{bodyweight}$) and one has to use the content of metabolically active component per bodyweight instead of the absolute amount $N_{in\ liver}^{hep/mp}$ in eq. (20). We thus introduce the variable *MPBW* (in number of hepatocytes or mg of S9/microsomes per kg bodyweight) for the content of metabolically active component per kg bodyweight. The metabolically active component per kg bodyweight, *MPBW*, in humans is given by commonly used scaling factors: in case of hepatocytes one multiplies the hepatocellularity ($99 * 10^6$ $\frac{\text{hepatocytes}}{g_{liver}}$) [17] with the liver weight per kg bodyweight ($20\ \frac{g_{liver}}{kg_{bodyweight}}$) to get *MPBW*, in case of microsomes one multiplies the microsomal protein content ($32\ \frac{mg_{microsomal\ protein}}{g_{liver}}$) [17] with the liver weight per kg bodyweight ($20\ \frac{g_{liver}}{kg_{bodyweight}}$) [3] to get *MPBW*. Using *MPBW* for calculation of blood clearance yields:

$$CL_{blood,bodyweight} = \frac{Q_{H,bodyweight} * \frac{f_{blood}^{unbound}}{f_{assay}^{unbound}} * CL_{assay} \frac{MPBW}{N_{in\ assay}^{hep/mp}} * \frac{w_{assay}}{w_{blood}}}{Q_{H,bodyweight} + \frac{f_{blood}^{unbound}}{f_{assay}^{unbound}} * CL_{assay} \frac{MPBW}{N_{in\ assay}^{hep/mp}} * \frac{w_{assay}}{w_{blood}}} \quad (21)$$

One has to note that the clearance calculated via eq. (21) still is a blood clearance (in $mL_{blood}/min/kg_{bodyweight}$) and not a whole-body clearance ($mL_{organism}/min$), i.e. $CL_{blood,bodyweight}$ describes the effect of hepatic metabolism on a compound concentration in blood. Note, that in the literature, the terms $CL_{assay} \frac{MPBW}{N_{in\ assay}^{hep/mp}}$ and $\frac{f_{blood}^{unbound}}{f_{assay}^{unbound}}$ are often substituted by the shorter but potentially unclear variable names '*in vivo* intrinsic clearance' and 'f_u' [5].

Of course, one can also create an alternative version of the equation for calculation of hepatic blood clearance with flow limitation that uses partition coefficients instead of unbound fractions. Details for the derivation of this alternative version can be found in SI section 5. The resulting equation for hepatic blood clearance in the unit mL_{blood}/min is:

$$CL_{blood} = \frac{Q_H * \ CL_{assay} \frac{K_{assay/water}}{K_{blood/water}} \frac{N_{in\ liver}^{hep/mp}}{N_{in\ assay}^{hep/mp}}}{Q_H + \ CL_{assay} \frac{K_{assay/water}}{K_{blood/water}} \frac{N_{in\ liver}^{hep/mp}}{N_{in\ assay}^{hep/mp}}} \quad (22)$$

and for the hepatic blood clearance in the unit $mL_{blood}/min/kg_{bodyweight}$:

$$CL_{blood,bodyweight} = \frac{Q_{H,bodyweight} * \ CL_{assay} \frac{K_{assay/water}}{K_{blood/water}} \frac{MPBW}{N_{in\ assay}^{hep/mp}}}{Q_{H,bodyweight} + \ CL_{assay} \frac{K_{assay/water}}{K_{blood/water}} \frac{MPBW}{N_{in\ assay}^{hep/mp}}} \quad (23)$$

RESULTS & DISCUSSION

Our stepwise derivation of the extrapolation mathematics yielded specific equations for all desired extrapolation goals: extrapolation from the assay clearance to the clearance in other well-stirred systems like hepatocytes, liver or a whole organism and extrapolation from the assay clearance to the clearance of blood that flows through a well-stirred liver. For better overview, Table 1 shows all extrapolation goals with the numbers of the corresponding final equations at a glance:

Table 1: Overview of extrapolation goals and corresponding equations for two types of sorption information.

sorption information in form of	extrapolation to			
	I) whole-body clearance	IIa) organ clearance	IIb) hepatic blood clearance	III) hepatocyte clearance
known unbound fraction	(13)	(12)	(20) or normalized to bodyweight with (21)	(14)
known partition coefficient	(16)	(15)	(22) or normalized to	(17)

			bodyweight with (23)	

In the supporting information of this manuscript, the generalized forms of the here derived equations for extrapolation between any two well-mixed systems are presented and a guide for equation selection is given in table S1 (SI section 1).

As described in the introduction, the extrapolation schemes available in the literature target only the scenario of extrapolation to the clearance of blood that flows through a well-stirred liver [1, 4, 8, 13, 15]. Comparison of the results above with the extrapolation schemes available in the literature is difficult, because the used variables differ in their normalizations and units and derivations of the used equations are often not provided in detail. One of the first extrapolation schemes that includes binding correction and that is frequently cited was presented by Obach [13] yielding three different equations for extrapolation to hepatic blood clearance in a well-stirred liver. These three extrapolation procedures presented by Obach differ in the way in which binding correction is performed. Among these, the procedure that considers unspecific binding in the assay as well unspecific binding *in vivo* comes closest to our extrapolation procedure. The equation that Obach provides for this extrapolation procedure is:

$$CL^{Obach}_{blood,body\ weight} = \frac{Q_{H,bodyweight} * \frac{f^{unbound}_{blood}}{f^{unbound}_{assay}} * CL'_{int}}{Q_{h,bodyweight} + \frac{f^{unbound}_{blood}}{f^{unbound}_{assay}} * CL'_{int}} \quad (24)$$

According to Obach, this equation yields a blood clearance in $mL_{blood}/min/kg_{bodyweight}$ that is calculated by using the hepatic blood flow $Q_{H,bodyweight}$ normalized to bodyweight and the scaled *in vivo* clearance CL'_{int} normalized to bodyweight. Scaling and normalization of CL'_{int} to bodyweight is achieved via multiplication of the measured assay clearance that is

normalized to mg of microsomal protein in the assay with the content of metabolically active component per kg bodyweight ($MPBW = 32\ \frac{\mathrm{mg_{microsomal\ protein}}}{\mathrm{g_{liver}}} * 20\ \frac{\mathrm{g_{liver}}}{\mathrm{kg_{bodyweight}}}$):

$$CL'_{int} = \frac{CL_{assay}}{N_{in\ assay}^{hep/mp}} * MPBW \tag{25}$$

Accordingly, eq. (24) can be rewritten to:

$$CL_{blood,body\ weight}^{Obach} = \frac{Q_{H,bodyweight} * \frac{f_{blood}^{unbound}}{f_{assay}^{unbound}} * CL_{assay} \frac{MPBW}{N_{in\ assay}^{hep/mp}}}{Q_{H,bodyweight} + \frac{f_{blood}^{unbound}}{f_{assay}^{unbound}} * CL_{assay} \frac{MPBW}{N_{in\ assay}^{hep/mp}}} \tag{26}$$

Eq. (26) representing the Obach approach can be directly compared to our eq. (21) because both are supposed to describe the same extrapolation scheme. The comparison reveals, though, that they are not exactly equivalent. Our eq. (21) contains a ratio of water contents that is not included in the Obach equation and the resulting units are different in both equations. The term $\frac{f_{blood}^{unbound}}{f_{assay}^{unbound}} * CL_{assay} \frac{MPBW}{N_{in\ assay}^{hep/mp}} * \frac{w_{assay}}{w_{blood}}$ in our eq. (21) has units of $mL_{blood}/min/kg_{bodyweight}$ as one would expect for blood clearance, whereas the corresponding term $\frac{f_{blood}^{unbound}}{f_{assay}^{unbound}} * CL_{assay} \frac{MPBW}{N_{in\ assay}^{hep/mp}}$ in eq. (26) from Obach has units of $mL_{assay}/min/kg_{bodyweight}$ which implies that this term represents an assay clearance. Numerically, however, both equations produce very similar numbers, because the term $\frac{w_{assay}}{w_{blood}}$ by which both equations differ is rather close to unity. More precisely, the water content of blood is 0.8 mL_{water}/mL_{blood} [18] and the water content of the assay will always be close to 1 mL_{water}/mL_{assay}. Accordingly, the ratio of the water contents, $\frac{w_{assay}}{w_{blood}}$, roughly yields 1.25 and the numerical difference between both equations thus won't exceed 25 %. This is not sufficient to explain the discrepancies that have been found in the past (see introduction). These might rather have their cause in the neglect of extrahepatic metabolism.

As an amendment to our equations shown above we have also set out to derive the extrapolation scheme based on rate constants instead of clearance and using an alternative starting point. This derivation (shown in SI section 6) is in fact shorter and – in our opinion – easier to understand intuitively than the one given in the main text that uses the clearance concept. The result derived from this alternative approach at the end is identical to the one shown above in eqs. (15), (16) and (17). Plausibility of the alternative derivation can be checked via a thought experiment for chemicals with a small unbound fraction (SI section 7). The fact that the alternative approach (SI section 6) for solving the extrapolation goals I-III gives the same results as in our main text and the plausibility check (SI section 7) was successful provides assurance that our presented extrapolation schemes are mathematically correct.

Sensitivity Analysis. Equations (12) - (14) constitute the central equations for IVIVE for those three scenarios that do not account for blood flow limitation (see Fig.1). Obviously, any error in the *in vitro* clearance value, CL_{assay}, will proportionally affect the extrapolated clearance value. For the most part, this error in the *in vitro* data is probably a systematic one that is connected to the quality and representativeness of the hepatocytes, S9 material or microsomes. If one compares normalized *in vitro* clearance values from different labs for given chemicals one finds quite high discrepancies. For pyrene Lee et al. [19] reported an assay clearance normalized to the amount of S9 (from trout liver) in assay of 0.4 mL/h/mg_{S9}, whereas Nichols reported a value of 10.1 mL/h/mg_{S9} [20] and for benzo(a)pyrene Han et al. [21] published a value of 0.07 mL/h/mg_{S9}, whereas Nichols determined a value of 16.7 mL/h/mg_{S9} [20]. Besides the *in vitro* metabolic information, input information on the amount of metabolically active components, on the unbound fractions and on the relative water contents of the assay and the target system (whole organism, liver or hepatocytes) is required. The amount of metabolically active components and the relative water contents are either well-known from the literature [3, 17, 18] or set by the investigator so that this information should have

high accuracy (the relative error is likely below 5%) and the error in the extrapolated clearance resulting from the uncertainties of these values is below 20 %. Errors in the information on fraction unbound can be considerably higher if estimated values are used due to the high uncertainties in the respective partition coefficients. However, in the two extreme situations (i.e. fraction unbound is very high or very low) the term $\frac{f_{liver}^{unbound}}{f_{assay}^{unbound}}$ is not determined by the partition coefficients of the chemical anymore but by the quotient of either the relative aqueous volumes in assay and organism/liver/hepatocytes or by relative volumes of sorbing tissue in assay and organism/liver/hepatocytes. Hence, only for chemicals in a moderate range of hydrophobicity the accurate knowledge of the partition coefficient is crucial for the quality of the term $\frac{f_{liver}^{unbound}}{f_{assay}^{unbound}}$ and thus for the quality of the whole extrapolation result. This qualitative conclusion can be supported by more quantitative information if one calculates $\frac{f_{liver}^{unbound}}{f_{assay}^{unbound}}$ based on the log of the octanol-water partition coefficients, log K_{ow}, of the chemicals (for details see SI section 9). In Fig. 2 we show the results for such a calculation for a range of octanol-water partition coefficients representing chemicals with different hydrophobicity. To include a calculation of the propagated error we assumed that the actual partition coefficients might be a factor 3 higher or lower than the value used and plotted the results against log K_{ow} (Figure 2).

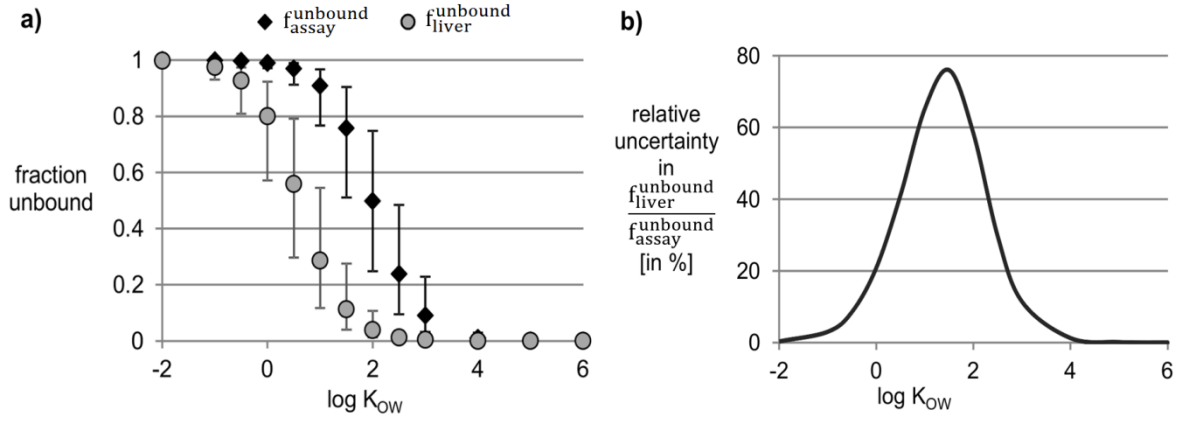


Figure 2: Uncertainties in the fractions unbound in assay and liver estimated using octanol-water partition coefficients (part a) and the resulting relative error in $\frac{f_{liver}^{unbound}}{f_{assay}^{unbound}}$ (part b).

Figure 2 indicates that the fractions unbound in assay and liver are most sensitive to potential errors for chemicals with log K_{OW} between 0 and 3 (Figure 2a). Accordingly, the relative uncertainty in the term $\frac{f_{liver}^{unbound}}{f_{assay}^{unbound}}$ is biggest for chemicals with log K_{OW} between 0 and 3 as well (Figure 2b). Information on the relevant partition coefficients of these chemicals thus needs to be known quite reliably or measurements of the fractions unbound are needed. For chemicals with log K_{OW} lower than 0 or higher than 3, the relative uncertainty in $\frac{f_{liver}^{unbound}}{f_{assay}^{unbound}}$ becomes small and the accuracy of the partition coefficient used to estimate $\frac{f_{liver}^{unbound}}{f_{assay}^{unbound}}$ is not relevant for the accuracy of the extrapolation result.

For the last extrapolation scenario, the extrapolation to hepatic blood clearance considering blood flow limitation, one needs to discriminate the not-blood flow-limited from the blood flow-limited case when discussing potential errors. For the first case, the extrapolation scheme simplifies to the scenarios discussed above. For the latter case, the blood flow-limited case, the extrapolation result simply approaches the blood flow rate and thus becomes insensitive to errors resulting from the extrapolation procedure.

Evaluation of perfused liver data

The presented toolbox for IVIVE offers solutions for various extrapolation goals. For a comparison of extrapolated clearances with measured clearances the level of the hepatic blood clearance determined in isolated perfused liver experiments is most suitable. In these experiments, the pure metabolic capacity of the liver is determined because other factors like extrahepatic metabolism or elimination via urine are excluded. Here, we extrapolate *in vitro* data from trout S9 for six PAHs (polycyclic aromatic hydrocarbons) to the level of hepatic

blood clearances and compare the extrapolation results with the clearances determined in isolated perfused trout liver experiments published by Nichols et al. [20]. In this study, isolated trout livers were perfused with perfusates containing the PAHs and either 1 mg/mL bovine serum albumin (BSA) or 10 mg/mL BSA. Via analysis of the PAH concentrations in the perfusate, hepatic blood clearance or, more precisely (because perfusate instead of blood is used in the experiments), hepatic perfusate clearance was determined. For comparison, we extrapolated *in vitro* data for the same PAHs to hepatic perfusate clearance using eq. (20) (for details see SI section 10). By using eq. (20) blood flow limitation is considered in the extrapolation. Additionally we calculated a second set of clearances where blood flow limitation is neglected (for details see SI section 10). The results are shown in Figure 3, a complete overview of the used data can be found in SI section 10.

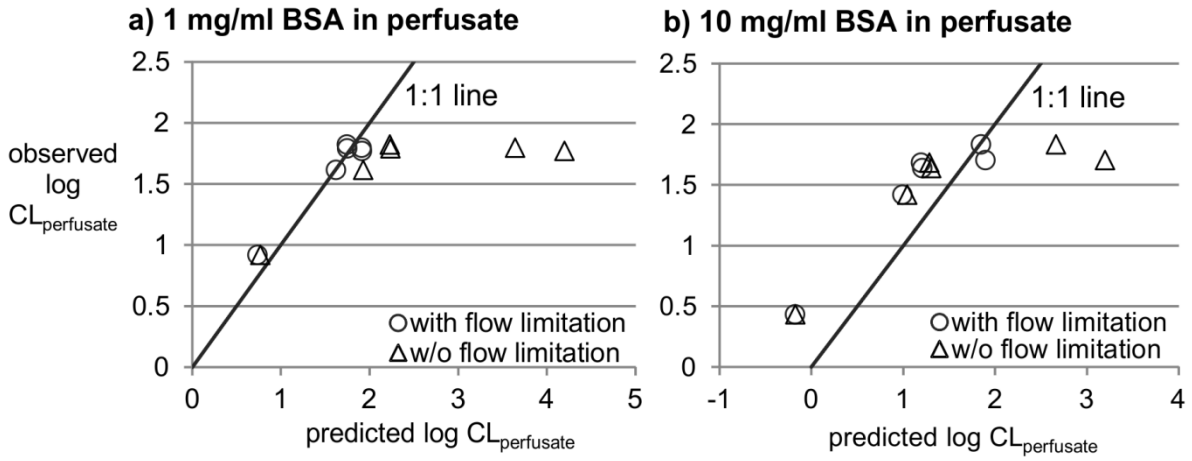


Figure 3: Comparison of predicted clearances with observed clearances from isolated perfused liver experiments for six PAHs. Part a) shows results for experiments with 1 mg/ml BSA in perfusate, part b) shows results for experiments with 10 mg/ml in perfusate.

In Figure 3, the observed log $CL_{perfusate}$ is plotted versus the predicted log $CL_{perfusate}$. The 1:1 line is shown for orientation. The triangles represent the extrapolation results when blood flow limitation is not considered; the circles represent extrapolation results when blood flow limitation is accounted for. For the best-metabolized PAHs (highest observed $CL_{perfusate}$), hepatic metabolism appears to be blood flow limited because the extrapolation results neglecting blood flow greatly exceed (up to 2 log-units) the extrapolation results considering

blood flow; and the latter are in fact in good agreement with the measured values. The largest discrepancy between measured and extrapolated $CL_{perfusate}$ occurs for naphthalene that lies in the hydrophobicity range (log K_{OW} = 3.3) where the highest error from estimated unbound fractions must be expected (see Figure 2b). Another explanation for the remaining discrepancies could be an altered metabolic capacity of the used S9-material in the *in vitro*-experiments compared to the intact liver used in perfused liver experiments. Such a systematic error could not be corrected for by the extrapolation scheme but could be avoided by optimization of the *in vitro* test system.

Conclusion

The here derived set of equations presents solutions for *in vitro*-*in vivo* extrapolation from all kinds of *in vitro* assays (hepatocytes, S9-material, microsomes) to various *in vivo* scales (whole organism, liver, blood, hepatocytes). With this extrapolation toolbox at hand it should now become easy to pick out the appropriate extrapolation pathway for any practical problem of *in vivo* – *in vitro* extrapolation for hepatic transformation kinetics.

ACKNOWLEDGEMENTS

The authors thank Nadin Ulrich for helpful discussions.

SUPPORTING INFORMATION

A guide for equation selection, a comparison of the variable names used here and used in the literature, detailed derivations of the presented equations for extrapolation, an alternative derivation of the presented IVIVE scheme using rate constants instead of clearances as an alternative starting point, a thought experiment as simple plausibility check, a derivation for inclusion of blood flow limitation and details for the calculations for the sensitivity analysis and the perfused liver data are shown in a supporting PDF file.

REFERENCES

(1) Rane, A., Wilkinson, G. R., and Shand, D. G. (1977) Prediction of hepatic extraction ratio from *in vitro* measurement of intrinsic clearance. *Journal of Pharmacology and Experimental Therapeutics 200,* 420-424.

(2) Chiba, M., Ishii, Y., and Sugiyama, Y. (2009) Prediction of hepatic clearance in human from *in vitro* data for successful drug development. *Aaps J 11,* 262-276.

(3) Obach, R. S. (1997) Nonspecific binding to microsomes: impact on scale-up of *in vitro* intrinsic clearance to hepatic clearance as assessed through examination of warfarin, imipramine, and propranolol. *Drug Metabolism and Disposition 25,* 1359-1369.

(4) Obach, R. S. (1999) Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: an examination of *in vitro* half-life approach and nonspecific binding to microsomes. *Drug Metabolism and Disposition 27,* 1350-1359.

(5) Nichols, J. W., Huggett, D. B., Arnot, J. A., Fitzsimmons, P. N., and Cowan-Ellsberry, C. E. (2013) Toward improved models for predicting bioconcentration of well-metabolized compounds by rainbow trout using measured rates of *in vitro* intrinsic clearance. *Environmental Toxicology and Chemistry 32,* 1611-1622.

(6) Brian Houston, J., and Carlile, D. J. (1997) Prediction of hepatic clearance from microsomes, hepatocytes, and liver slices. *Drug Metabolism Reviews 29,* 891-922.

(7) Jones, H., and Houston, J. B. (2004) Use of the substrate depletion approach for determining *in vitro* metabolic clearance: time dependencies in hepatocyte and microsomal incubations. *Drug Metabolism and Disposition 32,* 973-982.

(8) Poulin, P., and Haddad, S. (2013) Toward a new paradigm for the efficient *in vitro–in vivo* extrapolation of metabolic clearance in humans from hepatocyte data. *Journal of Pharmaceutical Sciences 102,* 3239-3251.

(9) Rowland, M., Benet, L. Z., and Graham, G. G. (1973) Clearance concepts in pharmacokinetics. *Journal of Pharmacokinetics and Biopharmaceutics 1,* 123-136.

(10) Stadnicka-Michalak, J., Weiss, F. T., Fischer, M., Tanneberger, K., and Schirmer, K. (2018) Biotransformation of benzo[a]pyrene by three rainbow trout (*Onchorhynchus mykiss*) cell lines and extrapolation to derive a fish bioconcentration factor. *Environmental Science & Technology 52,* 3091-3100.

(11) Larisch, W., Brown, T. N., and Goss, K.-U. (2017) A toxicokinetic model for fish including multiphase sorption features. *Environmental Toxicology and Chemistry 36,* 1538-1546.

(12) Krause, S., and Goss, K.-U. (2018) The impact of desorption kinetics from albumin on hepatic extraction efficiency and hepatic clearance: a model study. *Archives of Toxicology 92*, 2175-2182.

(13) Obach, R. S. (1996) The importance of nonspecific binding in *in vitro* matrices, its impact on enzyme kinetic studies of drug metabolism reactions, and implications for *in vitro-in vivo* correlations. *Drug Metabolism and Disposition 24*, 1047-1049.

(14) Poulin, P., Kenny, J. R., Hop, C. E. C. A., and Haddad, S. (2012) *In vitro–in vivo* extrapolation of clearance: modeling hepatic metabolic clearance of highly bound drugs and comparative assessment with existing calculation methods. *Journal of Pharmaceutical Sciences 101*, 838-851.

(15) Berezhkovskiy, L. M. (2011) The corrected traditional equations for calculation of hepatic clearance that account for the difference in drug ionization in extracellular and intracellular tissue water and the corresponding corrected PBPK equation. *Journal of Pharmaceutical Sciences 100*, 1167-1183.

(16) Endo, S., Brown, T. N., and Goss, K.-U. (2013) General model for estimating partition coefficients to organisms and their tissues using the biological compositions and polyparameter linear free energy relationships. *Environmental Science & Technology 47*, 6630-6639.

(17) Zoe, E. B., Martin, K. B., Philip, H. B., Alan, R. B., David, J. C., Robert, J. E., Houston, J. B., Brian, G. L., John, C. L., Olavi, R. P., Geoffrey, T. T., and Amin, R.-H. (2007) Scaling factors for the extrapolation of *in vivo* metabolic drug clearance from *in vitro* data: reaching a consensus on values of human microsomal protein and hepatocellularity per gram of liver. *Current Drug Metabolism 8*, 33-45.

(18) Snyder, W., Cook, M., Nasset, E., Karhausen, L., Howells, G., and Tipton, I. (1975) ICRP Publication 23: report of the task group on reference man, International Commission on Radiological Protection.

(19) Lee, Y.-S., Lee, D. H. Y., Delafoulhouze, M., Otton, S. V., Moore, M. M., Kennedy, C. J., and Gobas, F. A. P. C. (2014) *In vitro* biotransformation rates in fish liver S9: Effect of dosing techniques. *Environmental Toxicology and Chemistry 33*, 1885-1893.

(20) Nichols, J. W., Hoffman, A. D., ter Laak, T. L., and Fitzsimmons, P. N. (2013) Hepatic clearance of 6 polycyclic aromatic hydrocarbons by isolated perfused trout livers: prediction from *in vitro* clearance by liver S9 fractions. *Toxicological Sciences 136*, 359-372.

(21) Han, X., Nabb, D. L., Yang, C.-H., Snajdr, S. I., and Mingoia, R. T. (2009) Liver microsomes and S9 from rainbow trout (*Oncorhynchus mykiss*): Comparison of basal-level enzyme activities with rat and determination of xenobiotic intrinsic clearance in support of bioaccumulation assessment. *Environmental Toxicology and Chemistry 28*, 481-488.