Research Article

Impact of *In Vitro* Experimental Variation in Kinetic Parameters on Physiologically Based Kinetic (PBK) Model Simulations

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Abstract

In vitro toxicokinetic data are critical in meeting an increased regulatory need to improve chemical safety evaluations towards a better understanding of internal human chemical exposure and toxicity. *In vitro* intrinsic hepatic clearance (CL_{int}), the fraction unbound in plasma (fu_p), and the intestinal apparent permeability (P_{app}) are important parameters as input in a physiologically based kinetic (PBK) model to make first estimates of internal exposure after oral dosing. In the present study we explored the experimental variation in the values for these parameters as reported in the literature. Furthermore, the impact that this experimental variation has on PBK model predictions of maximum plasma concentration (C_{max}) and the area under the concentration time curve ($AUC_{0\text{-}24h}$) was determined. As a result of the experimental variation in CL_{int}, P_{app}, and fu_p, the predicted variation in C_{max} for individual compounds ranged between 1.4- to 28-fold, and the predicted variation in $AUC_{0\text{-}24h}$ ranged between 1.4- and 23-fold. These results indicate that there are still some important steps to take to achieve robust data that can be used in regulatory applications. To gain regulatory acceptance of *in vitro* kinetic data and PBK models based on *in vitro* input data, the boundaries in experimental conditions as well as the applicability domain and the use of different *in vitro* kinetic models need to be described in guidance documents.

1 Introduction

In 2020, the European Commission launched its EU Chemicals Strategy for Sustainability under the Green Deal. Key aspects of this strategy are to ban most harmful chemicals, to improve safe and sustainable chemicals by design, and to obtain a better account of potential "cocktail effects" (i.e., effects upon combined exposure) of chemicals (European Commission, 2019, 2020). Respective chemical safety data cannot only be obtained with traditional animal testing, which is costly and time-consuming and therefore not applicable to large numbers of compounds. Therefore, there is an increasing need for the regulatory use of animal-free testing strategies (Arnesdotter et al., 2021; Paul Friedman et al., 2020; de Boer et al., 2020). Absorption, distribution, metabolism and excretion of compounds, i.e., the kinetics, have a critical role in such animal-free testing strategies as understanding them can improve the interpretation of *in vitro* toxicity results, allowing to estimate the internal plasma and tissue concentrations in humans after oral, dermal, or inhalation exposures (Louisse et al., 2017; Blaauboer, 2014; Coecke et al., 2013). In addition, kinetic data are important in the interpretation of data from human biomonitoring studies, for example to translate measured urine concentrations of a compound or its metabolite(s) to related external exposures (Zare Jeddi et al., 2021). Finally, kinetic data are key to understanding dose-, species-, and route of exposure-dependent differences in internal exposure, as well as considerations of human interindividual variation and interactions between compounds (Punt et al., 2020; Paini et al., 2021).

Given that particularly human toxicokinetic data are generally scarcely available for non-pharmaceuticals, insights into kinetics are increasingly being obtained with *in vitro* test systems. These include approaches that assess, for example, the intestinal, dermal, or pulmonary permeability of compounds, or test systems that capture metabolic conversions, plasma or tissue binding, or influx or efflux transporter kinetics (Blaauboer, 2014; Punt et al., 2017; Wilk-Zasadna et al., 2015). Stand-alone data from such studies can, in general, not be used directly in safety evalu-

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ations, as the combined effects of different kinetic processes determine the internal exposure. Therefore, data obtained with the different test systems need to be integrated, for example by PBK modelling (Louisse et al., 2017; Bessems et al., 2014; Choi et al., 2019), while taking the uptake and kinetics of various ports of entry (oral, dermal and inhalation) into account. To gain confidence in the outcomes obtained with PBK models that rely on *in vitro* input data, it is important to assess the robustness of the *in vitro* input data that are used and the combined impact of experimental variation in each of the individual parameters on the model predictions. In addition, each *in vitro* kinetic assay has its own inherent boundaries with respect to the conditions under which the *in vitro* experiments should be performed, including, for example, limitations with respect to the applied substrate concentration, enzyme concentration, or incubation time (Hubatsch et al., 2007; Gouliarmou et al., 2018; Seibert and Tracy, 2014). There are further restrictions with respect to the applicability domain of different *in vitro* kinetic studies. For example, *in vitro* kinetic constants, measured under linear conditions, can only be used for predictions at dose levels that would not lead to saturation of enzymes or transporters (Peters, 2012). To achieve regulatory use of *in vitro* kinetic studies, the robustness, experimental conditions under which the *in vitro* experiments need to be performed, and applicability domain of different *in vitro* kinetic studies need to be better understood.

Recently, Louisse et al. (2020) collected reported intrinsic hepatic clearance (CL_{int}) values from the literature for 30 compounds obtained with primary human hepatocytes as well as information on the experimental set-ups applied. They observed differences of up to two orders of magnitude in reported *in vitro* hepatic CL_{int} values obtained from incubations with primary human hepatocytes and noticed that the experimental set-ups applied differed for many aspects between studies. Pooled hepatocytes were used in most studies, suggesting that differences between studies were not solely driven by interindividual differences in biotransformation activities (Louisse et al., 2020). Apart from the *in vitro* CL_{int} values, the fraction unbound in plasma (fu_p), and the intestinal apparent permeability (P_{app}) are important parameters with which first estimates of internal concentrations can be made for oral exposure using a PBK model (Jones and Rowland-Yeo, 2013). Experimental uncertainties related to small differences in experimental set-ups can be expected for these input parameters.

The goal of the present study was to obtain an insight into the experimental variation in CL_{int}, fu_p, and P_{app} and to explore the impact of this variation in the *in vitro* kinetic data on PBK model predictions. The results are discussed with respect to the importance of the development of guidance documents to 1) reduce experimental variation and 2) to equip regulatory bodies with the means to evaluate the quality of *in vitro* kinetic data and the adequacy of an *in vitro* study design.

2 Materials and methods

Data collection

A literature search was performed to obtain an indication of the experimental variation in *in vitro* measured CL_{int}, P_{app}, and fu_p. In case of CL_{int}, the *in vitro* data collected by Louisse et al. (2020) were included in the present study. In that study, a literature search was performed to obtain an indication of the experimental variation in intrinsic clearance values obtained with primary hepatocytes, predominantly following the substrate depletion protocol. Given that the clearance data from Louisse et al. (2020) mainly covered pharmaceuticals, an additional literature search was performed in the present study to expand the chemical domain to include non-pharmaceuticals. To this end, Scopus[1] was used to identify papers or databases that provide relatively large datasets on *in vitro* metabolic clearances measured with primary hepatocytes.

For non-pharmaceuticals, the R httk database[3] (EPA) and Black et al. (2021) were identified as major sources for hepatic clearance data. For compounds for which two independent clearance measurements were found in these initial selected data sources, an additional search was performed with Google Scholar[4] to obtain additional clearance data from individual scientific papers.

Literature data were also collected to obtain an indication of the experimental variation in Caco-2 P_{app}, and fu_p values. Scopus was used to identify papers or databases that contain relatively large datasets of Caco-2 P_{app} values or fu_p values. The final selection of Caco-2 P_{app} data was from Estudante et al. (2015), Gertz et al. (2010), Hallifax et al. (2012), Hou et al. (2004), Larregieu and Benet (2014), Lee et al. (2017), Li et al. (2007), and Neuhoff et al. (2003). In case of fu_p, the R httk database (EPA) and data from Ye et al. (2016), Wang et al. (2014), Srivastava et al. (2021), Jones et al. (2021), Ferguson et al. (2019), Chen et al. (2019), and Deshmukh and Harsch (2011) were selected. Table 1 provides a summary of the data obtained with the literature search on *in vitro* intrinsic hepatic clearance as well as Caco-2 P_{app} and fu_p values for compounds from different chemical domains (pharmaceutical, chemical, food, cosmetic). A more extensive overview of the data and references is provided in supplementary file 1[2].

PBK model predictions

For the compounds for which the experimental variation in all three parameters, i.e., CL_{int}, P_{app}, and fu_p, could be determined (see Tab. 1), simulations were performed to explore the impact of the experimental variation on predictions of the maximum plasma concentration (C_{max}) and the area under the concentration time curve ($AUC_{0\text{-}24h}$). For these simulations, a published generic human PBK model code by Jones and Rowland-Yeo (2013) was used. The original model code of Jones and Rowland-Yeo

[1] www.scopus.com
[2] doi:10.14573/altex.2202131s1
[3] https://cran.r-project.org/web/packages/httk/index.html
[4] https://scholar.google.nl/

Tab. 1: Model compounds and summary of *in vitro* kinetic data (mean, coefficient of variation (CV) and number of data entries (n)) collected for CL_{int}, P_{app}, and fu_p

Number	Compound[a]	CL_{int} (µL/min/10^6 cells)			P_{app} (10^{-6} cm/s)			fu_p		
		Mean	CV[b]	n	Mean	CV[b]	n	Mean	CV[b]	n
1	Antipyrine	0.19	75	8	48	93	8			
2	Disopyramide	0.28	41	8						
3	Lorazepam	0.51	74	7						
4	Dapsone	0.57	97	4						
5	Tolbutamide	1.1	120	11				0.044	50	5
6	**Diazepam**	1.4	110	15	38	50	5	0.028	86	9
7	**Caffeine**	1.6	130	10	38	19	5	0.97	42	3
8	Pindolol	1.9	29	7						
9	**S-warfarin**	1.9	150	5	30	29	3	0.013	46	9
10	Omeprazole	2.4	63	5						
11	Timolol	2.7	82	8						
12	Naproxen	4.1	160	6						
13	Metoprolol	4.8	77	11	32	112	12			
14	Ketoprofen	4.8	56	11						
15	Prazosin	5.2	68	6						
16	Ibuprofen	5.3	37	5						
17	**Diltiazem**	6.2	55	12	45	55	4	0.37	38	5
18	**Quinidine**	6.4	98	10	19	80	2	0.23	38	3
19	Bosentan	7	200	7				0.021	64	3
20	Clozapine	7	59	11				0.083	44	5
21	Prednisolone	7.2	130	8						
22	Sildenafil	7.6	54	15						
23	Lidocaine	8.8	78	6						
24	4-Nitroaniline	9.6	100	4						
25	**Midazolam**	14	91	18	39	46	3	0.034	46	8
26	Dextromethorphan	17	120	9				0.39	23	4
27	Imipramine	17	110	19				0.17	38	5
28	3,3',5,5' -Tetrabromobisphenol A	18	120	4						
29	Phecetin	19	110	11						
30	Buspirone	21	79	6				0.2	71	3
31	Nifedipine	21	88	6				0.042	5	2
32	Desipramine	21	96	9						
33	Ketanserin	25	82	6						
34	Carvidelol	29	43	8						
35	**Verapamil**	30	100	15	35	79	10	0.2	38	9
36	Diclofenac	31	120	15				0.0066	69	9

[a] For the compounds highlighted in bold, the experimental variation in all three parameters, i.e., CL_{int}, P_{app}, and fu_p could be determined.
[b] CV corresponds to the coefficient of variation (CV = SD/mean x 100%) and is used as indicator of the variation in the reported kinetic values.

Number	Compound[a]	CL_{int} (µL/min/10^6 cells)			P_{app} (10^{-6} cm/s)			fu_p		
		Mean	CV[b]	n	Mean	CV[b]	n	Mean	CV[b]	n
37	Bufuralol	33	110	5						
38	2,5-Di-tert-butylbenzene-1,4-diol	35	160	4						
39	Propanolol	37	220	12						
40	Chlorpromazine	52	140	10				0.04	38	2
42	Bisphenol A	76	70	3	36	87	3			
43	Ipcozole	120	80	4						
44	Benzylparaben	370	50	4						
45	Propranolol				36	89	9	0.23	36	9
46	Fluvastatin							0.0061	42	2
47	Rosuvastatin							0.13	7.8	2

[a] For the compounds highlighted in bold, the experimental variation in all three parameters, i.e., CL_{int}, P_{app}, and fu_p could be determined.
[b] CV corresponds to the coefficient of variation (CV = SD/mean x 100%) and is used as indicator of the variation in the reported kinetic values.

(2013) was converted to R (R Core Team, 2021) and is provided on GitHub[5]. A description of the PBK model according to the OECD harmonized template is provided in supplementary file 2[6]. The code was modified with respect to the definition of the freely available concentration in the liver that is available for metabolism (C_L*fu_p) to the more commonly used description (CV_L*fu_p), in which CV_L corresponds to the total concentration in the liver (C_L) divided by the liver:plasma partition coefficient (Grandoni et al., 2019). The generic PBK model consists of 13 compartments, corresponding to the major organs of the body and an arterial and venous blood compartment. The model requires chemical-specific parameters for 1) intestinal uptake, 2) partition coefficients, 3) the blood:plasma ratio, 4) the fraction unbound in plasma, and 5) hepatic clearance. Renal clearance is described in the model based on the glomerular filtration rate times the fraction unbound in plasma and does therefore not require any additional chemical-specific input parameter. The partition coefficients were calculated with the method of Rodgers and Rowland (2006). The blood:plasma ratio was assumed to be a fixed value of 1 for all compounds as there are currently insufficient data or calculators available to parameterize the blood:plasma ratio. The input parameters for the intestinal uptake, fraction unbound in plasma, and hepatic clearance were obtained from *in vitro* experiments as described above. To explore the impact of the variation in CL_{int}, P_{app}, and fu_p on the C_{max} and $AUC_{0\text{-}24h}$ predictions, simulations were performed with all possible combinations of CL_{int}, P_{app}, and fu_p for a specific compound. The codes to run these simulations are provided on GitHub[5]. The simulations were performed at a low single oral dose of 0.1 mg/kg bw at which linear clearance conditions can be expected for all compounds.

To determine which of the *in vitro* input parameters contributed most to the predicted variation in C_{max} and $AUC_{0\text{-}24h}$, a global sensitivity analysis was performed with RVis software (McNally et al., 2018; Loizou et al., 2021). To this end, for each compound, the R code of the PBK model was loaded into RVis[7]. Simulations were subsequently performed within the "Sensitivity" tab, using the e-FAST method, by adding the observed *in vitro* distributions (mean and CV) to the CL_{int}, fu_p, and P_{app} parameters. Additional details on how these simulations were performed are provided in supplementary file 2[6]. The input data for the RVis simulations are provided in supplementary file 1[2].

3 Results

3.1 Evaluation of the *in vitro* experimental variation in CL_{int} values

Figure 1 shows the experimental variation in data from *in vitro* metabolic clearance studies as obtained from the literature. For many of the compounds, the CL_{int} measurements vary over a 100-fold, generally ranging between values that are 5-fold higher and 20-fold lower than the mean of a specific compound. The results from Figure 1 reveal that the variation in CL_{int} is consistent over the different types of compounds and chemical domains. The highest variations in *in vitro* CL_{int} values are observed for the pharmaceuticals bosentan (19) and naproxen (12) with a respective 172-fold and 164-fold range in CL_{int} values. However, a large variation is also found for the food-related compounds, exemplified for caffeine (7) and the food preservative 2,5-di-tert-butylbenzene-1,4-diol (38), with a 63-fold and 43-fold variation in *in vitro* reported CL_{int} values, respectively.

[5] https://github.com/wfsrqivive/PBK_exp_variation.git
[6] doi:10.14573/altex.2202131s2
[7] https://github.com/GMPtk/RVis/releases, v0.15, using R 4.1.1

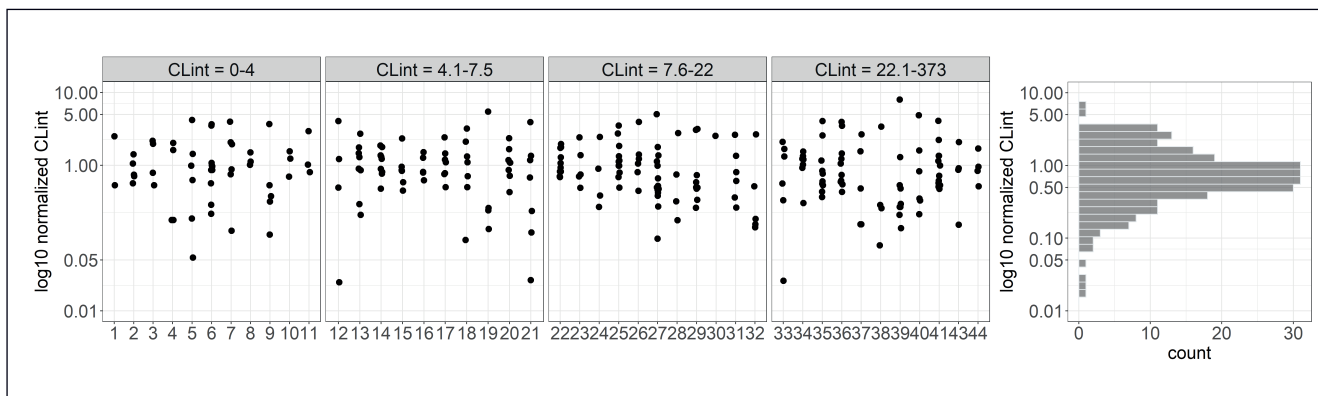

Fig. 1: Variation in *in vitro* CL_{int} (µL/min/10^6 cells) measurements
The histogram depicts the combined distribution of the variation over the different compounds. The values represent the normalized CL_{int} values, corresponding to the CL_{int} values obtained for a specific compound, divided by the mean of these values for the specific compound. The depicted compounds are numbered as described in Table 1 and grouped into four categories from low to high CL_{int} values.

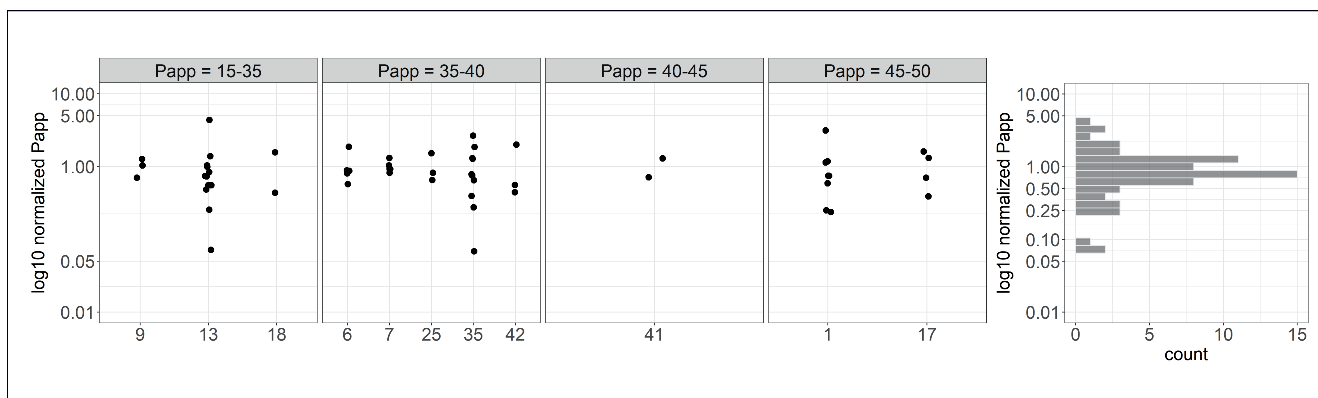

Fig. 2: Variation in *in vitro* Caco-2 P_{app} values (10^{-6} cm/s)
The histogram depicts the combined distribution of the variation over the different compounds. The values represent the normalized P_{app} values, corresponding to the P_{app} values obtained for a specific compound, divided by the mean of these values for the specific compound. The depicted compounds are numbered as shown in Table 1 and grouped into four categories from low to high P_{app} values.

This consistency in experimental variation over the range of different compounds provides an indication of the variation that can be expected from *in vitro* metabolic clearance studies with primary hepatocytes.

3.2 Evaluation of the *in vitro* experimental variation in Caco-2 P_{app} values

Figure 2 shows the experimental variation in *in vitro* reported P_{app} values. For the three compounds for which most Caco-2 P_{app} measurements are available (i.e., metoprolol (13), verapamil (35), and antipyrine (1)), the variation in P_{app} values appears to range over 13- to 60-fold, between values that are about 3- to 4-fold higher and about 4- to 15-fold lower than the mean P_{app} value of a specific compound. Less data was available for the remaining compounds, and the results reveal a 1.5- to 5-fold variation.

3.3 Evaluation of the *in vitro* experimental variation in fu_p values

Figure 3 reveals the experimental variation in *in vitro* derived fu_p values for a range of compounds. Given that the fu_p values can only range between 0 and 1, as the fu_p is a fraction, the extent of variation in the fu_p estimates is less than observed for CL_{int} and Caco-2 P_{app} values as described above. The largest experimental variation is observed for diclofenac (36) with fu_p values ranging from 0.0015-0.015, corresponding to a 10-fold range.

3.4 Impact of the combined variation in CL_{int}, P_{app}, and fu_p on the PBK model-predicted C_{max} and AUC_{0-24h}

For the seven compounds within the dataset for which CL_{int}, P_{app}, and fu_p data from different studies were available, the combined effects of the experimental variation in the three input parameters on the PBK model predictions were determined. The

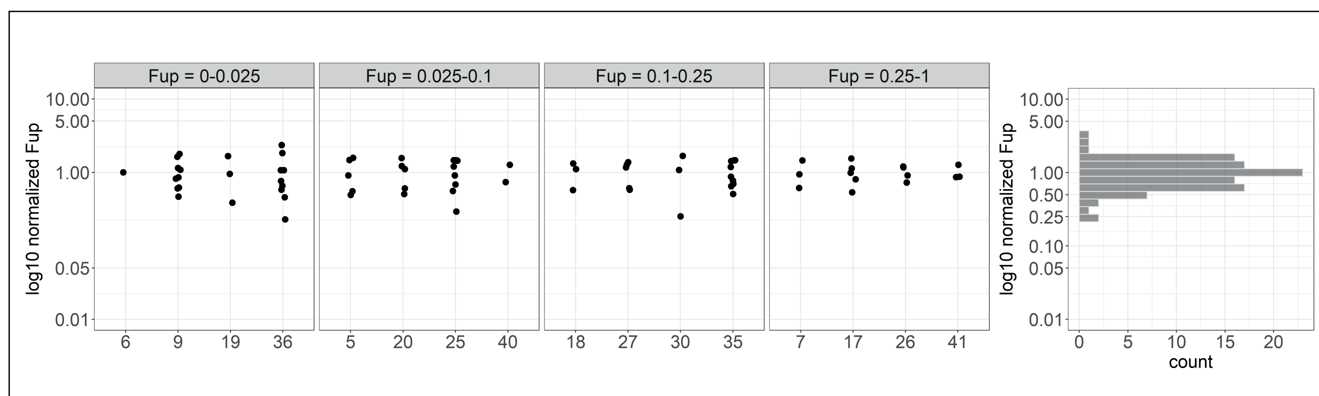

Fig. 3: Variation in *in vitro* fu_p (unitless) measurements
The histogram depicts the combined distribution of the variation over the different compounds. The presented values represent the normalized fu_p values, corresponding to the fu_p values obtained for a specific compound, divided by the mean of these values for the specific compound. The depicted compounds are numbered as shown in Table 1 and grouped into four categories from low to high fu_p values.

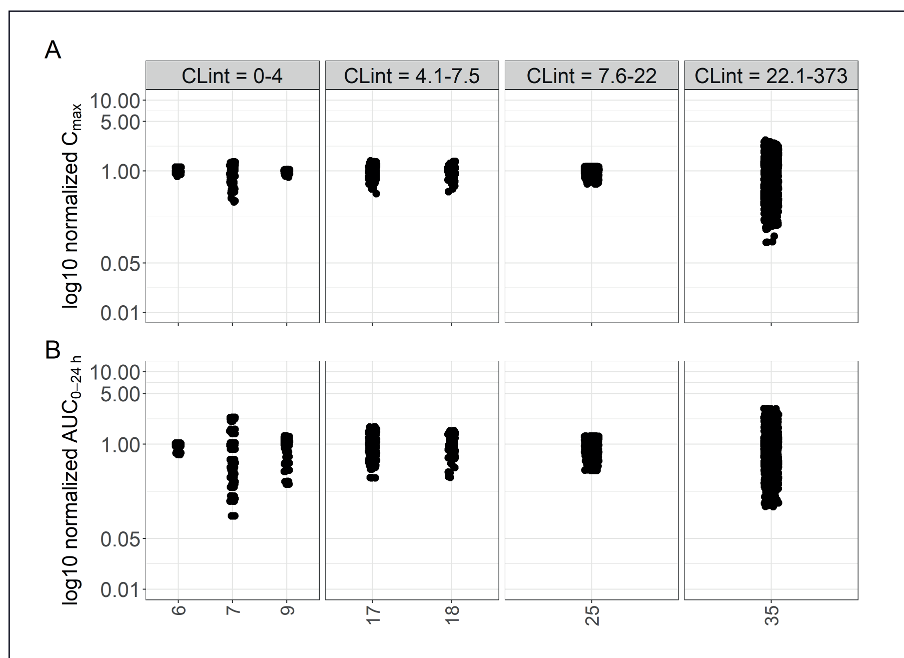

Fig. 4: Variation in PBK model-predicted C_{max} (A) and AUC_{0-24h} (B) as a result of the variation in reported *in vitro* CL_{int}, P_{app}, and fu_p values
The depicted compounds are numbered as described in Table 1.

results of these predictions are depicted in Figure 4. For every chemical, each available CL_{int} value was combined with each available P_{app} value, and each CL_{int}-P_{app} combination was in turn combined with each available fu_p value for a specific compound. Figure 4 reveals that the impact of the variation in experimental conditions on the PBK model predictions is different for each compound. The lowest variation in C_{max} and AUC_{0-24h} predictions occurs for the low-clearance compound diazepam (6), revealing a 1.4-fold range in both C_{max} and AUC_{0-24h} predictions. The highest variation in both C_{max} and AUC_{0-24h} predictions occurs for the high-clearance compound verapamil (35), revealing a 28-fold range in predicted C_{max} and a 23-fold range in predicted AUC_{0-24h}. A high variation in AUC_{0-24h} of 23-fold is also observed for the low-clearance compound caffeine (7).

3.5 Relative contribution of the different input parameters to the variation in predicted C_{max} and AUC_{0-24h} values

Figure 5 depicts the results of the global sensitivity analysis that was performed to determine which of the three input parameters (i.e., CL_{int}, P_{app}, or fu_p) contribute most to the variation in C_{max} and AUC_{0-24h} predictions as observed in Figure 4. Experimental variation in CL_{int} had the highest impact on AUC_{0-24h} predic-

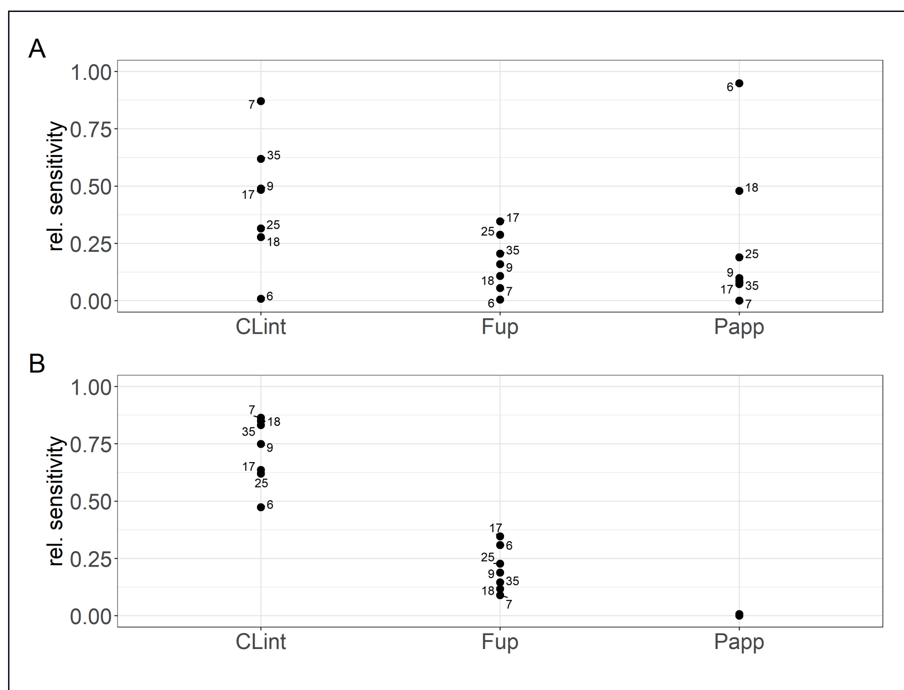

Fig. 5: Relative sensitivity of the C_{max} (A) and $AUC_{0\text{-}24h}$ (B) prediction to the variation in CL_{int}, P_{app}, and fu_p, as obtained with the RVis global sensitivity analysis
The relative sensitivity represents the relative contribution of each of the three parameters to the variation in C_{max} or $AUC_{0\text{-}24h}$ as observed in Figure 4. For example, in case of caffeine (7), the variation in CL_{int} accounted for 87% of the total variation in C_{max} predictions, whereas variation in fu_p and P_{app} contributed 6% and 1.8%, respectively. The remaining 5.2% variation is caused by the interaction between these different parameters as depicted in the supplementary file 2[6].

tions for all compounds and for four out of the seven compounds also on the C_{max} predictions (caffeine (7), diltiazem (17), S-warfarin (9), and verapamil (35)). The observed variation in C_{max} predictions for these compounds can thus largely be attributed to the variation in CL_{int}. The experimental variation in uptake parameter P_{app} has no influence on the $AUC_{0\text{-}24h}$ predictions but does have an impact on the C_{max} predictions of two out of the seven compounds (diazepam (6) and quinidine (18)). The relative sensitivity towards experimental variation in fu_p values was found to be lower than for CL_{int} (Fig. 5).

4 Discussion

With the present study we explored the experimental variation in *in vitro* CL_{int}, Caco-2 P_{app}, and fu_p measurements and the impact that this experimental variation has on PBK model predictions of C_{max} and $AUC_{0\text{-}24h}$. As a result of the observed experimental variation in CL_{int}, P_{app}, and fu_p, the PBK model-predicted C_{max} for the seven compounds for which all three parameters were available was found to range between 1.4- and 28-fold and the $AUC_{0\text{-}24h}$ to range between 1.4- to 23-fold. The large variation in C_{max} and $AUC_{0\text{-}24h}$ predictions, as observed for some of the compounds, indicates that the *in vitro* kinetic data are currently difficult to use in a regulatory context, since there are currently no means to evaluate the adequacy of a given *in vitro* kinetic experimental design used to obtain PBK model input parameters.

At present, insufficient data are available to elucidate the underlying causes for the experimental variation, as often critical experimental details, like solubility experiments and linearity checks (rate constants linear with time or concentration), are not reported in the publications. A more systematic analysis would be required to identify critical aspects of experimental designs, for example, by performing the *in vitro* kinetic studies with a full factorial design approach in which the impact of a number of variables in the experimental design is systematically studied (Maas et al., 2000). An incorrect design of *in vitro* kinetic experiments is expected to be one of the causes of the large variation in *in vitro* kinetic data present in the literature. For example, a critical aspect of *in vitro* clearance measurements with the substrate depletion protocol is that the applied concentration should be below the Michaelis-Menten constant K_m (Black et al., 2021). However, measurements are still being published in which this condition is not met or not considered (e.g., Fortaner et al., 2021). In case of Caco-2 absorption experiments, a critical aspect for obtaining relevant P_{app} values is that the experiments are performed under a concentration gradient, otherwise diffusion cannot take place. This means that the time-range in which the absorption studies are performed needs to be optimized to make sure that less than 10% of the compound is diffused to the basolateral compartment (also called sink conditions) (Usansky and Sinko, 2005). Such sink conditions provide the best representation of the physiological conditions as a concentration gradient between the gut lumen and the plasma will exist *in vivo* due to distribution of the chemical in the body after absorption. Examples are available in the literature in which the criterion of measuring under sink conditions is not met or not considered (e.g., Kulthong et al., 2018). In addition, factors that affect the concentration of a test item (solubility or plastic binding) will affect the results when not adequately taken into account (Fagerholm et al., 2021). Finally, data processing can also have a large effect on the derived kinetic constants. For example, mismatch-

es between the observed data points and mathematical fit were observed in the present study for the compound 2,5-di-tert-butylbenzene-1,4-diol (38) (Wambaugh et al., 2019). Additional background information on critical aspects that need to be considered with respect to the design of *in vitro* kinetic studies is provided in supplementary file 2[6].

Within a regulatory context, no guidance documents are currently available to be able to judge the quality of *in vitro* kinetic measurements, hampering the adequate performance of *in vitro* kinetic studies as well as the evaluation of data by end-users, including regulators. Recently, the OECD published a guidance document on a workflow for characterizing and validating PBK models (OECD, 2021). The quality of the *in vitro* input data is not yet explicitly taken into account in this guidance document. Nonetheless, effective protocols for performing *in vitro* kinetic studies to derive values for CL_{int}, P_{app}, and fu_p are available in the scientific literature (e.g., Watanabe et al., 2018; Cai and Shalan, 2021; Hubatsch et al., 2007; Black et al., 2021). We highly recommend that these high-quality protocols are formalized to describe the applicability domain/use in a regulatory context. However, it should be noted that most of the protocols have been developed within the pharmaceutical domain, and most experience with the predictive performance of the different *in vitro* kinetic studies comes from the pharmaceutical domain. Compounds like pesticides, biocides, industrial chemicals, cosmetic ingredients, and food-related compounds generally have a broader range of physicochemical properties than pharmaceuticals and can contain, for example, compounds that are highly lipophilic or volatile (Andersen et al., 2019; Ferguson et al., 2019).

At present, *in vivo* experimental animal or human kinetic data are still requested in various regulatory guidelines (e.g., SCCS, 2018; EMA, 2018; OECD, 2021) to evaluate the performance of PBK models and to obtain confidence in the model predictions. However, this approach of model evaluations against *in vivo* data is mainly successful within the pharmaceutical domain as sufficient clinical data are only available for pharmaceuticals (EMA, 2018; Punt et al., 2017). For many other chemical domains, the availability of experimental animal or human *in vivo* kinetic data is limited, and evaluation against *in vivo* kinetic data is often not possible. Given that the combination of *in vitro* kinetic input data with PBK models provides a promising strategy to simulate the fate of chemicals in a body in the absence of *in vivo* kinetic data, it becomes crucial to find other means to gain confidence in the *in vitro* kinetic data and related PBK model predictions. The quality of the *in vitro* input parameters is important, as the model predictions will only be as good as the input. Application of uncertainty factors to the *in vitro*-based PBK model predictions might be one way to take the uncertainties related to the *in vitro* experimental variation into account. The results of the present study indicate, however, that large uncertainty factors may then be required to cover the impact of potential experimental variation. Increasing robustness of *in vitro* kinetic data and improving the possibilities within regulatory risk evaluations to assess the quality of *in vitro* kinetic data are therefore important next steps.

Apart from guidance documents on the design of *in vitro* kinetic studies, guidance will also be needed on the applicability domain of different *in vitro* kinetic studies to meet specific regulatory needs. The *in vitro* kinetic data discussed in the present study can, for example, only be used to make first-tier estimates of plasma concentrations of the parent compound after oral exposure (Jones and Rowland, 2013). Simulations of inhalation and dermal exposure will require additional kinetic input data on *in vitro* lung and dermal absorption to mimic these respective exposure routes. The first-tier estimates of plasma C_{max} and AUC_{0-24h} in the present study after oral exposures also do not yet take the contribution of metabolites, possible saturation of biotransformation enzymes, possible involvement of transporters, or possible extrahepatic metabolism into account. At present it remains particularly difficult to determine when additional kinetic processes, like transporter kinetics or extrahepatic metabolism, need to be considered for a specific compound (Sager et al., 2015). Additional research is still needed to define the characteristics of chemicals that require the inclusion of these kinetic processes in PBK models (Punt et al., 2022).

Whereas the present study focused on the impact of variation in reported *in vitro* CL_{int}, fu_p, and P_{app} values on PBK model predictions, other *in vitro* kinetic input parameters could be relevant as well. Metabolic clearance is, for example, measured not only with primary hepatocytes but also with liver microsomes and S9. In addition, in situations where dose-dependent kinetics are of importance, the Michaelis-Menten constants (K_m and V_{max}) need to be derived from the *in vitro* metabolism studies. Moreover, *in vitro* transporter kinetic data (e.g., intestine, kidney, and liver transporters) are important for the kinetics of some compounds. A similar variability in experimental results may be expected for each of these *in vitro* methods if non-standardized approaches are used, and a description of experimental boundaries and the applicability domain will be needed. For example, the variability reported in the literature for metabolic clearance rates for bisphenol A with human liver microsomes ranges 30-fold (from 0.078 to 2.36 mL/min/mg microsomal protein) (Mazur et al., 2010; Elsby et al., 2001; Hanioka et al., 2020), which is similar to the overall variability in hepatocyte clearance data as observed in the present study. Apart from the *in vitro* kinetic data, *in silico* predictors of different kinetic parameters have been developed that may provide input data for PBK models. Particularly the prediction of partition coefficients (determining the distribution of compounds in different organs) depends on the use of these calculators, as these parameters are difficult to obtain with *in vitro* experiments. Recently, Punt et al. (2022) revealed that significant differences can occur as a result of the use of different calculators. For example, the calculation method of Berezhkovskiy (2004) frequently led to underpredictions of the C_{max} of acidic compounds ($pKa < 6$), whereas the calculation method of Schmitt (2008) appeared to perform less well for highly lipophilic compounds (Punt et al., 2022). The calculation method of Rodgers and Rowland (2006) performed best overall and was therefore applied in the present study to predict the partition coefficients of the different compounds.

Overall, the results of the present study indicate a strong impact of experimental variation in CL_{int}, P_{app}, and fu_p on PBK model-based C_{max} and AUC_{0-24h} predictions. This implies that

steps need to be taken to reduce experimental variation to increase the confidence in these *in vitro* kinetic data and related PBK model simulations for regulatory use. To this end, it will be crucial that the *in vitro* experiments are performed in a standardized way to meet regulatory needs. In addition, the chemical and regulatory applicability domains of the *in vitro* test systems and kinetic models need to be defined. Therefore, it is important that existing protocols are formalized in guidance documents to improve harmonization of testing procedures and correct usage of test results.

References

Andersen, M. E., McMullen, P. D., Phillips, M. B. et al. (2019). Developing context appropriate toxicity testing approaches using new alternative methods (NAMs). *ALTEX 36*, 523-534. doi:10.14573/altex.1906261

Arnesdotter, E., Rogiers, V., Vanhaecke, T. et al. (2021). An overview of current practices for regulatory risk assessment with lessons learnt from cosmetics in the European Union. *Crit Rev Toxicol 51*, 395-417. doi:10.1080/10408444.2021.1931027

Berezhkovskiy, L. M. (2004). Volume of distribution at steady state for a linear pharmacokinetic system with peripheral elimination. *J Pharm Sci 93*, 1628-1640. doi:10.1002/jps.20073

Bessems, J. G., Loizou, G., Krishnan, K. et al. (2014). PBTK modelling platforms and parameter estimation tools to enable animal-free risk assessment. Recommendations from a joint EPAA-EURL ECVAM ADME workshop. *Regul Toxicol Pharmacol 68*, 119-139. doi:10.1016/j.yrtph.2013.11.008

Blaauboer, B. J. (2014). In vitro approaches to predictive biokinetics. In A. Bal-Price and P. Jennings (eds), *In Vitro Toxicology Systems, Methods in Pharmacology and Toxicology* (521-530). New York, NY USA: Humana Press. doi:10.1007/978-1-4939-0521-8_23

Black, S. R., Nichols, J. W., Fay, K. A. et al. (2021). Evaluation and comparison of in vitro intrinsic clearance rates measured using cryopreserved hepatocytes from humans, rats, and rainbow trout. *Toxicology 457*, 152819. doi:10.1016/j.tox.2021.152819

de Boer, A., Krul, L., Fehr, M. et al. (2020). Animal-free strategies in food safety & nutrition: What are we waiting for? Part I: Food safety. *Trends Food Sci Technol 106*, 469-484. doi:10.1016/j.tifs.2020.10.034

Cai, J. and Shalan, H. (2021). Assessment of cytochrome P450 metabolic clearance using hepatocyte suspension. In Z. Yan and G. W. Caldwell (eds), *Cytochrome P450. Methods in Pharmacology and Toxicology* (243-259). New York, NY, USA: Humana. doi:10.1007/978-1-0716-1542-3_15

Chen, Y.-C., Kenny, J. R., Wright, M. et al. (2019). Improving confidence in the determination of free fraction for highly bound drugs using bidirectional equilibrium dialysis. *J Pharm Sci 108*, 1296-1302. doi:10.1016/j.xphs.2018.10.011

Choi, G.-W., Lee, Y.-B. and Cho, H.-Y. (2019). Interpretation of non-clinical data for prediction of human pharmacokinetic parameters: In vitro-in vivo extrapolation and allometric scaling. *Pharmaceutics 11*, 168. doi:10.3390/pharmaceutics11040168

Coecke, S., Pelkonen, O., Leite, S. B. et al. (2013). Toxicokinetics as a key to the integrated toxicity risk assessment based primarily on non-animal approaches. *Toxicol In Vitro 27*, 1570-1577. doi:10.1016/j.tiv.2012.06.012

Deshmukh, S. V. and Harsch, A. (2011). Direct determination of the ratio of unbound fraction in plasma to unbound fraction in microsomal system (fu_p/fu_{mic}) for refined prediction of phase I mediated metabolic hepatic clearance. *J Pharmacol Toxicol Methods 63*, 35-39. doi:10.1016/j.vascn.2010.04.003

Elsby, R., Maggs, J. L., Ashby, J. et al. (2001). Comparison of the modulatory effects of human and rat liver microsomal metabolism on the estrogenicity of bisphenol A: Implications for extrapolation to humans. *J Pharmacol Exp Ther 297*, 103-113.

EMA (2018). EMA Guideline on the reporting of physiologically based pharmacokinetic (PBPK) modelling and simulation. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-reporting-physiologically-based-pharmacokinetic-pbpk-modelling-simulation_en.pdf

Estudante, M., de Mello-Sampayo, C., Sahin, S. et al. (2015). The utility of in vitro trials that use Caco-2 cell systems as a replacement for animal intestinal permeability and human bioequivalence measurements in drug development. *J Biomed Biopharm Res 12*, 117-126. doi:10.19277/bbr.12.1.110

European Commission (2019). The European Green Deal. Communication from the Commission to the European Parliament, the European Council, the Council, the European Economic and Social Committee and the Committee of the Regions. Brussels, 11-12-2019 640 final. doi:10.54648/eerr1996017

European Commission (2020). Chemicals strategy for sustainability – Towards a toxic-free environment, communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions. Brussels, 14-10-2020 667 final. doi:10.54648/eerr1996017

Fagerholm, U., Spjuth, O. and Hellberg, S. (2021). Comparison between lab variability and in silico prediction errors for the unbound fraction of drugs in human plasma. *Xenobiotica 51*, 1095-1100. doi:10.1080/00498254.2021.1964044

Ferguson, K. C., Luo, Y. S., Rusyn, I. et al. (2019). Comparative analysis of rapid equilibrium dialysis (RED) and solid phase micro-extraction (SPME) methods for in vitro-in vivo extrapolation of environmental chemicals. *Toxicol In Vitro 60*, 245-251. doi:10.1016/j.tiv.2019.06.006

Fortaner, S., Mendoza-De Gyves, E., Cole, T. et al. (2021). Determination of in vitro metabolic hepatic clearance of valproic acid (VPA) and five analogues by UPLC-MS-QTOF, applicable in alternatives to animal testing. *J Chromatogr B Anal Technol Biomed Life Sci 1181*, 122893. doi:10.1016/j.jchromb.2021.122893

Gertz, M., Harrison, A., Houston, J. B. et al. (2010). Prediction of human intestinal first-pass metabolism of 25 CYP3A substrates from in vitro clearance and permeability data. *Drug Metab Dispos 38*, 1147-1158. doi:10.1124/dmd.110.032649

Gouliarmou, V., Lostia, A. M., Coecke, S. et al. (2018). Establishing a systematic framework to characterise in vitro methods for human hepatic metabolic clearance. *Toxicol In Vitro 53*, 233-244. doi:10.1016/j.tiv.2018.08.004

Grandoni, S., Cesari, N., Brogin, G. et al. (2019). Building in-house PBPK modelling tools for oral drug administration from literature information. *ADMET DMPK 7*, 4-21. doi:10.5599/admet.638

Hallifax, D., Turlizzi, E., Zanelli, U. et al. (2012). Clearance-dependent underprediction of in vivo intrinsic clearance from human hepatocytes: Comparison with permeabilities from artificial membrane (PAMPA) assay, in silico and caco-2 assay, for 65 drugs. *Eur J Pharm Sci 45*, 570-574. doi:10.1016/j.ejps.2011.12.010

Hanioka, N., Isobe, T., Tanaka-Kagawa, T. et al. (2020). In vitro glucuronidation of bisphenol A in liver and intestinal microsomes: Interspecies differences in humans and laboratory animals. *Drug Chem Toxicol 45*, 1565-1569. doi:10.1080/01480545.2020.1847133

Hou, T. J., Zhang, W., Xia, K. et al. (2004). ADME evaluation in drug discovery. 5. Correlation of Caco-2 permeation with simple molecular properties. *J Chem Inf Comput Sci 44*, 1585-1600. doi:10.1021/ci049884m

Hubatsch, I., Ragnarsson, E. G. E. and Artursson, P. (2007). Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nat Protoc 2*, 2111-2119. doi:10.1038/nprot.2007.303

Jones, H. and Rowland-Yeo, K. (2013). Basic concepts in physiologically based pharmacokinetic modeling in drug discovery and development. *CPT Pharmacometrics Syst Pharmacol 2*, e63. doi:10.1038/psp.2013.41

Jones, R. S., Chang, J. H., Flores, M. et al. (2021). Evaluation of a competitive equilibrium dialysis approach for assessing the impact of protein binding on clearance predictions. *J Pharm Sci 110*, 536-542. doi:10.1016/j.xphs.2020.09.012

Kulthong, K., Duivenvoorde, L., Mizera, B. Z. et al. (2018). Implementation of a dynamic intestinal gut-on-a-chip barrier model for transport studies of lipophilic dioxin congeners. *RSC Adv 8*, 32440-32453. doi:10.1039/c8ra05430d

Larregieu, C. A. and Benet, L. Z. (2014). Distinguishing between the permeability relationships with absorption and metabolism to improve BCS and BDDCS predictions in early drug discovery. *Mol Pharm 11*, 1335-1344. doi:10.1021/mp4007858

Lee, J. B., Zgair, A., Taha, D. A. et al. (2017). Quantitative analysis of lab-to-lab variability in Caco-2 permeability assays. *Eur J Pharm Biopharm 114*, 38-42. doi:10.1016/j.ejpb.2016.12.027

Li, C., Liu, T., Cui, X. et al. (2007). Development of in vitro pharmacokinetic screens using Caco-2, human hepatocyte, and Caco-2/human hepatocyte hybrid systems for the prediction of oral bioavailability in humans. *J Biomol Screen 12*, 1084-1091. doi:10.1177/1087057107308892

Loizou, G., McNally, K., Dorne, J.-L. C. M. et al. (2021). Derivation of a human in vivo benchmark dose for perfluorooctanoic acid from ToxCast in vitro concentration – Response data using a computational workflow for probabilistic quantitative in vitro to in vivo extrapolation. *Front Pharmacol 12*, 630457. doi:10.3389/fphar.2021.630457

Louisse, J., Beekmann, K. and Rietjens, I. M. C. M. (2017). Use of physiologically based kinetic modeling-based reverse dosimetry to predict in vivo toxicity from in vitro data. *Chem Res Toxicol 30*, 114-125. doi:10.1021/acs.chemrestox.6b00302

Louisse, J., Alewijn, M., Peijnenburg, A. A. C. M. et al. (2020). Towards harmonization of test methods for in vitro hepatic clearance studies. *Toxicol In Vitro 63*, 104722. doi:10.1016/j.tiv.2019.104722

Maas, W. J. M., de Graaf, I. A. M., Schoen, E. D. et al. (2000). Assessment of some critical factors in the freezing technique for the cryopreservation of precision-cut rat liver slices. *Cryobiology 40*, 250-263. doi:10.1006/cryo.2000.2246

Mazur, C. S., Kenneke, J. F., Hess-Wilson, J. K. et al. (2010). Differences between human and rat intestinal and hepatic bisphenol a glucuronidation and the influence of alamethicin on in vitro kinetic measurements. *Drug Metab Dispos 38*, 2232-2238. doi:10.1124/dmd.110.034819

McNally, K., Hogg, A. and Loizou, G. (2018). A computational workflow for probabilistic quantitative in vitro to in vivo extrapolation. *Front Pharmacol 9*, 508. doi:10.3389/fphar.2018.00508

Neuhoff, S., Ungell, A. L., Zamora, I. et al. (2003). pH-dependent bidirectional transport of weakly basic drugs across Caco-2 monolayers: Implications for drug-drug interactions. *Pharm Res 20*, 1141-1148. doi:10.1023/a:1025032511040

OECD (2021). Guidance Document on the Characterisation, Validation and Reporting of Physiologically Based Kinetic (PBK) Models for Regulatory Purposes. *Series on Testing and Assessment, No. 331*.

Paini, A., Tan, Y. M., Sachana, M. et al. (2021). Gaining acceptance in next generation PBK modelling approaches for regulatory assessments – An OECD international effort. *Comput Toxicol 18*, 100163. doi:10.1016/j.comtox.2021.100163

Paul Friedman, K., Gagne, M., Loo, L.-H. et al. (2020). Utility of in vitro bioactivity as a lower bound estimate of in vivo adverse effect levels and in risk-based prioritization. *Toxicol Sci 173*, 202-225. doi:10.1093/toxsci/kfz201

Peters, S. A. (2012). Review of pharmacokinetic principles. In S. A. Peters, *Physiologically-Based Pharmacokinetic (PBPK) Modeling and Simulations* (Chapter 3, 17-42). Hoboken, NJ, USA: John Wiley & Sons, Inc. doi:10.1002/9781118140291.ch3

Punt, A., Peijnenburg, A. A. C. M., Hoogenboom, R. L. A. P. et al. (2017). Non-animal approaches for toxicokinetics in risk evaluations of food chemicals. *ALTEX 34*, 501-514. doi:10.14573/altex.1702211

Punt, A., Bouwmeester, H., Blaauboer, B. J. et al. (2020). New approach methodologies (NAMs) for human-relevant biokinetics predictions. Meeting the paradigm shift in toxicology towards an animal-free chemical risk assessment. *ALTEX 37*, 607-622. doi:10.14573/altex.2003242

Punt, A., Louisse, J., Beekmann, K. et al. (2022). Predictive performance of next generation human physiologically based kinetic (PBK) models based on in vitro and in silico input data. *ALTEX 39*, 221-234. doi:10.14573/altex.2108301

R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Rodgers, T. and Rowland, M. (2006). Physiologically based phar-

macokinetic modelling 2: Predicting the tissue distribution of acids, very weak bases, neutrals and zwitterions. *J Pharm Sci 95*, 1238-1257. doi:10.1002/jps.20502

Sager, J. E., Yu, J., Ragueneau-Majlessi, I. et al. (2015). Physiologically based pharmacokinetic (PBPK) modeling and simulation approaches: A systematic review of published models, applications, and model verification. *Drug Metab Dispos 43*, 1823-1837. doi:10.1124/dmd.115.065920

SCCS (2018). The SCCS Notes of Guidance for the Ttesting of Cosmetic Ingredients and their Safety Evaluation. 10th revision. Section 3-4.12.1.

Schmitt, W. (2008). General approach for the calculation of tissue to plasma partition coefficients. *Toxicol In Vitro 22*, 457-467. doi:10.1016/j.tiv.2007.09.010

Seibert, E. and Tracy, T. S. (2014). Fundamentals of enzyme kinetics. *Methods Mol Biol 1113*, 9-22. doi:10.1007/978-1-62703-758-7_2

Srivastava, A., Pike, A., Williamson, B. et al. (2021). A novel method for preventing non-specific binding in equilibrium dialysis assays using Solutol® as an additive. *J Pharm Sci 110*, 1412-1417. doi:10.1016/j.xphs.2020.11.018

Usansky, H. H. and Sinko, P. J. (2005). Estimating human drug oral absorption kinetics from Caco-2 permeability using an absorption-disposition model: Model development and evaluation and derivation of analytical solutions for k_a and F_a. *J Pharmacol Exp Ther 314*, 391-399. doi:10.1124/jpet.104.076182

Wambaugh, J. F., Wetmore, B. A., Ring, C. L. et al. (2019). Assessing toxicokinetic uncertainty and variability in risk prioritization. *Toxicol Sci 172*, 235-251. doi:10.1093/toxsci/kfz205

Wang, H., Zrada, M., Anderson, K. et al. (2014). Understanding and reducing the experimental variability of in vitro plasma protein binding measurements. *J Pharm Sci 103*, 3302-3309. doi:10.1002/jps.24119

Watanabe, R., Esaki, T., Kawashima, H. et al. (2018). Predicting fraction unbound in human plasma from chemical structure: Improved accuracy in the low value ranges. *Mol Pharm 15*, 5302-5311. doi:10.1021/acs.molpharmaceut.8b00785

Wilk-Zasadna, I., Bernasconi, C., Pelkonen, O. et al. (2015). Biotransformation in vitro: An essential consideration in the quantitative in vitro-to-in vivo extrapolation (QIVIVE) of toxicity data. *Toxicology 332*, 8-19. doi:10.1016/j.tox.2014.10.006

Ye, M., Nagar, S. and Korzekwa, K. (2016). A physiologically based pharmacokinetic model to predict the pharmacokinetics of highly protein-bound drugs and the impact of errors in plasma protein binding. *Biopharm Drug Dispos 37*, 123-141. doi:10.1002/bdd.1996

Zare Jeddi, M., Hopf, N. B., Viegas, S. et al. (2021). Towards a systematic use of effect biomarkers in population and occupational biomonitoring. *Environ Int 146*, 106257. doi:10.1016/j.envint.2020.106257

Conflict of interest

The authors declare that they have no conflicts of interest.

Data availability

The collected literature data used in the present study is provided in supplementary file 1[2]. The model code of the PBK model and input parameters are provided on GitHub[5].

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