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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 (CLint), the fraction unbound in plasma (Fup), and the intestinal apparent permeability (Papp) 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 (Cmax) and the area under the concentration time curve (AUC_{0-24h}) was determined. As a result of the experimental variation in CLint, Papp, and Fup, the predicted variation in Cmax for individual compounds ranged between 1.4- to 28-fold and the predicted variation in AUC_{0-24h} 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). Such additional insights in chemical safety 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). Insights in the absorption, distribution, metabolism and excretion of compounds, i.e. the kinetics, have a critical role in such animal-free testing strategies, particularly to 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, that can be related to the *in vitro* effect concentrations (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 obtain better insights in 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 in kinetics are increasingly obtained with *in vitro* test systems. These include approaches that capture, 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 directly be used in safety evaluations, as the combined effects of different kinetic processes determines the internal exposure. Therefore, data obtained with the different test systems need to be integrated, for example with help of 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 have insight into the robustness of the *in vitro*

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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, boundaries 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 furthermore 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 become more apparent.

Recently, Louisse et al. (2020), collected reported intrinsic hepatic clearance (CLint) values from the literature for 30 compounds obtained with human hepatocytes, as well as information on the experimental set-ups applied. They observed up to two orders of magnitude differences in literature reported *in vitro* hepatic CLint values as obtained from incubations with primary human hepatocytes and noticed that the experimental set-ups applied differed for many aspects between studies. In most studies, pooled hepatocytes were used, suggesting that differences between studies are not solely driven by interindividual differences in biotransformation activities (Louisse et al., 2020). Apart from the *in vitro* CLint values, the fraction unbound in plasma (Fup), and the intestinal apparent permeability (Papp) are also important parameters with which first estimates of internal concentrations can be made for oral exposure, upon using these data as input in a PBK model (Jones and Rowland-Yeo, 2013). Experimental uncertainties related to small differences in experimental set-ups can also be expected for these input parameters. The goal of the present study was to obtain an insight in the experimental variation in CLint, Fup, and Papp, and to explore the impact of this experimental 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

2.1 Data collection

A literature search was performed to obtain an indication of the experimental variation in *in vitro* measured CLint, Papp, and Fup. In case of CLint, the *in vitro* data as collected by Louisse et al. (2020) were included in the present study. In that study, Louisse et al. (2020) performed a literature search to obtain an indication of the experimental variation in intrinsic clearances values obtained with primary hepatocytes, predominantly obtained with 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 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 (EPA) and Black et al. (2021) were identified as major source 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, to determine if additional clearance data could be obtained from individual scientific papers.

In addition to the collection of CLint data, literature data were also collected to obtain an indication of the experimental variation in Caco-2 Papp and Fup values. To this end, Scopus was used to identify papers or databases that contain relatively large datasets of Caco-2 Papp values or Fup values. The final selection of Caco-2 Papp data were obtained from Estudante et al. (2015), Gertz et al. (2010), Hallifax et al. (2012), Hou et al. (2004), Larregieu & Benet (2014), Lee et al. (2017), Li et al. (2007), and Neuhoff et al. (2003). In case of Fup, 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, Caco-2 Papp and Fup 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].

2.2 PBK model predictions

For the compounds for which the experimental variation in all three parameters, i.e. CLint, Papp, and Fup, could be determined (see Table 1), simulations were performed to explore the impact of the experimental variation on predictions of the maximum plasma concentration (Cmax) 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 (2013) was converted to R (R Core Team, 2021) and is provided on GitHub[3]. A description of the PBK model according to the OECD harmonized template is provided in supplementary file 2[4]. The code was modified with respect to definition of the freely available concentration in the liver that is available for metabolism (CL*Fup), to the more commonly used description (CVL*Fup) in which CVL corresponds to the total concentration in the liver (CL) divided by the liver:plasma partition coefficient (Grandoni et al., 2019). The generic PBK model consists of 13 compartments, corresponding to the major organs in 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.

[1] www.scopus.com
[2] doi:10.14573/altex.2202131s1
[3] https://github.com/wfsrqivive/PBK_exp_variation.git
[4] doi:10.14573/altex.2202131s2

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Tab. 1: Model compounds and summary of *in vitro* kinetic data (mean, coefficient of variation (CV) and number of data entries (n)) collected for CLint, Papp and Fup

Number	Compound[a]	CLint (µL/min/10^6 cells)			Papp (10^{-6} cm/s)			Fup		
		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	Sildefil	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
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. CLint, Papp and Fup 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.

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 calculation 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 CLint, Papp, and Fup on the Cmax and $AUC_{0\text{-}24h}$ predictions, simulations were performed with all possible combinations of CLint, Papp, and Fup for a specific compound. The codes to run these simulations are provided on[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 Cmax and $AUC_{0\text{-}24h}$, a global sensitivity analysis was performed with RVis (McNally et al., 2018; Loizou et al., 2021). To this end, for each compound, the R code of the PBK model was loaded into the RVis software[6]. 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 CLint, Fup and Papp parameters. Additional details on how these simulations were performed are provided in supplementary file 2[4]. 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 CLint 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 CLint measurements varied 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 also reveal that the variation in CLint is consistent over the different types of compounds and chemical domains. The highest variation in *in vitro* CLint values is observed for the pharmaceuticals bosentan (19) and naproxen (12) with a respective 172-fold and 164-fold range in CLint values. However, also for the food related compounds a large variation is found, 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 CLint values, respectively. 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 Papp values

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

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

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

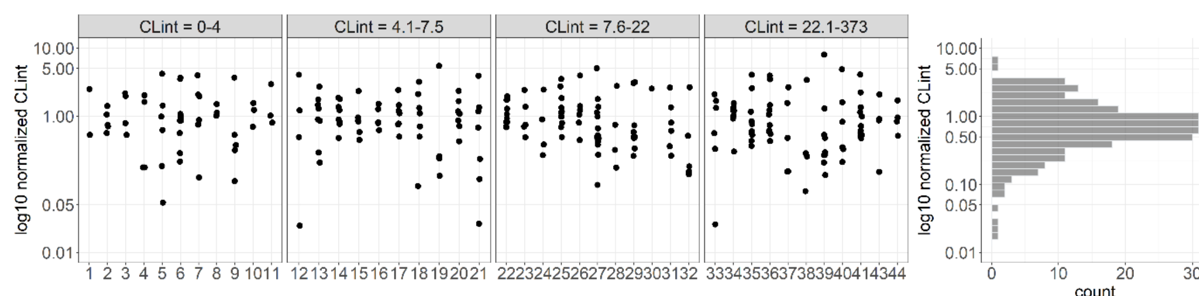

Fig. 1: Variation in *in vitro* CLint (µL/min/10^6 cells) measurements
The histogram depicts the combined distribution of the variation over the different compounds. The values represent the normalized CLint values, corresponding to the CLint values obtained for a specific compound, divided by the mean 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 CLint values.

[5] https://github.com/wfsrqivive/PBK_exp_variation.git
[6] https://github.com/GMPtk/RVis/releases, v0.15, using R 4.1.1

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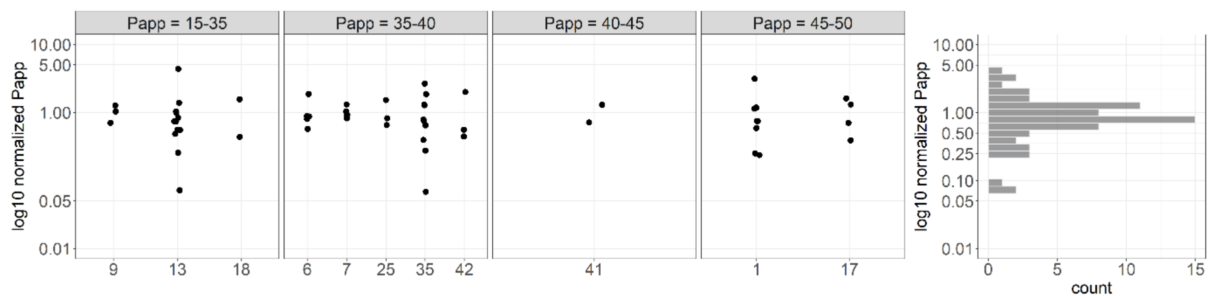

Fig. 2: Variation in reported *in vitro* Caco-2 Papp values (10^{-6} cm/s)
The histogram depicts the combined distribution of the variation over the different compounds. The values represent the normalized Papp values, corresponding to the Papp values obtained for a specific compound, divided by the 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 Papp values.

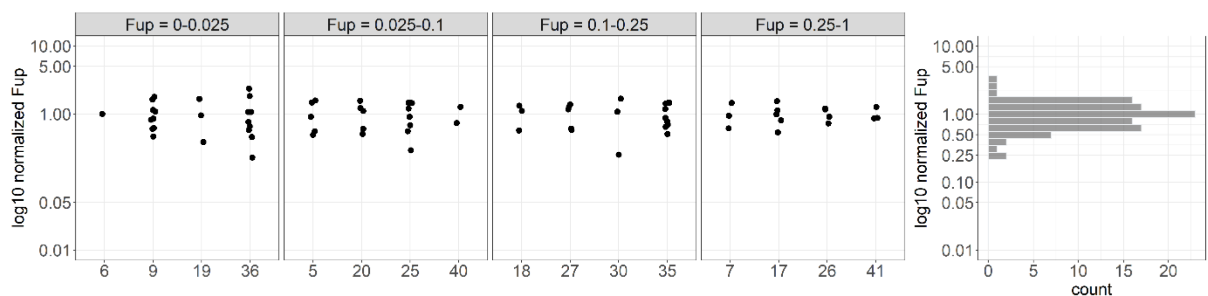

Fig. 3: Variation in *in vitro* Fup (unitless) measurements
The histogram depicts the combined distribution of the variation over the different compounds. The presented values represent the normalized Fup values, corresponding to the Fup values obtained for a specific compound, divided by the mean of 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 Fup values.

3.4 Impact of the combined variation in CLint, Papp and Fup on the PBK model-predicted Cmax and $AUC_{0\text{-}24h}$

For the seven compounds within the dataset for which CLint, Papp and Fup 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 results of these predictions are depicted in Figure 4. For every chemical, each available CLint value was combined with each available Papp value, and each CLint-Papp combination was in turn combined with each available Fup 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 Cmax and $AUC_{0\text{-}24h}$ predictions occurs for the low-clearance compound diazepam (6), revealing both a 1.4-fold range in Cmax and $AUC_{0\text{-}24h}$ predictions. The highest variation in both Cmax and $AUC_{0\text{-}24h}$ predictions occurs for the high-clearance compound verapamil (35), revealing a 28-fold range in predicted Cmax and a 23-fold range in predicted $AUC_{0\text{-}24h}$. A high variation in $AUC_{0\text{-}24h}$ of 23-fold is also observed for the low-clearance compound caffeine (7).

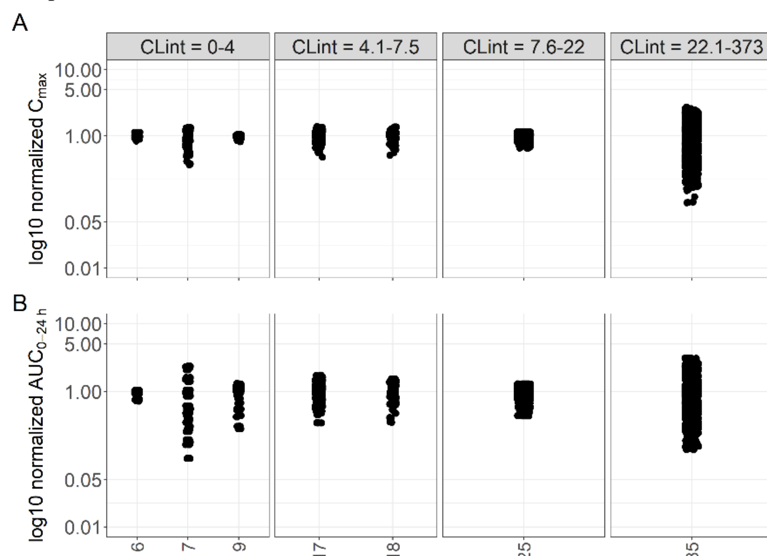

Fig. 4: Variation in PBK model-predicted Cmax (A) and $AUC_{0\text{-}24h}$ (B) as a result of the variation in reported *in vitro* CLint, Papp and Fup values
The depicted compounds are numbered as described in Table 1.

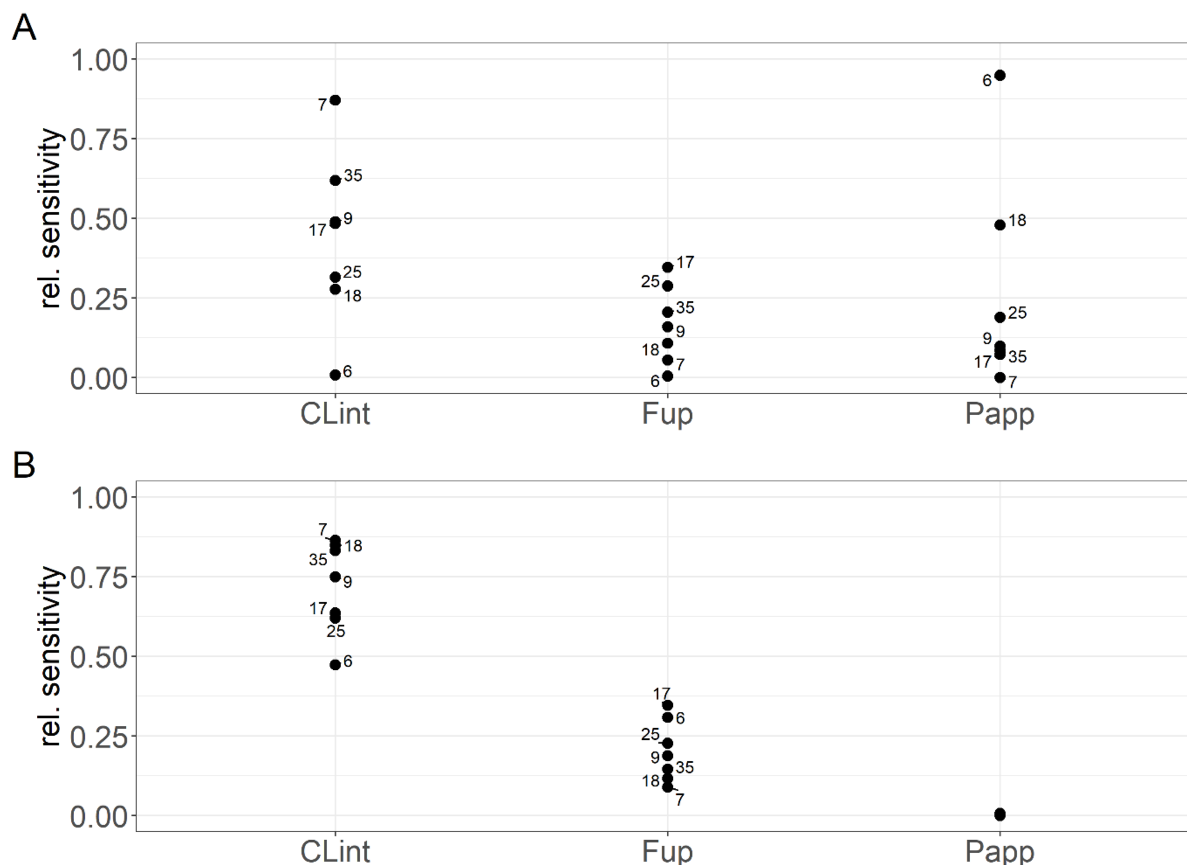

Fig. 5: Relative sensitivity of the Cmax (A) and AUC_{0-24h} (B) prediction to the variation in CLint, Papp and Fup, as obtained with the RVis global sensitivity analysis
The relative sensitivity represents the relative contribution of each of the three parameters to the variation in Cmax or AUC_{0-24h} as observed in Figure 4. For example, in case of caffeine (7), the variation in CLint accounted for 87% of the total variation in Cmax predictions, whereas variation in Fup and Papp contributed with 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[4].

3.5 Relative contribution of the different input parameters to the variation in predicted Cmax 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., CLint, Papp, or Fup) contribute most to the variation in Cmax and AUC_{0-24h} predictions as observed in Figure 4. Experimental variation in CLint had the highest impact on AUC_{0-24h} predictions for all compounds and for four out the seven compounds also on the Cmax predictions (caffeine (7), diltiazem (17), S-warfarin (9) and verapamil (35)). The observed variation in Cmax predictions for these compounds can thus largely be attributed to the variation in CLint. The experimental variation in uptake parameter Papp has no influence on the AUC_{0-24h} predictions but does have an impact on the Cmax predictions of two out of the seven compounds (diazepam (6) and quinidine (18)). The relative sensitivity towards experimental variation in Fup values was found to be lower than for CLint (Figure 5).

4 Discussion

With the present study we explored the experimental variation *in vitro* CLint, Caco-2 Papp and Fup measurements and the impact that this experimental variation has on PBK model predictions of Cmax and AUC_{0-24h}. As a result of the observed experimental variation in CLint, Papp, and Fup, the PBK model-predicted Cmax for the seven compounds for which these three parameters were available, was found to range between 1.4- to 28-fold and the AUC_{0-24h} to range between 1.4- to 23-fold. The large variation in Cmax and AUC_{0-24h} 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 obtain insights in 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 often not reported in the scientific literature. 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 Km (Black et al., 2021). However, measurements are still available in the literature in which this condition is not met or not considered (for example, Fortaner et al., 2021). In case of Caco-2 absorption experiments, a critical aspect of obtaining relevant Papp 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 this criterium to measure under sink-conditions is not met or not considered (for example, 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, mismatches between the observed data points and mathematical fit was 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[4].

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 explicitly taken into account in this guidance document yet. Nonetheless, effective protocols for performing *in vitro* kinetic studies to derive values for CLint, Papp, and Fup are available in the scientific literature (For example Watanabe et al., 2018; Cai and Shalan, 2021; Hubatsch et al., 2007; Black et al., 2021) . We highly recommend that these high-quality protocols would be 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 also 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 being requested in various regulatory guidelines (for example 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 only for pharmaceuticals sufficient clinical data are available (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 evaluations 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 an important aspect in this respect, 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 evaluate the quality of *in vitro* kinetic data are therefore an important next step.

Apart from guidance documents on the design of *in vitro* kinetic studies, guidance documents will also be needed with respect to the applicability domain of different *in vitro* kinetic studies with respect to meeting 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 Cmax and $AUC_{0\text{-}24h}$ in the present study after oral exposures do also 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 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 additional kinetic processes in PBK models (Punt et al., 2022).

Whereas the present study focussed on the impact of variation in reported *in vitro* CLint, Fup and Papp values on PBK model predictions, other *in vitro* kinetic input parameters could be relevant as well. Metabolic clearance is, for example, not only measured 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 (Km and Vmax) need to be derived from the *in vitro* metabolism studies. Moreover, *in vitro* transporter kinetic data (for example 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 in literature reported 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 as well 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 (Berezhkovskiy, 2004) led frequently to underpredictions of the C_{max} of acidic compounds (pKa<6), whereas the calculation method of Schmitt (Schmitt, 2012) appeared to perform less well for highly lipophilic compounds (Punt et al., 2022). The calculation method of Rodgers and Rowland (Rodgers and Rowland, 2006) performed overall best, which 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 CLint, Papp and Fup on PBK model-based Cmax and $AUC_{0\text{-}24h}$ 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, thereby meeting the 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 harmonisation of testing procedures and correct usage of test results.

Conflict of interest

The authors declare that they have no conflicts of interest

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Data availability statement

The collected literature data that are used in the present study are provided in supporting information 1. The model code of the PBK model and input parameters are provided on GitHub (https://github.com/wfsrqivive/PBK_exp_variation.git).

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