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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 describe the key aspects that need to be considered when performing in vitro kinetic studies and explored the experimental variation in the values for these parameters as reported in 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 ($AUC_{0\text{-}24h}$) was determined. As a result of the experimental variation in CLint, Papp, and Fup, the predicted variation in Cmax for individual compounds ranged between 1.5- to 79-fold and the predicted variation in $AUC_{0\text{-}24h}$ ranged between 1.3- 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 it will be important that the boundaries in experimental conditions and the applicability domain, and of use of different in vitro kinetic models are clearly 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 (Blaauboer, 2010; Jochem Louisse *et al.*, 2017; 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, e.g. with help of PBK modelling (Bessems *et al.*, 2014; J. Louisse *et al.*, 2017; 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 understand 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 to 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 needs 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 100-fold differences in literature reported in vitro hepatic CLint values as obtained from incubations with primary human hepatocytes, and noticed that 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 1) provide an overview of the aspects that need to be taken into account when performing in vitro kinetic studies to derive these parameters for oral exposure and 2) to explore the impact of experimental variation in the in vitro kinetic results on PBK model predictions. The results are discussed with respect to the potential impact of the in vitro experimental variation on regulatory decision making and the steps required for standardization of the in vitro methods.

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 this 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 (www.scopus.com) 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

(www.scopus.com) 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 Hallifax *et al.* (2012), Neuhoff *et al.* (2003), Gertz *et al.* (2010), Estudante *et al.* (2015), Larregieu and Benet (2014), Li *et al.* (2007), and Lee *et al.* (2017). In case of Fup, the R httk database (EPA) and Srivastava *et al* (2021), Ye *et al.* (2016), and Ferguson *et al.* (2019) were identified as major sources. 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 the supporting information.

Table 1. Model compounds and the distribution of the collected CLint, Papp, and Fup values.

number	compound[a]	CLint			Papp			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	29	21	3	0.028	86	9
7	**Caffeine**	1.6	130	10	37	22	4	0.97	42	3
8	Pindolol	1.9	29	7						
9	S-warfarin	1.9	150	5				0.013	46	9
10	Omeprazole	2.4	63	5						
11	Timolol	2.7	82	8						
12	proxen	4.1	160	6						
13	Metoprolol	4.8	77	11	33	120	10			
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	36	81	9	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.

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, n.d.) as described by Punt et al. (2021) and is provided on GitHub (https://github.com/wfsrqivive/PBPK_exp_variation.git). 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. 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. 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 https://github.com/wfsrqivive/PBPK_exp_variation.git. 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. To this end, for each compound, the R code of the PBK model was loaded into the RVis software (v0.15, using R 4.1.1). 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 the supporting information.

3. Results

3.1 Background CLint measurements and evaluation of the in vitro experimental variation.

Different protocols exist to measure in vitro kinetic constants for metabolism. Incubations are most frequently performed with primary hepatocytes or sub-cellular liver fractions like microsomes or S9 (in the presence of relevant cofactors) (Gouliarmou *et al.*, 2018; Lipscomb and Poet, 2008; Pelkonen and Turpeinen, 2007). Primary hepatocytes are considered the gold standard for performing in vitro metabolism studies. Generally, experiments are performed with cryopreserved primary hepatocytes, as these can be stored for a longer period making them more readily available than freshly prepared hepatocytes. Cryopreserved hepatocytes retain most of the activity of freshly prepared hepatocytes (Lipscomb and Poet, 2008). With a so-called "metabolite formation protocol", in vitro incubations are performed at different substrate concentrations at a fixed incubation time and cell concentration (Figure 1A). The formation of metabolites is then measured in these experiments, which follows the Michaelis-Menten equation (Seibert and Tracy, 2014):

$$v = \frac{Vmax * [compound]}{Km + [compound]} \quad \text{eq. 1}$$

In this equation, the Vmax is the maximum velocity (e.g. µmol/min/10^6 hepatocytes) and the Km is the Michaelis-Menten constant with unit µM (Peters, 2012). A key advantage of this approach is that the kinetic constants obtained can be used to describe the formation of metabolites, and allows to account for concentration dependent saturation of the enzymes. A disadvantage of this protocol is that standards of the metabolites are required for quantification. Given that such standards cannot be easily obtained for most compounds, the metabolic conversion of compounds is more frequently measured with a so-called "substrate depletion approach" in which the disappearance of a compound is measured over time to derive CLint based on the slope of the substrate depletion curve (Jones and Houston, 2004). One of the most critical aspects of substrate depletion experiments is that the substrate concentration should be well below the Km, as only then the rate, *v*, can be simplified as depicted in Equation 2 (Seibert and Tracy, 2014).

$$v = \frac{Vmax * [compound]}{Km} = CLint*[compound] \quad \text{eq. 2}$$

The obtained CLint values can therefore only be used in situations where no saturable metabolism is to be expected. This can be explored with in vitro range finding experiments at different concentrations to determine at which concentrations saturation of metabolism occurs (Sjögren *et al.*, 2012; Nichols *et al.*, 2018). First estimates of the internal concentrations with a PBK model can be used to determine if these saturable conditions are likely to be reached in the liver. Other aspects that need to be considered when performing in vitro metabolic clearance studies are e.g. the protein amount in the incubation mixture, whether or not serum is added to the incubation, number of time points and sampling schedule, the percentage of test item consumption at the end of the incubation, and aspects related to the analytical techniques that are used to analyse the sample (Louisse *et al.*, 2020a; Gouliarmou *et al.*, 2018).

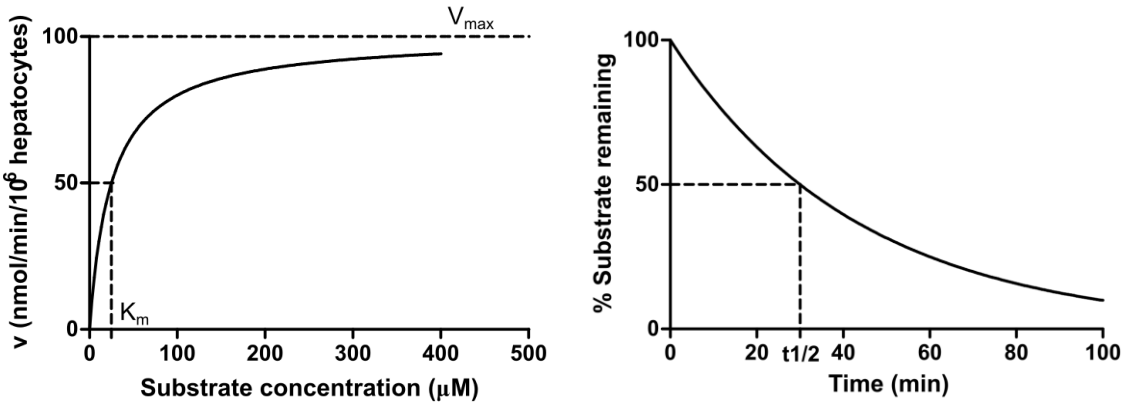

Figure 1. Examples of A) Michaelis–Menten kinetics with a Km of 25 µM and Vmax values of 100 nmol/min/10^6 hepatocytes and B) a metabolic clearance study with a t1/2 of 30 min and a CLint of ln(2)/30 = 0.02 ml/min/$10^{\wedge 6}$ hepatocytes when performed in an incubation that contains 10^6 hepatocytes per ml.

Figure 2 shows the experimental variation in in vitro metabolic clearance studies as obtained from the literature. For the majority 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 2, also reveals that the variation in CLint is consistent over the different compounds. This is corroborated by a Levene's test on equal variances, revealing no statistical differences in the variance for most of the compounds (Results provided in the supporting information). This consistency in experimental variation over a range of different compounds provides an indication of the variation that can be expected from in vitro metabolic clearance studies with primary hepatocytes.

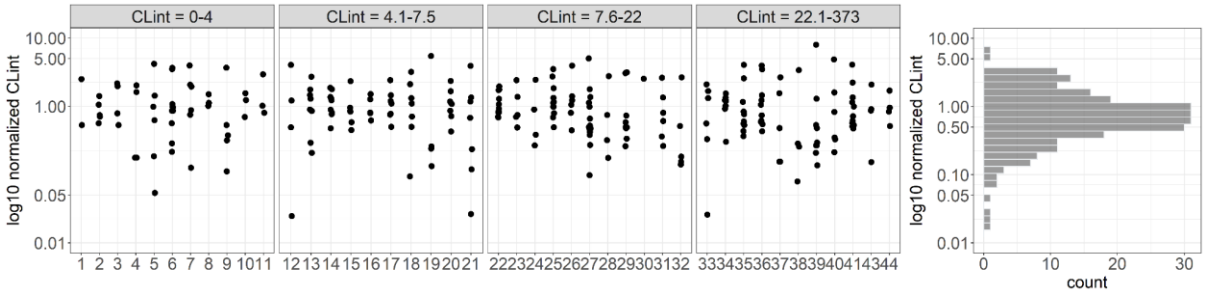

Figure 2. Variation in in vitro CLint (µL/min/10^6 cells) measurements. The histogram depicts the combined distribution of the variation over the different compounds. The values represent the normalized CLint values, corresponding to the CLint values obtained for a specific compound, divided by the mean of these values for the specific compound.

3.2 Background Caco-2 Papp measurements and evaluation of the in vitro experimental variation.

The Caco-2 cellular model of intestinal absorption is one of the most frequently used in vitro cell models to study the rate of transport of compounds over the intestinal cell membrane. Although Caco-2 cells are derived from a human colon carcinoma, the cells mimic the epithelial barrier of the small intestine when cultured in a monolayer (Hubatsch *et al.*, 2007). For in vitro Papp measurements, the cells are grown in a so-called Transwell system, in which the cells are seeded on a permeable filter

insert and are cultured for about 21 days to form a monolayer. To measure the cellular transport of a compound, the cell culture medium at the apical compartment of the Transwell is replaced by a transport buffer in which the compound is dissolved and the cell culture medium at the basolateral compartment is replaced by the transport buffer, often containing bovine serum albumin to mimic the blood compartment (Hubatsch *et al.*, 2007). A critical aspect of Papp measurements 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 need 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. In addition, it should be noted that Caco-2 experimental results often vary between labs and with batches of cells. Therefore, a range of reference substrates should be included in the experimental setup to normalize the results. A final important experimental aspect that can affect the Papp measurement is the pH gradient that is applied between the apical and basolateral compartment. A pH gradient of 6.5-7.4 provides the best representation of the physiological conditions between the intestine and blood (Neuhoff *et al.*, 2003).

Figure 3 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 40 to 60-fold, ranging between values that about 3 to 4-fold higher and about 15-fold lower than the mean Papp of a specific compound. For the remaining compounds, less data was available and the results revealed a 1.5 to 5-fold variation.

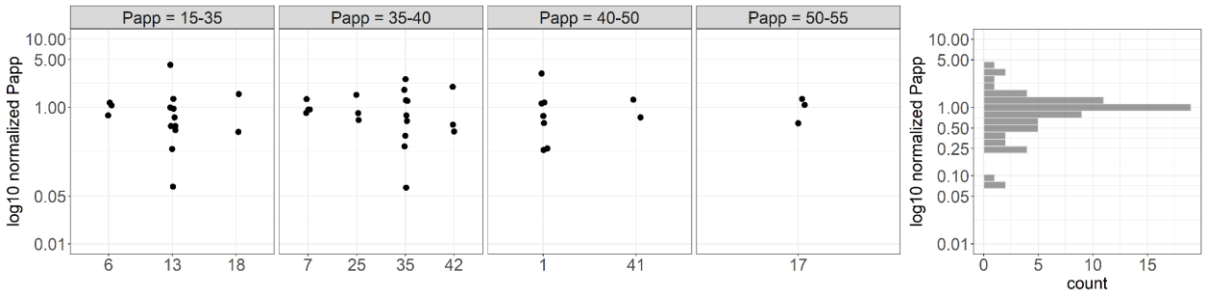

Figure 3. Variation in in vitro Caco-2 Papp (cm^{-6}/s) measurements. The histogram depicts the combined distribution of the variation over the different compounds. The values represent the normalized Papp values, corresponding to the Papp values obtained for a specific compound, divided by the mean of these values for the specific compound.

3.3 Background Fup measurements and evaluation of the in vitro experimental variation.

Various methods have been developed to measure Fup, of which the equilibrium dialysis test system being most commonly applied. For these experiments, so-called equilibrium dialysis devices are used, which consists of a bas plate and different dialysis inserts. Each of dialysis inserts consists of two chambers separated by a dialysis membrane. The human plasma, generally containing 2 to 5 µM of the substrate, is added to one chamber and phosphate-buffered saline (PBS) to the other (Ryu *et al.*, 2021). The concentrations in the two chambers is monitored until an equilibrium is reached. The equilibrium dialysis techniques particularly poses challenges with measuring the fraction unbound for highly bound compounds. For these compounds the levels in the receiving PBS chamber will be close to the limit of detection, resulting in unmeasurable Fup values. For highly bound compounds, modified equilibrium dialysis have therefore been proposed, including bidirectional equilibrium dialysis, dilution methods and pre-saturation methods (Ferguson *et al.*, 2019; Wambaugh *et al.*, 2019).

To obtain insights in the variation in Fup measurements, a literature search was performed to find reported Fup values for a range of compounds. Figure 4 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 appears to be less than observed for CLint and Caco-2 Papp values as described above. The largest experimental variation is observed for diclofenac (34) with Fup values ranging from 0.0015-0.02, corresponding to a 13-fold range.

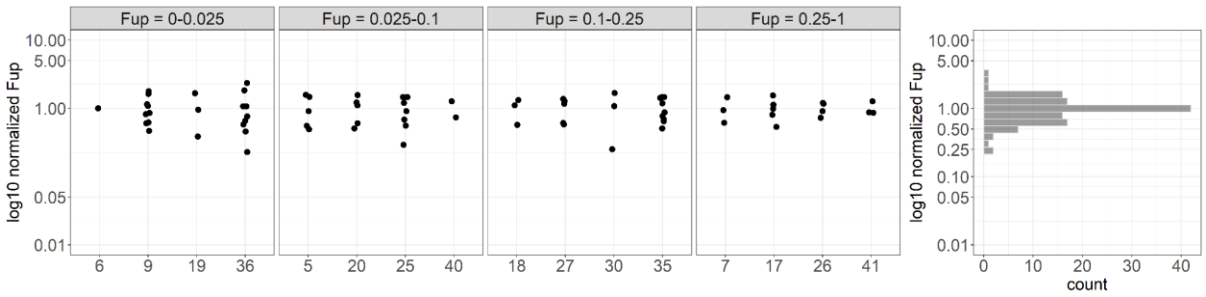

Figure 4. Variation in in vitro Fup (unitless) measurements. The histogram depicts the combined distribution of the variation over the different compounds. The values represent the normalized Fup values, corresponding to the Fup values obtained for a specific compound, divided by the mean of these values for the specific compound.

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 six 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 5. 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 5 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 only a 1.5-fold range in predicted Cmax values and 1.3-fold range in predicted $AUC_{0\text{-}24h}$. The highest variation in both Cmax and $AUC_{0\text{-}24h}$ predictions occurs for the high-clearance compound verapamil (35), revealing a 79-fold

range in predicted Cmax and a 16 -fold range in predicted $AUC_{0\text{-}24h}$. A high variation in $AUC_{0\text{-}24h}$ of 23-fold is also observed for caffeine (7).

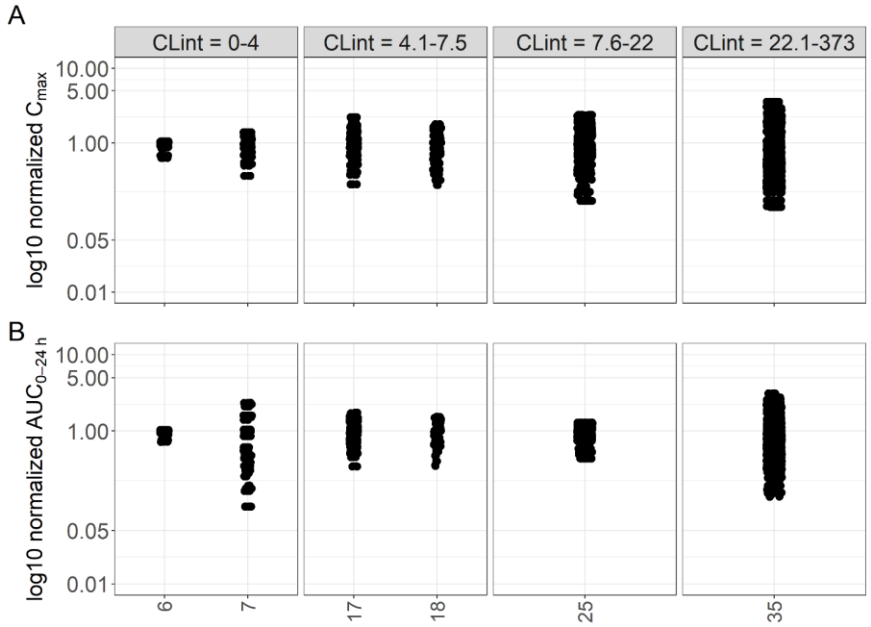

Figure 5. Variation in PBK model-predicted Cmax (A) and $AUC_{0\text{-}24h}$ (B) as a result of the variation in reported in vitro CLint, Papp and Fup values.

3.5 Relative contribution of the different input parameters to the variation in predicted Cmax and $AUC_{0\text{-}24h}$ values.

Figure 6 depicts the results of the global sensitivity analysis that was performed to determine which of the three input parameters (i.e. CLint, Papp, and Fup) contribute most to the variation in Cmax and $AUC_{0\text{-}24h}$ predictions as observed in Figure 5. Experimental variation in CLint had the highest impact on $AUC_{0\text{-}24h}$ predictions for all compounds and for three out the six compounds also on the Cmax predictions (caffeine (7), diltiazem (17) 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 had no influence on the $AUC_{0\text{-}24h}$ predictions, but does have an impact on the Cmax predictions of two out of the six compounds (diazepam (6) and quinidine (18)). The relative sensitivity towards experimental variation in Fup values was found to be lower than for CLint.

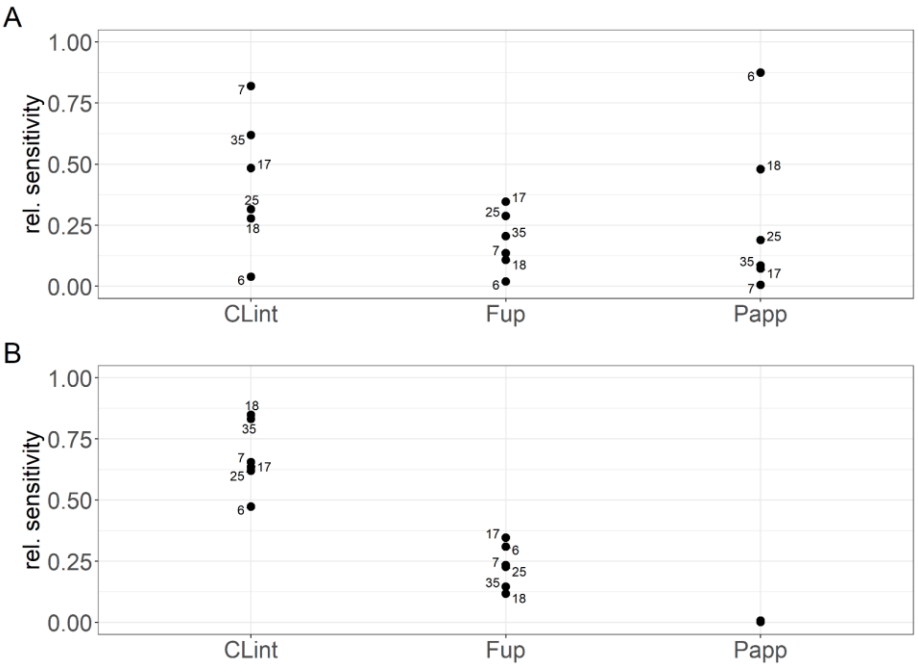

Figure 6 . Relative sensitivity of the Cmax (A) and $AUC_{0\text{-}24h}$ (B) prediction to the variation in CLint, Papp and Fup, as obtained with the RVis global sensitivity analysis. The relative sensitivity respresents the relative contribution of each of the three parameters to the variation in Cmax or $AUC_{0\text{-}24h}$ as observed in Figure 5. For example, in case of caffeine (7), the variation in CLint accounted for 82% of the total variation in Cmax predictions, whereas variation in Fup and Papp contributed with 14% and 0.6%, respectively. The remaining 3.4% variation is caused by the interaction between these different parameters as depicted in the supporting information.

4. Discussion

With the present study we described various aspects that need to be taken into account when performing in vitro CLint, Caco-2 Papp and Fup kinetic studies and explored the experimental variation observed with these parameters and the impact that this experimental variation has on PBK-model predictions of Cmax and $AUC_{0\text{-}24h}$. As a result of the observed experimental variation in CLint, Papp, and Fup, the PBK-predicted Cmax for compounds for which these three parameters were available, was found to range between 1.5- to 79-fold and the $AUC_{0\text{-}24h}$ ranged between 1.3- and 23-fold. The extensive variation in Cmax and $AUC_{0\text{-}24h}$ predictions, as observed for some of the compounds, indicates that the in vitro kinetic data are currently not robust enough to make reliable predictions of the in vivo kinetics of a compound without means to evaluate the adequacy of a specific in vitro kinetic parameter or PBK-model prediction.

To gain confidence in in vitro kinetic parameters and PBK model predictions, comparisons with in vivo experimental animal or human kinetic data are currently being requested in various regulatory guidelines (e.g. SCCS, 2018; EMA, 2018; OECD, 2021). This approach of model evaluations against in vivo data is at present, however, 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. For a transition to next generation (animal-free) regulatory risk evaluations to happen, other means to gain confidence in the in vitro kinetic data and PBK-model predictions are therefore urgently needed. 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. It will therefore be more critical to improve the robustness of in vitro kinetic data and to improve the possibilities within regulatory risk evaluations to evaluate the quality of in vitro kinetic data and the adequacy of an in vitro study design.

As described in the present study, each in vitro kinetic test system has its own set of inherent boundaries and restrictions with respect to how the data can be used in a regulatory context. Critical to the in vitro experimental setup are e.g. the selection of the substrate concentration, the incubation time, or concentration of enzyme that is used (Louisse *et al.*, 2020). In addition, considerations of the loss of a chemical due to sticking to the plastic or from evaporation are important to take into account in the experimental setup (Groothuis *et al.*, 2015). At present, various protocols for performing in vitro kinetic studies to derive values for CLint, Papp, and Fup are available in the scientific literature (e.g. Watanabe *et al.*, 2018; Cai and Shalan, 2021; Hubatsch *et al.*, 2007) and describe these critical aspects that need to be considered. Our study shows that a next step would be to formalize these literature available protocols and to describe the applicability domain/use in a regulatory context. It should, however, 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). Recently, Black et al. (2021) also observed that a starting concentration of 1 µM, that is generally applied for pharmaceutical compounds for metabolic clearance studies, may be insufficient to achieve first-order reaction conditions for some non-pharmaceuticals. These are all aspects that need to be considered when formalizing existing protocols. At present, there are no guidance documents available that arrange these type of considerations. Recently, the OECD published a guidance document on a workflow for characterising 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. The results of the present study indicate that the quality of the model predictions will be as good as the quality of the input data. The development of guidelines on the design of in vitro

kinetic studies will therefore be a critical follow up step to this guidance document to gain confidence in PBK model predictions.

Apart from guidance documents on the design of in vitro kinetic studies, guidance documents will also be needed with respect to the applicability 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 $AUC_{0\text{-}24h}$ 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 (Punt et al., submitted).

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 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 (e.g. intestine, kidney and liver transporters) are important for the kinetics of some compounds. A similar description of experimental boundaries and the applicability domain will be needed for each of these studies. Apart from the in vitro kinetic data, in silico predictors of different kinetic parameters have been developed as well. 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. (submitted) revealed that significant differences can occur as a result of the use of different calculators. For example, the calculation method of Berezhkovskiy (Berezhkovskiy, 2008) 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., submitted). The calculation method of Rodgers and Rowland (Rodgers and Rowland, 2006) performed overall best, and was also 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 Cmax and $AUC_{0\text{-}24H}$ predictions. This implies that steps need to be taken to reduce experimental variability and the associated uncertainty in order to increase the confidence in these in vitro kinetic data for regulatory use. To this end, it will be crucial that the in vitro experiments are performed in a standardized way and thereby meet the regulatory needs. In addition, the chemical and regulatory applicability domains of the in vitro test systems and kinetic models need to be clearly described. Therefore, it is important that existing protocols are formalized in guidance documents to improve harmonisation of testing procedures and correct usage of test findings.

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