Research Article

Predictive Performance of Next Generation Human Physiologically Based Kinetic (PBK) Models Based on *In Vitro* and *In Silico* Input Data

Ans Punt[1], Jochem Louisse[1], Karsten Beekmann[1], Nicole Pinckaers[1], Eric Fabian[2], Bennard van Ravenzwaay[2], Paul L. Carmichael[3], Ian Sorrell[3] and Thomas E. Moxon[3]

[1]Wageningen Food Safety Research, Wageningen, The Netherlands; [2]BASF SE, Experimental Toxicology and Ecology, Ludwigshafen, Germany; [3]SEAC, Unilever, Colworth Science Park, Sharnbrook, Bedford, UK

Abstract

The goal of the present study was to assess the predictive performance of a generic human physiologically based kinetic (PBK) model based on *in vitro* and *in silico* input data and the effect of using different input approaches for chemical parameterization on those predictions. For this purpose, a dataset was created of 38,772 C_{max} predictions for 44 compounds by applying different combinations of *in vitro* and *in silico* approaches for chemical parameterization, and these predicted C_{max} values were compared to reported *in vivo* data. Best results were achieved when the hepatic clearance was parameterized based on *in vitro* (i.e., hepatocytes or liver S9) measured intrinsic clearance values, the method of Rodgers and Rowland for calculating tissue:plasma partition coefficients, and the method of Lobell and Sivarajah for calculating the fraction unbound in plasma. With these parameters, the median C_{max} values of 34 out of the 44 compounds were predicted within 5-fold of the observed C_{max}, and the C_{max} values of 19 compounds were predicted within 2-fold. The median C_{max} values of 10 compounds were more than 5-fold overestimated. Underestimations (> 5-fold) did not occur. A comparison of the current generic PBK model structure with chemical-specific PBK models available in the literature was made to identify possible kinetic processes not included in the generic PBK model that might explain the overestimations. Overall, the results provide crucial insights into the predictive performance of PBK models based on *in vitro* and *in silico* input and the influence of different input approaches on the model predictions.

1 Introduction

Physiologically based kinetic (PBK) modelling has a crucial role in next-generation (non-animal) risk evaluations to predict internal human plasma (or tissue) concentrations and to relate these concentrations to *in vitro* biological effect concentrations (Blaauboer, 2010; Fabian et al., 2019; Louisse et al., 2017; Punt et al., 2019, 2021; Wetmore et al., 2015; Yoon et al., 2015). The ultimate applicability of PBK models in next generation (non-animal) risk evaluations will, however, depend on being able to make predictions without the support of animal *in vivo* kinetic data (e.g., plasma or tissue concentrations) (Paini et al., 2019; Peters and Dolgos, 2019). When available, human kinetic data as well as data from chemical analogues may be applied for PBK model development (OECD, 2021), but such data are generally scarce or unavailable for non-pharmaceuticals. Even though PBK models are increasingly parameterized based on *in vitro* and *in silico* input data, *in vivo* data are currently still needed to assess the validity of the model predictions for a given chemical. Moreover, when certain kinetic processes cannot be parameterized based on *in vitro* or *in silico* experiments, they are usually obtained by fitting model predictions to *in vivo* data (Peters and Dolgos, 2019; Tsamandouras et al., 2015). Given that human kinetic data are difficult to obtain, other strategies to evaluate the adequacy of *in vitro*- and *in silico*-based PBK models to estimate *in vivo* kinetics are needed.

Initial estimates of plasma and tissue concentrations of orally consumed compounds can effectively be made with minimal generic PBK models that are defined based on 1) a first order intes-

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Correspondence: Ans Punt, PhD
Wageningen Food Safety Research
P.O. Box 230, 6700 AE, Wageningen, The Netherlands
(ans.punt@wur.nl)

tinal absorption rate (ka) and a fraction absorbed (fa), 2) intrinsic hepatic clearance (CLint), 3) tissue:plasma partition coefficients, 4) the fraction unbound in plasma (fu_p), and 5) passive renal excretion (defined as the glomerular filtration rate times the unbound concentration of the compound in plasma (GFR × cPlasma × fu_p) (Jones and Rowland-Yeo, 2013). Gaining confidence in PBK model predictions requires confidence in the quality of the input data that are used to parameterize these kinetic processes (Gouliarmou et al., 2018; Louisse et al., 2020; Utsey et al., 2020). In addition, it is important to determine for which compounds a minimal PBK model as described above provides a sufficient level of prediction, and for which compounds additional kinetic processes need to be added to the model (for example, extrahepatic metabolism and/or transporter-mediated kinetics).

Recently, we evaluated the predictions made for C_{max} in rats (upon a single exposure) for 44 compounds with a minimal rat PBK model, applying different methods to obtain chemical-specific input parameters (Punt et al., 2022). This analysis revealed that C_{max} predictions ranged up to six orders of magnitude for some of the compounds as a result of the input parameters that were used. C_{max} predictions were typically found to be within 5-fold of the observed C_{max} for those compounds for which the unbound intrinsic clearance was low (< 1 L/h) or relatively high (> 20 L/h). However, one must be cautious with generalization of these findings to other compounds with different physicochemical properties, and findings obtained with the generic rat PBK model may not be applicable to a generic human PBK model. Since within next generation risk assessment predominantly human PBK model predictions will be required, it is crucial to find means to specifically evaluate human PBK model predictions in the absence of human *in vivo* data.

The goal of the present study was to assess the predictive performance of a minimal generic human PBK model, as described above, based on *in vitro* and *in silico* input data to predict plasma C_{max}. The C_{max} was selected as the most critical kinetic parameter that is most frequently used in PBK modelling-based reverse dosimetry approaches to predict *in vivo* toxicity from *in vitro* data (Louisse et al., 2017), particularly when the biological effect has a threshold-related mechanism of action (Groothuis et al., 2015). To evaluate the predictive performance of the minimal human generic PBK model to predict C_{max} values of a chemical, a literature search was performed to gather human *in vivo* plasma concentrations for a range of compounds. C_{max} predictions were subsequently made upon a single oral dose based on a variety of input approaches for estimating the chemical-specific parameters (CLint, fu_p, partition coefficients, ka, and fa) and compared to the observed C_{max} values as reported in the collected literature. Based on the data obtained, we characterized the contribution of different input approaches to the variation in C_{max} outcomes for individual compounds. In addition, for the compounds that were not predicted within 5-fold of the observed C_{max}, a literature study was performed to find existing chemical-specific PBK models that have been developed for these compounds, allowing us to evaluate which additional kinetic processes may need to be added to the generic PBK models to better describe their *in vivo* kinetics and improve plasma C_{max} estimations.

2 Materials and methods

Chemical selection and collection of human in vivo C_{max} values

A dataset of 44 model compounds was created to evaluate the performance of the C_{max} predictions by the PBK model based on different *in vitro* and/or *in silico* input approaches. The starting point for the selection of the model compounds was the availability of *in vitro* human intrinsic hepatic metabolic clearance data as key input to PBK model development. The R httk package (version 2.0.4.) by Pearce et al. (2017) was used as primary source for these *in vitro* metabolic clearance data, based on which the clearance data for 40 compounds could be derived. The dataset was extended to include rosuvastatin and fluvastatin, which are structurally related compounds for which transporter-mediated processes in liver and kidney play a main role in the kinetics (Chan, 2019). Further, ochratoxin A and coumarin were added to extend the dataset to also include non-pharmaceuticals. *In vivo* human kinetic studies are available for the latter 4 compounds, and their intrinsic clearance was measured in the present study in incubations with human liver S9.

A literature search for the selected compounds was performed in Scopus™[1] to identify human kinetic studies that report peak plasma concentrations of the compounds after a single oral dose or within the first 24 hours of a repeated oral dose study. The following key words were used for this literature study: ((TITLE ("compound name") AND ALL (bioavailability OR pharmacokinetics OR kinetics)) AND ((human OR man OR volunteer OR subject)) AND (C_{max} OR "c max" OR "maximal concentration" OR "maximum concentration" OR "peak concentration")). The studies that were identified for each compound were subsequently filtered to exclude 1) results obtained for specific patient groups like patients with renal impairment or gastric by-pass, 2) studies with children, and 3) studies using slow or extended-release formulations. Description of kinetics in such situations requires specific model adjustments, which was beyond the scope of the present study which focusses on the applicability domain of a generic PBK model for an average healthy adult. The final list of model compounds and related *in vivo* C_{max} data (and related oral doses) from 421 different published studies that were obtained in the search are provided in the supplementary Excel file[2] (SM1).

Generic PBK model code and input parameters

PBK model predictions were performed based on a published generic human PBK model code by Jones and Rowland-Yeo (2013) that was implemented as R script[3] by Punt et al. (2021) with mi-

[1] www.scopus.com (last accessed 22.12.2021)

[2] doi:10.14573/altex.2108301s1

[3] https://www.R-project.org/

nor modifications as summarized in the model code provided on GitHub[4]. The model consists of 13 compartments, corresponding to the major organs in the body and the arterial and venous blood compartment. The model requires chemical-specific parameters for intestinal uptake, distribution (i.e., partition coefficients, blood:plasma ratio (assumed to be 1 in the present study for all compounds), fraction unbound in plasma), hepatic clearance, and renal clearance (assumed to be the glomerular filtration rate times the free plasma concentration). Table 1 provides an overview on how these different input parameters were parameterized using a range of *in vitro* and/or *in silico* methods. Further details on these input approaches are given in the text below. The differential equations of the model are solved with the deSolve package (Soetaert et al., 2010).

Absorption from the gastrointestinal tract was described in the model by a first-order uptake process from the intestine to the liver compartment and requires an absorption rate constant (ka) and fraction absorbed (fa) as input (Jones and Rowland-Yeo, 2013). For the parameterization of these input constants, an *in silico* approach based on a QSAR from Hou et al. (2004) was applied that predicts the Caco-2 apparent permeability (P_{app}) based on the topological polar surface area (TPSA) of the compounds (Equation 1). For 30 of the 44 compounds, the QSAR-based approach was compared with *in vitro* measured P_{app} coefficients in Caco-2 transwell absorption experiments. These *in vitro* measured Caco-2 absorption data were partly obtained from Punt et al. (2022) and partly generated in the present study. Details of the QSAR calculations and Caco-2 experiments are provided in the supplementary information (SM2[5]). Both the QSAR-derived P_{app} values and the *in vitro* measured values were scaled to ka and fa based on the following equations.

$$\text{Log } P_{app} \text{ (cm/s)} = -4.36 - 0.01*\text{TPSA} \quad \text{Eq. 1}$$

$$\text{Log } P_{eff} (10^{-4} \text{ cm/s}) = 0.4926*\log P_{app} (10^{-6} \text{ cm/s}) - 0.1454 \quad \text{Eq. 2}$$

$$ka \text{ (/h)} = P_{eff}*2 \text{ (cm/s) /R (cm) * 3600 (s/h)} \quad \text{Eq. 3}$$

$$fa = 1 - (1 + (2*P_{eff}*\langle T_{si}\rangle)/(7*R))^{-7} \quad \text{Eq. 4,}$$

in which Equation 2 scales the Caco-2 apparent permeability to an human effective permeability based on Sun et al. (2002), and Equations 3 and 4 describe how the effective permeabilities are converted to ka and fa as described by Yu and Amidon (1999). For the calculation of ka and fa with Equations 2 and 3, an intestinal radius (R) of 1 cm and a small intestinal transit time $\langle T_{si}\rangle$ of 3.32 h were used (Grandoni et al., 2019).

Physicochemical data (log P, log D and pKa values, TPSA), which are used as input to calculate the fu_p and tissue:plasma partition coefficients and intestinal uptake were derived with ADMET Predictor[6] (v9.0, Simulation Plus, Lancaster, CA, USA) and with ChemAxon[7] (ChemAxon Ltd., Budapest, Hungary). Given that slight differences occur between the results of these two software packages with respect to the log P and pKa estimates, the influence of these differences on the PBK model predictions was evaluated. The log P, log D and pKa(s) that were obtained for the 44 compounds with each of the two software packages are provided in the Github repository[4].

For the parameterization of fu_p values, two *in silico* approaches were compared with *in vitro* measured values. One *in silico* approach for the calculation of fu_p is a method of Lobell and Sivarajah (2003). Log P and information on the pKa(s) are required input parameters for this calculation. The R code for this calculation is provided in the Github repository[4]. As second *in silico* approach, the fu_p calculations were obtained with the ADMET Predictor software[5]. The *in vitro*-derived human-specific fu_p values for 39 compounds were taken from the httk package with the original data measured by Wambaugh et al. (2019), Sohlenius-Sternbeck et al. (2012), Obach (1999), Lombardo et al. (2018), Ito et al. (2004), Wetmore et al. (2015), and Shibata et al. (2000).

In case of the calculation of partition coefficients, three approaches were compared, including the *in silico* approaches of (i) Rodgers and Rowland (2006), (ii) Berezhkovskiy (2004), which corresponds to the corrected method of Poulin and Theil (2002), and (iii) the *in silico* approach of Schmitt (2008). Log P and information on the pKa(s) and fu_p are required input parameters for these calculations. The R codes for these different calculations were obtained from Utsey et al. (2020) and were adjusted to fit the pipeline of the PBK model calculations of the current study. The codes can be found in the Github repository[4]. In case of the method of Rodgers and Rowland, the effect of including lysosomal trapping in the calculation method, as described by Schmitt et al. (2021), was tested. However, as this adjustment did not lead to substantial differences in predicted partition coefficients (SM1[2]), only the original approach of Rodgers and Rowland was included in the final dataset. In case of the method of Schmitt (2008), the membrane affinity (log MA) was calculated from the log P based on a QSAR from Yun and Edginton (2013) as provided in the code. The harmonized tissue composition data from Utsey et al. (2020) were used as input.

Three different approaches to obtain model parameter values for hepatic intrinsic clearance were compared. These included i) an *in silico* approach using ADMET Predictor[5], ii) an *in vitro* approach based on clearance studies with primary human hepatocytes, and iii) an *in vitro* approach based on clearance studies with human liver S9. For the *in silico* based approach, the cytochrome P450-dependent human hepatic clearance rates of the 44 compounds (CYP_HLM_CLint) were predicted with the ADMET Predictor[5]. These predicted clearance rates, expressed as µL/min/mg microsomal protein, were scaled in the model to the whole liver based on a microsomal protein yield of 40 mg microsomes/gram liver (Barter et al., 2007). The primary human hepatocyte clearance data were derived from the database of the R httk package containing the values that were originally

4 https://github.com/wfsrqivive/human_PBK.git (last accessed 22.12.2021)

5 doi:10.14573/altex.2108301s2

6 www.simulations-plus.com (last accessed 22.12.2021)

7 www.chemaxon.com (last accessed 22.12.2021)

Tab. 1: Input approaches applied in the PBK model predictions

Applied input	Method reference	Method name used in the figures	Number of compounds for which the respective data are available
Intestinal uptake (ka and fa)			
QSAR based on the topological surface area (TPSA)	Hou et al., 2004	QSAR	44
Caco-2 P_{app}	This work and Punt et al., 2022	*In vitro*	30
Physicochemical characteristics			
log P, log D, pKa, TPSA	ADMET Predictor	ADMET	44
log P, log D, pKa, TPSA	ChemAxon	ChemAxon	44
Tissue:plasma partition coefficients			
Berezhkovskiy	Berezhkovskiy, 2004	Berezhkovskiy	44
Rodgers and Rowland	Rodgers and Rowland, 2006	RodgersRowland	44
Schmitt	Schmitt, 2008	Schmitt	44
Intrinsic hepatic clearance (CL_{int})			
(Cryopreserved) primary hepatocytes	Data derived from the httk package (Pearce et al., 2017)	Hep	40
S9	This work	S9	18
In silico predicted CYP clearance	ADMET predictor	*In silico*	44
Fraction unbound plasma			
In vitro with rapid equilibrium dialysis	Data derived from the httk package (Pearce et al., 2017)	*In vitro*	44
In silico predicted based on log P, log D and pKa	Lobell and Sivarajah, 2003	Lobell and Sivarajah	44
In silico predicted based on the SMILES	ADMET predictor	ADMET	44

measured by Wambaugh et al. (2019), Sohlenius-Sternbeck et al. (2012), Obach (1999), Lombardo et al. (2018), Ito et al. (2004), Wetmore et al. (2015), and Shibata et al. (2000). For 18 out of the 44 compounds, the intrinsic hepatic clearance was also measured in incubations with human liver S9 in the present study. The protocol for these incubations is provided in the supplementary file (SM2[5]). The primary hepatocyte intrinsic clearance data were scaled to the whole liver based on a hepatocellularity (number of hepatocytes per gram liver) of 117.5 x 10^6 (Barter et al., 2007), whereas the S9-derived clearance data were scaled based on an S9 protein yield of 121 mg/g liver (Houston and Galetin, 2008). Corrections for unspecific binding of the compounds to the hepatocytes or S9 in the *in vitro* incubations was applied based on a calculation method of Kilford et al. (2008) for primary hepatocytes and a method of Hallifax and Houston (2006) for the S9. Although the latter calculation method was developed to predict the unbound intrinsic clearance in microsomal incubations, it was assumed to also be applicable to S9 incubations.

Human PBK model predictions and data analysis

By combining different input approaches, a total of 38,772 C_{max} predictions were made for the different model compounds at the same exposure conditions as applied in the *in vivo* studies from which the reported C_{max} values were obtained. For each chemical, the predicted C_{max} was divided by the observed C_{max} as marker of the quality of the PBK model prediction for that compound. As a result of the different input combinations, a range of predicted:observed ratios was obtained for each chemical of which the median was calculated. Median predicted C_{max} values that were more than 5-fold higher than the observed C_{max} values were considered overestimated, and median C_{max} values that were more than 5-fold lower than the observed C_{max} values were considered underestimated, though the latter did not occur in the present data set (see Results section). We also assessed the number of chemicals that were predicted within 2-fold, which is often used as a requirement when a PBK model is used within a regulatory context to demonstrate that the proposed model is fit for purpose (Shebley et al., 2018). The effect of different input approaches on the C_{max} predictions was determined by comparing for each input approach and compound the median C_{max} and predicted:observed ratios and determining the differences between the input approaches in predicted median C_{max} values.

A sensitivity analysis was performed for the predictions by changing the input value of a parameter by 1% and determining

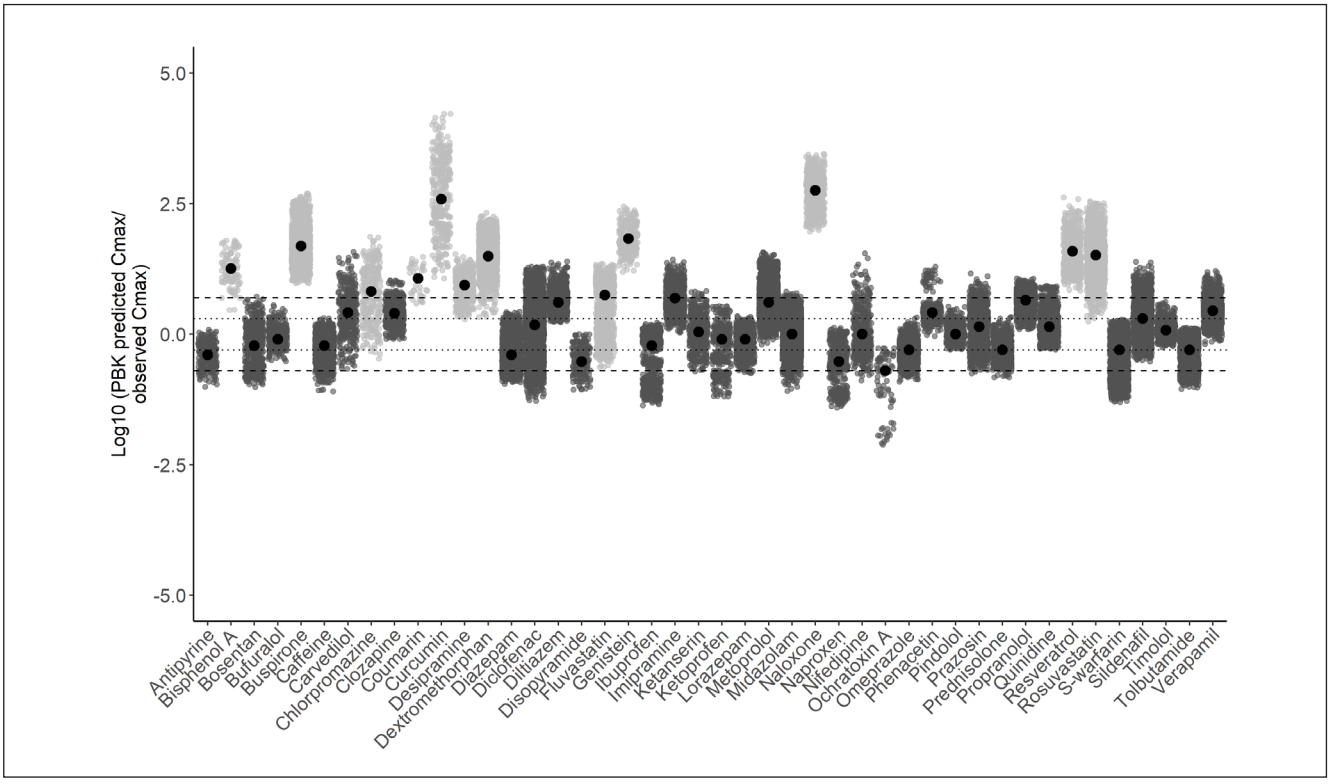

Fig. 1: Ratios between PBK model-predicted C_{max} values and *in vivo* observed C_{max} values observed for 44 reference compounds in humans

Per chemical, different predicted C_{max} values are obtained by running simulations with the different input approaches (*in vitro* or *in silico* approaches to parameterize input parameters) as presented in Table 1. Each predicted C_{max} is then compared with the *in vivo* C_{max} values for the chemical in the dataset. The median of these predicted:observed ratios is depicted along the individual datapoints. Datapoints between the dashed horizontal lines are within 5-fold of the observed C_{max} and the datapoints between the dotted horizontal lines are within 2-fold of the observed C_{max}. Compounds for which the median predicted C_{max} is more than 5-fold overestimated are depicted in light grey.

the relative change in C_{max}, expressed as the normalized sensitivity coefficient (NSC) according to Equation 5.

$$NSC = (C' - C)/(P' - P) \times (P/C) \quad \text{Eq. 5,}$$

where C is the initial value of the model output, C′ is the modified value of the model output resulting from an increase in parameter value, P is the initial parameter value, and P′ is the modified parameter value. The sensitivity analysis was performed at an equal oral dose of 1 mg/kg bw for all compounds and input combinations. The R codes for the above analyses can be found in the Github repository[4].

To better understand the potential causes of why the C_{max} of certain compounds could not be predicted within 5-fold of the observed C_{max}, a literature study was performed to explore critical differences between the current minimal PBK model and existing PBK models for the different compounds. For this literature study, the following keywords were used: ((ALL ("compound name") AND ALL (PBPK OR PBK OR PBBK OR PBTK)). The obtained results were manually screened for the presence of PBK model codes for the given substance.

3 Results

3.1 Evaluation of the collected data on human plasma concentrations

For 41 of the 44 compounds, two or more *in vivo* studies were available from which the peak plasma concentrations could be derived. For these compounds, the variation in reported plasma concentrations between studies was assessed by calculating the normalized C_{max} (C_{max}/dose) for each study and assessing the distribution of these normalized C_{max} values. For 22 of the 44 compounds, the difference in normalized C_{max} values between the different studies ranged between 1.3- and 3-fold and for 10 compounds between 3 and 5-fold (see SM1[2]). For the remaining 12 compounds, larger differences between studies are observed, with the highest difference occurring for metoprolol (16-fold difference normalized C_{max} values between studies) and dextromethorphan (13-fold difference normalized C_{max} values between studies). These compounds are both CYP2D6 substrates (Frank et al., 2007) and are therefore prone to large interindividual variation in humans. The PBK model predictions (based on combinations of input approaches) were com-

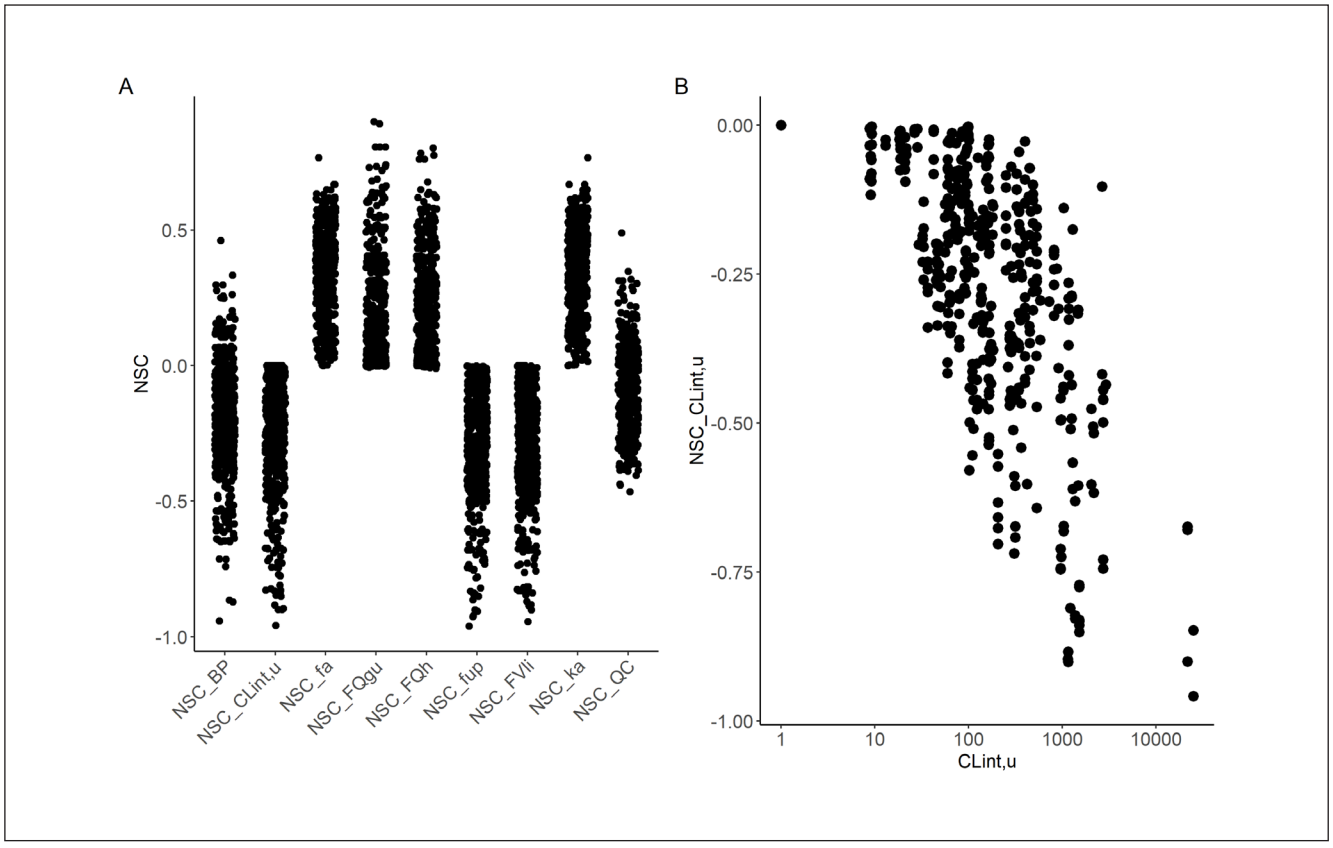

Fig. 2: (A) Normalized sensitivity coefficients (NSCs) of the C_{max} predictions to different input parameters for the different compounds. (B) NSCs for CLint,u plotted against CLint,u values used as input

The datapoints in the figures correspond to the NSCs for a random selection of 12 C_{max} simulations based on different input approaches per chemical. BP, blood plasma ratio; CLint,u, unbound intrinsic liver clearance; fa, fraction absorbed; FQgu, blood flow fraction to the gut; FQh, blood flow fraction to the liver; fup, fraction unbound in plasma; FVli, volume fraction liver; ka, intestinal uptake rate; QC, cardiac output

pared to each of the *in vivo* reported C_{max} from all the different studies of the 44 compounds.

3.2 Performance of the generic PBK model based on different input approaches

In Figure 1, the ratios between PBK model-predicted C_{max} values and *in vivo* observed C_{max} values are shown. A large variation (1-4 orders of magnitude) in predicted:observed ratios can be observed, which is predominantly the result of the different input approaches that were applied and to a lesser extent reflects the experimental variation between the *in vivo* studies to which the PBK-model predictions are compared (as described above). The highest range in predicted:observed ratios is observed for curcumin. Depending on the input combinations, a 12- to 16,654-fold overprediction of the reported C_{max} values is obtained. Despite the variation in predicted:observed ratios for the different model compounds in the dataset, the median C_{max} of the majority of the compounds (32 out of the 44) is within 5-fold of the observed C_{max} values, and the C_{max} of 19 out of 44 compounds could be predicted within 2-fold. The C_{max} values of 12 compounds were predominantly overestimated (i.e., having a median predicted C_{max} that is > 5-fold higher than the observed C_{max}). Although some of the input combinations led to significant underpredictions for various compounds, the median of none of the compounds was more than 5-fold underpredicted. In figure 1, ochtratoxin is on the border of 5-fold underprediction, but not more than 5-fold underpredicted.

3.3 Sensitivity analysis

Improving the predictive value of the minimal PBK model requires insight into the critical input parameters that affect the model predictions. To this end, a sensitivity analysis was performed for the predictions by changing the input value of a parameter by 1% and determining the relative change in C_{max}, expressed as the normalized sensitivity coefficient (NSC) at a dose of 1 mg/kg bw. Figure 2A shows the results for the most influential input parameters that affect the C_{max} predictions (max. NSCs > 0.5 in absolute value). The NSCs of remaining input parameters can be found in Figure S1[5]. Figure 3 reveals that the scaled unbound intrinsic clearance (CLint,u), the fraction unbound (fu_p), and parameters that determine the blood flow to and the volume of the liver (FVli, FQh, Fqgu) are among the most crit-

ical input parameters. All these parameters determine the availability of the compounds for metabolic clearance. Other important input parameters that affect the C_{max} predictions relate to the oral absorption (ka and fa) and the blood flow (QC). The B:P ratio also was identified as a sensitive parameter. This parameter was set to a default value of 1 for all compounds in the present study since measured data on B:P ratios are generally lacking and no *in silico* tools are available to estimate the B:P ratio. Altogether, these most influential parameters can be summarized as factors that affect the bioavailability of the compound, such as the fraction absorbed and the liver clearance, and factors that influence the volume of distribution, such as the fu_p, B:P, and to a lesser extent the tissue:plasma partition coefficients (Fig. S1[5]). Figure 2A reveals that not all compounds are equally sensitive to the different input parameters. The observed differences between compounds in sensitivity were found to relate to the extent of metabolic clearance: the sensitivity increases with increasing CLint,u until a maximum sensitivity is reached for compounds with a high CLint,u (Fig. 2B). A similar relation is observed between the CLint,u of the compounds and the sensitivity towards the other input parameters like ka, fa, and QC (Fig. S2[5]).

3.4 Effect of the input approaches on C_{max} predictions

Given the sensitivity of the C_{max} predictions to chemical-specific input parameters like CLint, fu_p, fa, and ka, and to a lesser extent to the partition coefficients, the quality of the input approaches for these parameters can have a substantial effect on the C_{max} predictions. It is therefore important to understand if certain input approaches perform better than others. To this end, we determined whether differences occur in median C_{max} predictions between the different input approaches. In Figure 3, the C_{max} predictions with more than 3-fold differences between the applied input approaches are highlighted. These results reveal that differences in C_{max} predictions (and corresponding predicted:observed ratios) occur most frequently as a result of differences in calculation methods for the partition coefficients (Fig. 3A), with the method of Rodgers and Rowland performing best. The median C_{max} of 19 compounds was predicted within 2-fold of the observed C_{max} values with the Rodgers and Rowland method. With the methods of Berezhkovskiy and Schmitt, 12 and 13 compounds were predicted within 2-fold of the observed C_{max}, respectively. Particularly for acidic compounds (pKa < 6), like bosentan, diclofenac, fluvastatin, ibuprofen, ochratoxin A, naproxen, S-warfarin, and tolbutamide, the method of Berezhkovskiy resulted in relatively high partition coefficients and low C_{max} predictions compared with the other input approaches (Fig. 3A). The method of Schmitt resulted in overpredictions of the C_{max} of particularly lipophilic compounds, like bisphenol A, bosentan, clozapine, and midazolam.

In case of the parameterization of the intrinsic hepatic clearance, the *in silico* calculated clearance values were found to only provide rough estimates of the intrinsic clearance values as no direct correlation was observed between the *in vitro* (hepatocyte) measured clearance data and *in silico* (ADMET microsomal) predicted ones ($R^2 = 0.06$, Fig. S3A[5]). Yet, substantial differences (> 3-fold) in median C_{max} predictions between the *in silico* and *in vitro* input values for intrinsic clearance only occurred for chlorpromazine, coumarin, curcumin, and naloxone (Fig. 3B). For those compounds, the *in silico* calculated clearance values led to (higher) overestimations of the observed C_{max} values compared with the *in vitro* measured clearance values. For the compounds for which S9 data were available, the clearance values strongly correlated with the hepatocyte clearance data ($R^2 = 0.75$, Fig. 3C). Significant differences (> 3-fold) in C_{max} predictions between the latter two approaches were only observed for naloxone (Fig. 3B). A relatively lower number of compounds could be predicted within 2-fold when parameterized based on S9 clearance data. This was, however, expected as the S9 clearance data are not equally distributed over the dataset and contain a relatively high number of chemicals that are > 5-fold overpredicted (irrespective of the input approach).

Figure 3C reveals that the highest number of compounds was predicted within 2-fold when the *in silico* predicted fu_p values based on the method of Lobell and Sivarajah (2003) or the *in vitro* measured fu_p values were used. Although these two approaches appeared to perform equally well, the use of *in vitro* measured unbound fractions particularly led to overestimations of the C_{max} of buspirone, carvedilol, coumarin, prazosin, and resveratrol. The C_{max} predictions based on the ADMET predicted fu_p frequently led to underpredictions. Regarding the sources for the physicochemical characteristics (ADMET versus ChemAxon) and intestinal uptake data (*in vitro* Caco-2 data versus *in silico* predictions), no substantial (> 3-fold) differences in C_{max} predictions occurred between the different input approaches.

Figure 4 depicts the results of the dataset in which the most significant outliers described above have been removed. This includes removal of the simulations based on 1) the methods of Berezhkovskiy and Schmitt for the partition coefficients, 2) the *in silico* intrinsic hepatic clearance data, and 3) the ADMET predicted and *in vitro* measured fu_p values. The results of the reduced dataset show a significant reduction in the variation in C_{max} predictions and the related predicted:observed C_{max} ratios. Within the reduced dataset, the median C_{max} values of 34 out of the 44 compounds are predicted within 5-fold, and the C_{max} values of 19 compounds are predicted within 2-fold. Ten out of the 12 initially overestimated reference compounds remain overestimated by more than 5-fold. The median C_{max} predictions for chlorpromazine and coumarin now fall within 5-fold of the observed C_{max}. No underestimations of more than 5-fold occur.

3.5 Characteristics of the compounds for which the C_{max} is more than 5-fold overpredicted

Irrespective of the input method applied, the majority of the C_{max} predictions for bisphenol A, buspirone, curcumin, desipramine, dextromethorphan, fluvastatin, genistein, naloxone, resveratrol, and rosuvastatin were more than 5-fold overpredicted. Underpredictions of more than 5-fold did not occur. To obtain insight into the common causes for the overpredictions, a literature search was performed to explore available chemical-specific PBK models for all model compounds. The overpredictions either indicate that the bioavailability of the compounds is predicted to be too high or that the volume of distribution is predicted to be too low.

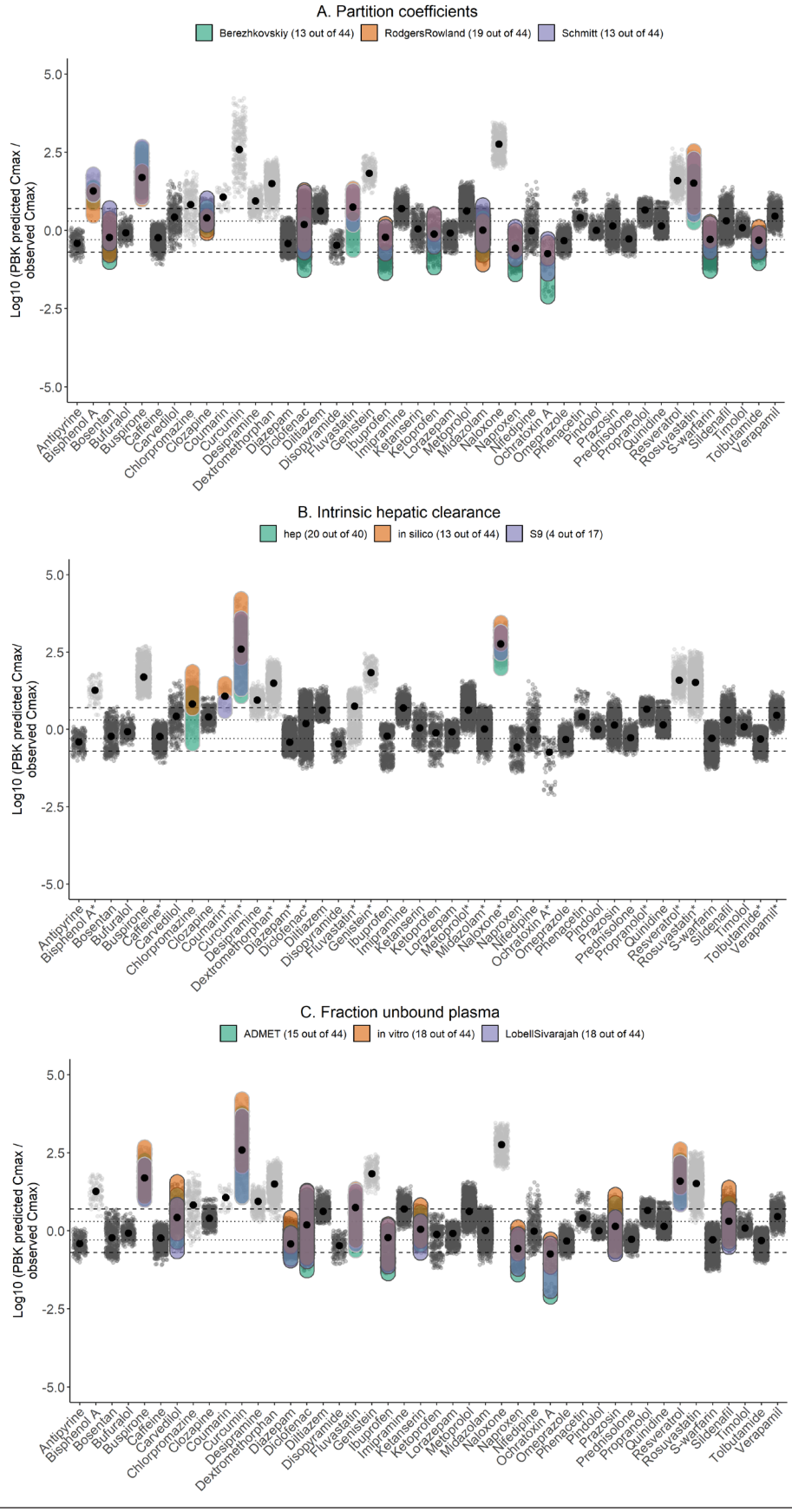

Fig. 3: Differences in predicted:observed C_{max} ratios related to the applied input approaches
Results for which a more than 3-fold difference in median C_{max} predictions occurs between the applied input approaches are highlighted. (B) S9-derived intrinsic clearance data are included in the comparison of input approaches for compounds marked with an asterisk. For each approach, the number of compounds that was predicted within 2-fold of the observed C_{max} is provided between brackets.

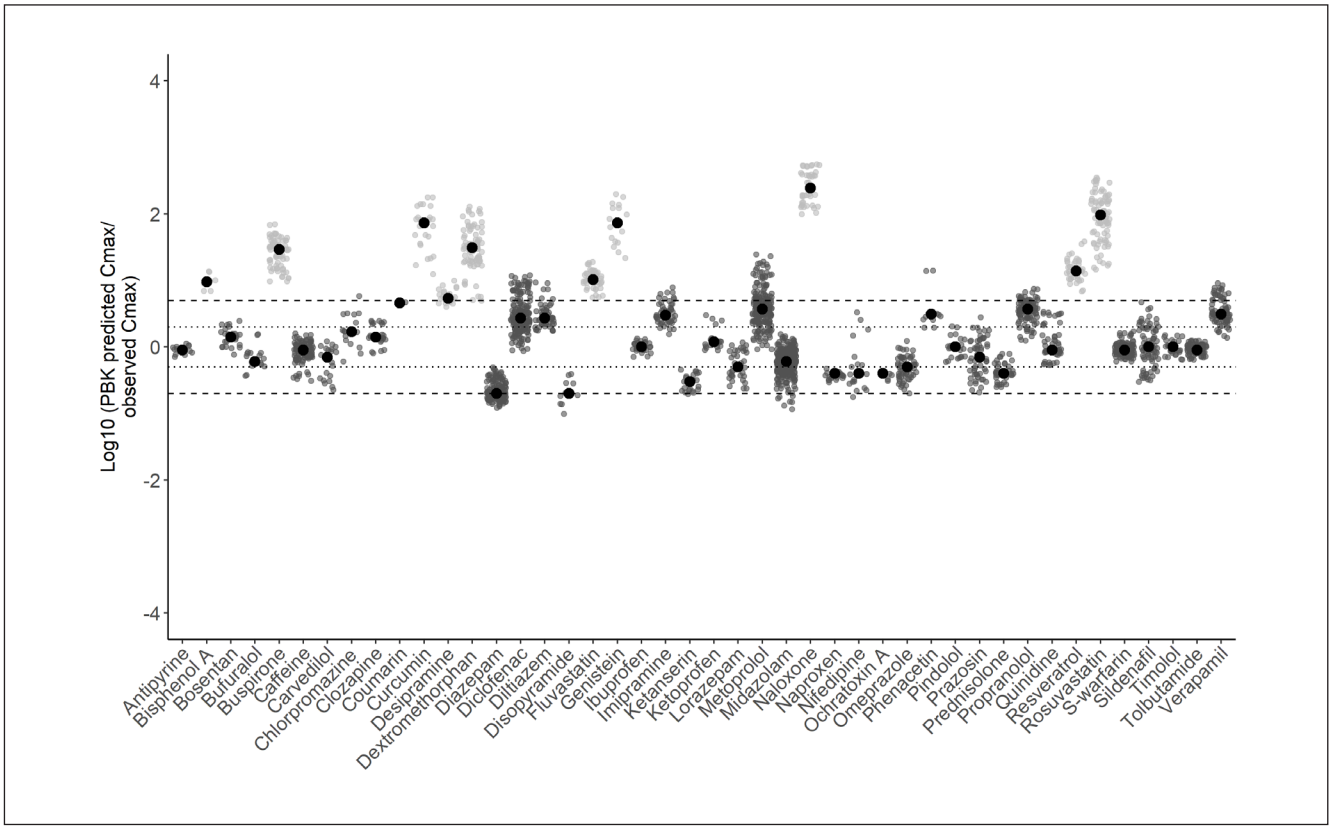

Fig. 4: Ratios between PBK model-predicted C_{max} values and *in vivo* C_{max} values observed for 44 reference compounds in humans as obtained after removal of the simulations based on input methods that led to significant differences between predicted and observed ratios as described in the main text

This means that additional kinetic processes need to be considered that are related to either a reduced bioavailability or an increased volume of distribution. The PBK model predictions of the present study were performed based on a minimal PBK model that includes the intrinsic liver clearance, partition coefficients, and the fraction unbound in plasma as main input parameters, lacking description of kinetic processes that could reduce the bioavailability like, for example, extrahepatic metabolism or active efflux transport. In addition, parameters that affect the volume of distribution, like the fu_p, partition coefficients, or tissue uptake transport, might be different in the current model compared with the chemical-specific PBK models. The key differences between the chemical-specific PBK models for the different compounds available in the literature and the current generic model were explored. Table 2 reveals the results of this comparison and shows that phase I metabolism or glucuronidation by intestinal epithelial cells is most frequently considered in the chemical-specific PBK models. This is particularly true for the compounds that were substantially overpredicted in the present study, but also for some of the compounds that were predicted within 5-fold (i.e., lorazepam, midazolam, sildenafil, and verapamil). Apart from intestinal metabolism, hepatic uptake and biliary excretion by active transport have been considered for the kinetic models of bosentan, bisphenol A, fluvastatin, rosuvastatin, and S-warfarin. In this context, the inclusion of active hepatic uptake in the PBK model may increase the liver concentration, resulting in enhanced metabolism and/or biliary excretion and reduced (initial) peak plasma concentrations. Biliary excretion might increase plasma concentrations again at later time points due to enterohepatic circulation. Intestinal efflux transport and renal active reabsorption and excretion have only been considered for rosuvastatin in the identified PBK models.

4 Discussion

Adequate predictions of internal dosimetry, such as C_{max}, are crucial in the transition towards next generation (animal-free) testing strategies for chemical safety evaluations to convert *in vitro* toxicity data into *in vivo* dose-response or at least potency information (e.g., Fabian et al., 2019; Louisse et al., 2017; Punt et al., 2019; Wetmore et al., 2015). To facilitate the application of PBK models in next generation risk assessment, it is important to gain confidence in the predictive performance of these models without case-by-case validation against *in vivo* data (e.g., plasma concentrations). The goal of the present study was to assess the predictive performance of a minimal generic human PBK model based on *in vitro* and *in silico* input data to predict the C_{max}.

Tab. 2: Overview of identified kinetic processes included in chemical-specific PBK models that were not included in the generic PBK model of the present study

Predicted within 5-fold[a]			More than 5-fold overpredicted		
Compound	**Kinetic process(es) included in the chemical-specific PBK model**	**References**	**Compound**	**Kinetic process(es) included in the chemical-specific PBK model**	**References**
Bosentan	Hepatic uptake transport and biliary efflux	Li et al., 2015; Yang et al., 2020; Jones et al., 2012; Posada et al., 2020	Bisphenol A	– Intestinal glucuronidation – Hepatic uptake transport	Kawamoto et al., 2007; Teeguarden et al., 2005
Lorazepam	Intestinal glucuronidation	Docci et al., 2020	Buspirone	Intestinal phase I metabolism (CYP3A4)	Heikkinen et al., 2012; Gertz et al., 2011; Karlsson et al., 2013
Midazolam	Intestinal phase I metabolism (CYP3A4)	Karlsson et al., 2013; Heikkinen et al., 2012; Cao and Jusko, 2012; Gertz et al., 2011; Nguyen et al., 2016; Jamei et al., 2009	Curcumin	Intestinal glucuronidation	Adiwidjaja et al., 2020
Metoprolol	Intestinal phase I metabolism (CYP2D6)	Chow et al., 2016	Desipramine	Intestinal phase I metabolism (CYP2D6)	Barter et al., 2013
Quinidine	Intestinal efflux	Harwood et al., 2013	Dextromethorphan	Intestinal phase I metabolism (CYP2D6)	Barter et al., 2013
Sildenafil	Intestinal phase I metabolism (CYP3A4, CYP2C9)	Ghoneim and Mansour, 2020; Karlsson et al., 2013	Diltiazem	Intestinal phase I metabolism (CYP3A4)	Zhang et al., 2009
S-warfarin	Hepatic uptake transport	Bi et al., 2018	Fluvastatin	Hepatic uptake transport and biliary efflux	Chan, 2019; Jones et al., 2012
Verapamil	Intestinal phase I metabolism (CYP3A4)	Karlsson et al., 2013; Heikkinen et al., 2012; Cao and Jusko, 2012	Genistein	Intestinal glucuronidation	Boonpawa et al., 2017
			Naloxone	Intestinal glucuronidation	Docci et al., 2020; German et al., 2019
			Resveratrol	ND	
			Rosuvastatin	– Intestinal efflux – Hepatic uptake and biliary efflux – Active renal excretion	Bowman et al., 2021; Emami Riedmaier et al., 2016; Chan, 2019

[a] For the compounds antipyrine, caffeine, carvedilol, clozapine, diazepam, diclofenac, ibuprofen, imipramine, ketanserin, naproxen, omeprazole, phenacetin, propranolol, and tolbutamide no additional kinetic processes like extrahepatic metabolism or transporter activity were identified in the explored chemical-specific PBK models. For the compounds bufuralol, chlorpromazine, disopyramide, ketoprofen, ochratoxin A, pindolol, prazosin, prednisolone, and timolol no chemical-specific PBK models were found. NA, not applicable (no specific additional kinetic processes (like extrahepatic metabolism or transporter activity) included in the PBK model; ND, not determined (no PBK models available for the compound)

Two cut-off values (2-fold and 5-fold) were used as performance indicators in the current study. Discussions on what level of deviation between predicted and observed kinetics is acceptable within a regulatory context are presently still ongoing (Shebly et al., 2018). The 2-fold cut-off value is frequently requested within a regulatory context to demonstrate that the proposed model is fit for purpose (Peters and Dolgos, 2019; Sager et al., 2015). A key challenge with this 2-fold value is that the differences between *in vivo* studies to which the model predictions are compared tend to be higher than 2-fold themselves, possibly related to inter-individual differences in biology or technical aspects (Shebly et al., 2018). This was also observed in the present study, revealing that the variation in normalized *in vivo* C_{max} values generally ranges between 1.3 to 5-fold, but can also be as high as 16-fold. Based on these results, a 5-fold cut-off value was selected to determine whether a compound fits the applica-

bility domain of the applied minimal PBK model. Overall, the medians of the predicted C_{max} values for 34 out of the 44 compounds, corresponding to 77%, were within 5-fold of the observed C_{max} values, whereas 19 compounds could be predicted within 2-fold, corresponding to 43%. The medians of the predicted C_{max} values of 10 out of 44 compounds (23%) were more than 5-fold overestimated. Underestimations of the median C_{max} (higher than 5-fold) did not occur. A bias towards overestimations has been reported before for PBK model predictions based on minimal input (i.e., liver clearance, partition coefficients, and passive intestinal uptake) and might indicate that such minimal PBK model predictions represent a worst case approach for predicting C_{max} values (Wambaugh et al., 2018). The choice of the calculation method for tissue:plasma partition coefficients had a high impact on the C_{max} predictions. In addition, occasional differences in C_{max} predictions were observed as a result of differences between input approaches (*in silico*, *in vitro*) for the fraction unbound in plasma and the different input approaches (*in silico*, primary hepatocytes, or S9) for intrinsic hepatic clearance. In case of the calculation methods for the partition coefficients, the calculation method of Berezhkovskiy particularly led to underpredictions of the C_{max} of acidic compounds (pKa < 6). For these compounds, the predicted partition coefficients for the different organs (except for the adipose tissue) were higher than with the method of Rodgers and Rowland or the method of Schmitt, resulting in lower plasma concentrations. The method of Berezhkovskiy takes the charge of the molecules into account for the calculation of the partition coefficient of the adipose tissues, but not for the other organs. This might explain the differences between the method of Berezhkovskiy and the calculation methods of Rodgers and Rowland and Schmitt, with the latter specifically focusing on the impact of drug ionization on partitioning (Utsey et al., 2020). The calculation method of Schmitt appeared to work less well for lipophilic compounds. This was previously also observed by Punt et al. (2022) and might be due to the fact that the method of Schmitt largely depends on the membrane affinity as input, which is difficult to obtain for different chemicals. The applied QSAR from Yun and Edginton (2013) to calculate the membrane affinity might not be applicable to lipophilic compounds. Overall, the calculation method of Rodgers and Rowland performed best in the current study, resulting in the highest number of compounds for which the C_{max} predictions were within 2-fold (and 5-fold) of the observed C_{max}.

The *in silico* input approaches that were used in the present study for the calculation of the apparent Caco-2 permeability, fraction unbound in plasma (particularly the method of Lobell and Sivarajah), and intrinsic hepatic clearance provided relevant C_{max} predictions for the majority of the compounds. However, particularly in case of the *in silico* calculated intrinsic clearance values and the *in silico* calculated Caco-2 permeability, care should still be taken with using these *in silico* approaches for the parameterization of PBK models as direct comparisons of the *in silico* and *in vitro* measured values revealed poor correlations between the *in vitro* and *in silico* data (Fig. S3[5]). It should also be noted that the *in silico* intrinsic clearance results[5] that were obtained with the ADMET Predictor only cover the cytochrome P450-dependent human hepatic clearance, and not sulfation or glucuronidation reactions. In general, *in vitro* input parameters therefore remain the preferred input approach for these input parameters. In contrast, the values of the *in silico* calculated fractions unbound in plasma were found to correlate better with the *in vitro* measured ones ($R^2 = 0.6$) and even provided better C_{max} predictions in the present study than the *in vitro* measured values. This might be due to challenges with measuring this parameter *in vitro*, particularly for highly lipophilic or chemically unstable compounds (Bowman et al., 2021; Emami Riedmaier et al., 2016; Wambaugh et al., 2019).

Given the importance of the *in vitro* measured input data for PBK model development, the quality of these measurements is crucial. This is true for the measurements of the fraction unbound as described above as well as for *in vitro* intrinsic hepatic clearance measurements and Caco-2 permeability studies. Recently, Louisse et al. (2020) evaluated the influence of experimental conditions of clearance studies on intrinsic clearance (CLint) values obtained from literature data. The CLint values for the majority of compounds differed by more than one order of magnitude, which is expected to partly depend on the *in vitro* protocol that was used. Such variation can have a large impact on the C_{max} predictions. These results highlight the importance of obtaining harmonized *in vitro* approaches (Gouliarmou et al., 2018; Louisse et al., 2020; Paini et al., 2019) to parameterize PBK models.

The frequently observed overpredictions indicate that particularly kinetic processes that are related to reduced predicted bioavailability or increased predicted volume of distribution are missing in the minimal PBK model. Comparison of the current model structure with chemical-specific PBK models reported in literature revealed that particularly the intestinal first pass metabolism (CYP3A4-mediated oxidation or glucuronidation), which was not included in the current generic PBK model structure, is frequently considered as an additional kinetic process. In addition, active hepatic uptake and biliary excretion have been included in the literature on chemical-specific PBK models of some of the compounds of the present study (Emami Riedmaier et al., 2016). Intestinal efflux and active renal excretion and/or reabsorption are less frequently considered, yet might also be important. Solubility and dissolution issues might also be relevant, resulting in a poorer intestinal fraction absorbed. New approaches to describe passive renal excretion based on Caco-2 permeability data might also help in obtaining better estimates of plasma concentrations (Scotcher et al., 2016). Further work will be needed to determine whether the C_{max} predictions can be improved by inclusion of some of these additional kinetic processes.

The current study focused on C_{max} predictions with the minimal PBK model. This is a critical kinetic parameter within PBK modelling-based reverse dosimetry approaches to predict *in vivo* toxicity from *in vitro* data (Louisse et al., 2017). Further work will, however, be needed to also evaluate the predictive performance with respect to other relevant kinetic descriptors like the area under the plasma concentration-time curve (AUC) and steady state plasma concentrations (Css). Together these different parameters describe the full kinetic profile of a chemical, and it remains to be elucidated to what extent the optimized se-

lection of input parameters, as obtained in the present study for C_{max}, will also work for other biokinetic parameters. In addition, it should be noted that the 44 reference compounds only represent a limited chemical space. Even though the chemicals within the dataset are diverse (e.g., log P between -0.55 and 5.3, MW between 179 and 552, and different ionization characteristics), the observations made and conclusions drawn within the present study cannot be directly extrapolated to a larger dataset.

Overall, the results of the current study provide relevant insights into the predictive performance of a minimal PBK model and the influence of different input approaches on the model predictions. Further work will be needed, in particular to find ways to determine when and which additional kinetic processes (like liver metabolism or transporter-mediated processes) need to be added in the absence of prior knowledge on the chemical's *in vivo* toxicokinetics.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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