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Tutorial on physiologically based kinetic modeling in molecular nutrition and food research

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Studies in the field of molecular nutrition and food research often aim at identifying effects of bioactive ingredients on living organisms. When data from human studies are difficult to obtain, effects are often studied in relevant animal or cellular in vitro models. This poses the need for adequate extrapolation from the in vitro to the in vivo situation, from high-dose levels to realistic low-dose levels and from experimental animals to humans. Furthermore, effects of genetic polymorphisms or lifestyle factors may have to be taken into account. Physiologically based kinetic (PBK) modeling provides a means to support these kinds of extrapolations. The present paper illustrates the basic concepts of PBK modeling. PBK modeling includes six steps: (i) definition of the conceptual model, (ii) translation into a mathematical model, (iii) defining parameter values, (iv) solving the equations, (v) evaluation of model performance and (vi) making predictions. The paper provides an overview of these basic steps and presents examples to illustrate how PBK modeling can be applied. This reveals that PBK modeling provides an important tool in the field of the 3Rs aiming at Replacement, Reduction and Refinement of animal studies and may also be a useful tool for risk assessment.

Keywords:
Basic steps / Educational / Examples / Physiologically based kinetic modeling / Tutorial

1 Introduction: what is a physiologically based kinetic model?

Studies in the field of molecular nutrition and food research often aim at identifying effects of bioactive ingredients on living organisms. This can relate to both beneficial as well as adverse health effects of functional food ingredients, food additives, environmental contaminants and/or natural toxins present in food. When data from human studies are difficult or even impossible to obtain, such as in cases of safety evaluation of toxic or newly developed compounds, effects are often studied in relevant animal or cellular in vitro models. This poses the need for methods for adequate extrapolation from the in vitro to the in vivo situation, from high-dose levels to realistic low-dose levels and from experimental animals to humans. Uncertainties about the shape of the dose–response curve at dose levels relevant for dietary human intake, about species differences in metabolic activation and detoxification, and about effects of genetic polymorphisms and lifestyle factors make it difficult to perform such extrapolations. Physiologically based kinetic (PBK) modeling can provide a means to obtain a mechanistic basis for extrapolations of data obtained in experimental animal studies to the human situation [1–3]. In the present tutorial we use the term PBK modeling to summarize a range of terms used in physiologically based modeling studies including physiologically based pharmacokinetic, physiologically based toxicokinetic or physiologically based biokinetic modeling.

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Abbreviations: ADME, absorption, distribution, metabolism and excretion; **AP,** 4-allylphenol; **BMD,** benchmark dose; **BMDL,** lower confidence limit of the benchmark dose; **bw,** body weight; **EGME,** ethylene glycol monomethyl ether; **EC $_{10, 20, 30, etc}$ values,** effective concentrations causing 10, 20, 30, etc percent response; **ED $_{10, 20, 30, etc}$ values,** effective doses causing 10, 20, 30, etc percent response; **EST,** embryonic stem cell test; **HE,** 1′-hydroxyestragole; **HEG,** 1′-hydroxyestragole glucuronide; **HES,** 1′-sulfooxyestragole; **OE,** 1′-oxoestragole; **oHPA,** *o*-hydroxyphenylacetaldehyde; **PBD,** physiologically based dynamic; **PBK,** physiologically based kinetic

A PBK model is a set of mathematical equations that together describe the absorption, distribution, metabolism and excretion (ADME) characteristics of a compound within an organism on the basis of three types of parameters [3–6]. These parameters include (i) physiological and anatomical parameters (e.g. cardiac output, tissue volumes and tissue blood flows), (ii) physico-chemical parameters (e.g. blood/tissue partition coefficients) and (iii) kinetic parameters (e.g. kinetic constants for metabolic reactions) [3–6]. Simultaneous solution of the PBK equations produces outcomes that are an indication of, for example, the tissue concentration of a compound or its metabolite(s) in any tissue over time at any dose, allowing analysis of effects at both high-, but also more realistic low-dose levels. Furthermore, such PBK models can be developed for different species, which can facilitate interspecies extrapolation. In addition, by incorporating equations and kinetic constants for metabolic conversions by individual human samples and/or specific isoenzymes, modeling of interindividual variations and genetic polymorphisms becomes feasible [7–9].

The PBK modeling approach includes six steps, namely, (i) definition of the conceptual model, (ii) translation into a mathematical model, (iii) defining parameter values, (iv) solving equations, (v) evaluation of the model performance and (vi) making predictions. The present tutorial describes these basic steps and provides examples illustrating the concepts.

2 Step 1: Defining a conceptual model: the basic structure of a PBK model

The basic structure of a PBK model assumes that the whole body can be described as a set of basic compartments. These basic compartments of a PBK model can be depicted each as a single box. In the PBK modeling approach it is assumed, as a generally accepted simplification, that a compartment is a single region of the body with a uniform xenobiotic concentration. It may be a particular functional or anatomical portion of an organ, a single blood vessel with surrounding tissue, an entire discrete organ such as the liver or kidney, or a widely distributed tissue type such as fat or skin. As a first step in developing a PBK model for a specific compound one has to decide what organs are relevant for the ADME characteristics of that compound and its biological effects. These compartments need to be specifically described, whereas all other organs can be lumped together in either a slowly or richly perfused tissue compartment. A reason to include a specific organ can be that this organ represents the site of uptake or elimination, a major site of metabolism or a target organ for the beneficial or adverse effect of the compound of interest. Figure 1 shows an example of a basic PBK model for a compound that is absorbed upon oral intake. This model includes separate compartments for liver, lung and kidney, since these organs are involved in metabolism and excretion of the compound.

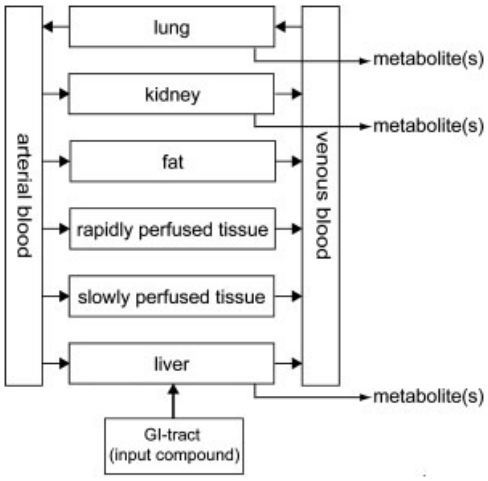

Figure 1. Schematic diagram of a PBK model for a compound that is absorbed upon oral intake and metabolized in liver, kidney and lung.

The uptake from the gastrointestinal tract is assumed to result from direct entry from the intestine to the liver compartment. Furthermore, a separate compartment for fat tissue can be included if a compound has a relatively higher partition in fat tissue. All other tissues can be lumped into either a rapidly perfused tissue group, comprising tissues such as adrenals, brain and heart or a slowly perfused tissue group, comprising tissues such as bone, muscle and skin [10].

3 Step 2: Translation into a mathematical model: example of a mathematical PBK equation

The second step in PBK modeling is the translation of the conceptual model into a mathematical model. As an example, Fig. 2 shows a schematic overview of the mathematical equation used to describe the liver compartment in a PBK model we developed for estragole [11, 12]. Estragole is an alkenylbenzene that occurs in different herbs such as tarragon, basil and fennel and is present in products derived from these herbs such as pesto and essential oils [13]. There is interest in the safety assessment of estragole as a food constituent, since estragole has been identified to be genotoxic in vitro and carcinogenic in rodent studies performed at high dose levels; http://ec.europa.eu/food/fs/sc/scf/out104_en.pdf [14–16]. Figure 3 shows an overview of estragole metabolism including pathways for bioactivation to the proximate and ultimate carcinogenic metabolite and pathways for detoxification [17, 18]. The main phase I metabolic pathways include 1′-hydroxylation, *O*-demethylation, epoxidation and 3′-hydroxylation of estragole. The main metabolic pathways of the proximate carcinogen 1′-hydroxyestragole (HE) are sulfonation to the ultimate carcinogen 1′-sulfooxyestragole (HES), and detoxification

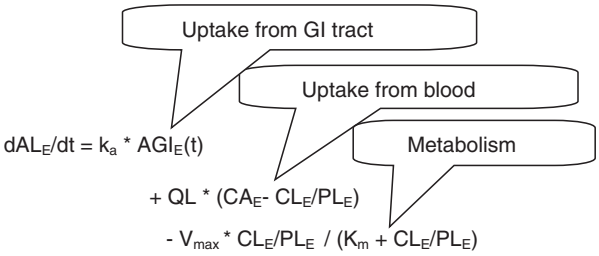

Figure 2. Schematic presentation of the mathematical equation for the liver compartment in a PBK model for estragole [11, 12]. The figure schematically presents that for defining a differential equation different processes have to be described mathematically. The uptake of estragole from the gastrointestinal tract can be described by a first-order process, with k_a (in h^{-1}) representing the first-order absorption rate constant and $AGI_E(t)$ (in μmol) being the amount of estragole remaining at a certain timepoint in the gastrointestinal tract, with AGI_E at time = 0 being equal to the oral dose. Assuming a perfusion-limited process the uptake of estragole in the liver from the blood equals the blood flow rate to the liver (QL in L/h) times the difference in estragole concentration between the arterial blood (CA_E) and the effluent venous blood of the liver (CL_E/PL_E). The metabolic conversion of estragole in the liver can be modeled by a Michaelis–Menten equation with the negative sign reflecting that this contribution results in a decrease in the amount of estragole in the liver. Since estragole can be converted to a variety of metabolites (Fig. 3), one needs to add a separate Michaelis–Menten equation for each metabolic conversion, one for each important metabolic route. For further details see text.

through glucuronidation to HE glucuronide or oxidation to 1′-oxoestragole (OE) (Fig. 3). The ultimate carcinogenic metabolite HES is unstable and degrades in aqueous environment to a reactive carbocation that is capable of forming DNA adducts [19–22].

The schematic overview of the equation shown in Fig. 2 indicates that the change in the amount of estragole (A_E) in the liver (L) in time (*t*), represented as dAL_E/dt, is a function of (i) the uptake of estragole from the gastrointestinal tract directly into the liver and the uptake of estragole from the blood, both resulting in an increase in the amount and (ii) the conversion of estragole into its different metabolites due to metabolism resulting in a net decrease in the amount of estragole in the liver.

The uptake of estragole from the gastrointestinal tract can be described by a first-order process, assuming direct entry from the intestine to the liver compartment. This implies that uptake can be described by $k_a * AGI_E(t)$, with k_a (in h^{-1}) representing the first-order absorption rate constant and $AGI_E(t)$ (in μmol) being the amount of estragole remaining at a certain timepoint in the gastrointestinal tract, with AGI_E at time = 0 being equal to the oral dose.

The uptake of estragole in the liver from the blood equals the blood flow rate to the liver (QL in L/h) times the difference in estragole concentration between the arterial blood (CA_E) and the effluent venous blood of the liver (CVL_E); thus $QL(CA_E - CVL_E)$, assuming a perfusion-limited and not a diffusion-limited process [5]. This implies that the

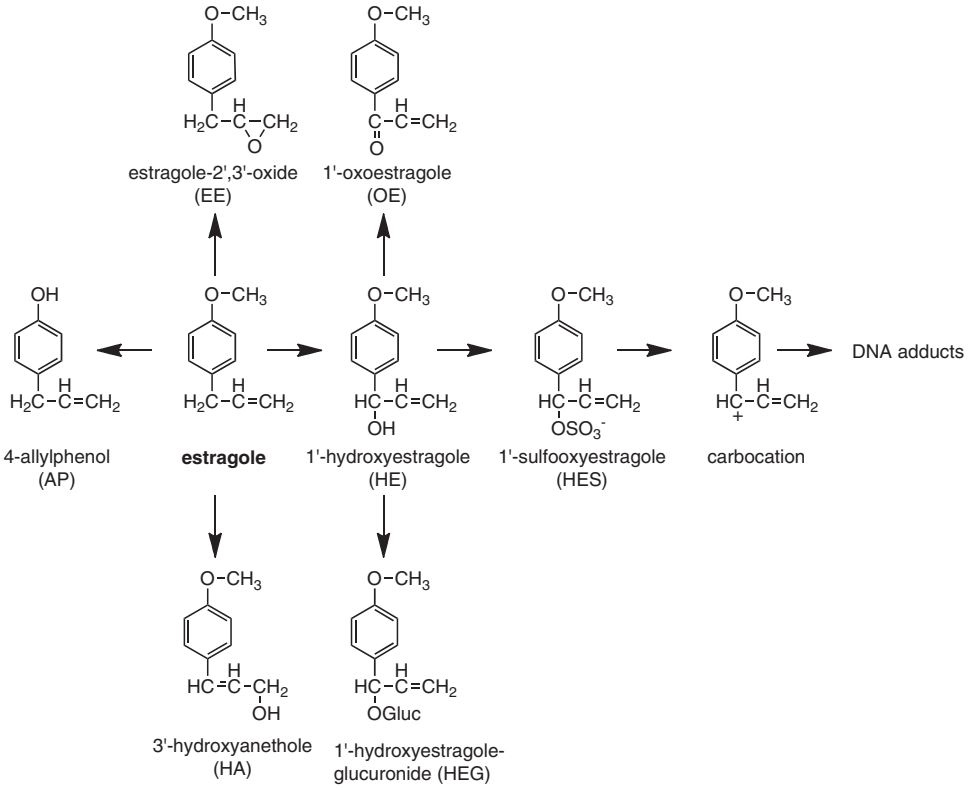

Figure 3. Metabolism of estragole with the bioactivation pathway proceeding by formation of the proximate carcinogen HE and the ultimate carcinogen HES. Formation of the other metabolites leads to detoxification and excretion.

uptake of estragole in the liver from the blood is dominated by the arterial and venous blood flow (perfusion) of the liver and not by diffusion of estragole itself.

This equation is based on Fick's law of simple diffusion ($dCt/dt = k^*\Delta C$), which states that the flux of a compound is proportional to its concentration gradient [5]. Assuming the equilibrium between the estragole concentration in venous blood leaving the liver (CVL_E) and the blood concentration of estragole in the liver (not bound to liver tissue), CVL_E in the equation equals CL_E/PL_E, with CL_E representing the total estragole concentration in the liver (bound and not bound to liver tissue; in μmol/L), and PL_E representing the liver/blood partition coefficient of estragole [4–6].

The metabolic conversion of estragole in the liver can be modeled by a Michaelis–Menten equation leading to a contribution equal to $-V_{max,L}{}^*CL_E/PL_E/(K_{m,L}+CL_E/PL_E)$ with the negative sign reflecting that this contribution results in a decrease in the amount of estragole in the liver and CL_E again representing the estragole concentration in the liver (in μmol/L), PL_E representing the liver/blood partition coefficient of estragole, and $V_{max,L}$ and $K_{m,L}$ representing the maximum rate and Michaelis–Menten constant for a specific conversion as determined using relevant liver samples. The Michaelis–Menten equation expresses the concentration of estragole in the liver as CL_E/PL_E, corresponding to the concentration of estragole in the liver that is not bound to liver tissue, being available for metabolism. Since estragole can be converted to a variety of metabolites (Fig. 3), one needs to add a separate Michaelis–Menten equation for each metabolic conversion, one for each important metabolic route. For metabolism of estragole in the rat liver this implies conversion to four major metabolites including 4-allylphenol (AP), HE, estragole-2′,3′-oxide, (EE) and 3′-hydroxyanethole (HA). All together this results in the following detailed description of the change in the amount of estragole in the live in time:

$$\begin{aligned} dAL_E/dt = {} & K_a * AGI_E(t) \\ & + QL^*(CA_E - CL_E/PL_E) \\ & - V_{max,L\_AP}{}^*CL_E/PL_E/(K_{m,L\_AP}+CL_E/PL_E) \\ & - V_{max,L\_HE}{}^*CL_E/PL_E/(K_{m,L\_HE}+CL_E/PL_E) \\ & - V_{max,L\_EE}{}^*CL_E/PL_E/(K_{m,L\_EE}+CL_E/PL_E) \\ & - V_{max,L\_HA}{}^*CL_E/PL_E/(K_{m,L\_HA}+CL_E/PL_E) \end{aligned}$$

With

$dUptake_E/dt = -dAGI_E/dt = K_aAGI_E(t)$,

$AGI_E(0) =$ oral dose

$CL_E = AL_E/VL$, with VL being the volume of the liver.

In a similar way equations for all other compartments in the model can be defined [11, 12].

Furthermore, in the same way equations for formation of relevant metabolites can be defined. As an example the formation of the proximate carcinogenic metabolite of estragole, HE, can be modeled by describing its formation from estragole and its decrease due to its conversion to phase II metabolites: HEG, HES, and OE leading to:

$$\begin{aligned} \text{Liver}: dAL_{HE}/dt = {} & V_{max,L\_HE} * CL_E/PL_E/ \\ & (K_{m,L\_HE}+CL_E/PL_E) \\ & - V_{max,L\_HEG}{}^*CL_{HE}/PL_{HE}/(K_{m,L\_HEG}+CL_{HE}/PL_{HE}) \\ & - V_{max,L\_HES}{}^*CL_{HE}/PL_{HE}/(K_{m,L\_HES}+CL_{HE}/PL_{HE}) \\ & - V_{max,L\_OE}{}^*CL_{HE}/PL_{HE}/(K_{m,L\_OE}+CL_{HE}/PL_{HE}) \end{aligned}$$

where AL_{HE} is the amount of HE in the liver (μmol), CL_{HE} is the HE concentration in the liver (μmol/L), PL_{HE} is the liver/blood partition coefficient of 1′-hydroxestragole, and V_{max,L_M} and K_{m,L_M} are the maximum rate of formation and the Michaelis–Menten constant for the formation of the different phase II metabolites: HEG, HES and OE in the liver. A full quantitative intrahepatic conversion of HE by phase II enzymes was assumed; therefore only intraorgan distribution of HE was taken into account and no distribution of this metabolite over the body was modeled [11, 12].

4 Step 3: Defining parameter values: nature, sources and scaling of parameters

In step 2, based on the basic structure of the PBK model a set of mathematical equations is defined, one set for each compartment. For the further development of a PBK model for a specific compound, in a next step model parameters need to be obtained. Model parameters required include (i) physiological and anatomical parameters (e.g. cardiac output, tissue volumes and tissue blood flows), (ii) physicochemical parameters (e.g. tissue/blood partition coefficients) and (iii) kinetic parameters (e.g. kinetic constants for transport as well as for biotransformation reactions of the compound and/or its metabolites) [4–6].

4.1 Physiological and anatomical parameters

The size of each of the compartments in the physiological model must be known. The size is generally specified as a volume (milliliters or liters), even though weights are most frequently obtained experimentally, but then a unit density is assumed. If a compartment contains sub-compartments, those volumes must also be known. Furthermore, the blood flow (Q in volume per unit time) to individual compartments and information on the total blood flow rate or cardiac output (Q_c) are needed. If inhalation is the route of exposure to the compound or is a route of elimination, the alveolar ventilation rate must also be known. The values for physiological and anatomical parameters of a species (e.g. blood flow rates and tissue volumes) can usually be obtained from the literature. Representative reference

physiological parameters for different laboratory animals as well as humans can, for instance, be obtained from Brown et al. [23]. Recently, Schroeder et al. [24] encouraged the use of publicly available tools such as the Model Equation Generator (MEGen, http://xnet.hsl.gov.uk/megen), a free web-based PBK model equation generator and parameter database developed at the UK Health and Safety Laboratory as part of a joint industry project promoting the rapid generation of PBK models.

4.2 Physico-chemical parameters

Physico-chemical parameters mainly refer to the tissue/blood partition coefficients, which represent the ratio of the concentration of a compound in the tissue relative to that in the blood under steady-state conditions [4, 5]. The extent to which a compound partitions into a tissue is directly dependent on the composition of the tissue and is independent of the concentration of the compound. When tissues are regarded as perfusion-limited, the relation between the tissue blood concentration and total tissue concentration becomes one of proportionality in which the two are linked by the tissue/blood partition coefficient. An example was already given above where the liver blood concentration of estragole was described as CL_E/PL_E. Assuming perfusion-limitation of tissues, the concentration of a compound in the tissue available for, for instance, metabolism is equal to the tissue blood concentration of a compound and can therefore also be depicted as CL/PL [4, 5].

Tissue/blood partition coefficients can be obtained from in vivo studies by comparing tissue/blood levels at steady state [6]. However, several in vitro and in silico methods are also available based on which the tissue/blood partition coefficients can be estimated. In vitro methods include vial equilibrium, equilibrium dialysis and ultrafiltration techniques [25, 26]. In silico methods include quantitative structure activity relationship/quantitative structure property relationship approaches with which tissue/blood partition coefficients can be estimated based on octanol/water partition coefficients [27–29]. One should be aware that the use of these in vitro and in silico methods needs validation or may otherwise increase uncertainties in the PBK model output.

4.3 Kinetic parameters

Kinetic parameters for PBK models relate to the rates of absorption, biotransformation, macromolecular binding and excretion of compounds. Kinetic parameters for PBK models, including metabolic parameters, are, at present, often obtained by optimizing the fit of the model to an informative data set [4–6]. Alternatively, kinetic parameters might also be derived from in vitro experiments. Parameters for oral absorption in kinetic models can, for instance, be determined in a first approximation using in vitro oral absorption studies utilizing cultured CaCo-2 cell monolayers in Transwell plates. This approach has been applied to predict serum folate concentrations after intake of fortified milk products [30]. To characterize biotransformation, several in vitro methods are available, including incubations with over-expressed or purified enzymes (e.g. supersomes or isolated enzymes), subcellular fractions (e.g. microsomes, S9 or cytosol), isolated cells (e.g. primary hepatocytes or cyropreserved hepatocytes) or tissue slices. The advantages and disadvantages of each of these systems have been reviewed [31–33]. In vitro methods can be performed with preparations derived from test animal species but also allow the use of human material that enables to make model predictions that are relevant for the human situation as well. Lipscomb and Poet [31] have pointed out some advantages of using in vitro metabolic parameters to define PBK models, which include the ability to separately define and analyze individual metabolic processes, such as phase I metabolism and phase II metabolism, or bioactivation and detoxification, and to compare contributions from individual conversions to the overall metabolism across species and between individuals [31].

When metabolic parameters are derived from in vitro experiments with tissue fractions, primary cell cultures or tissue slices of organs involved in the metabolism of the compound, the V_{max} values obtained, expressed for example in nmol/min per mg microsomal protein, need to be scaled to the respective tissue and expressed in nmol/min per g tissue. For humans, this can be done using a microsomal protein yield of for example 32 mg per g liver [33]. In a similar way V_{max} values expressed as nmol/min per 10^6 cells can be scaled using a hepatocellularity of 99×10^6 cells per gram tissue [33]. Scaling factors for other model systems may be applied accordingly using scaling factors obtained from the literature. The apparent in vitro K_m values are generally not scaled and are assumed to correspond to the apparent in vivo K_m values. Renal excretion may be modeled by specific equations including diffusion, perfusion or Michaelis–Menten equations, the latter in the case of rate-limiting transporter-mediated specific processes or kidney-specific metabolism. As a first approximation, however, it may be assumed that renal excretion is not rate-limiting when compared to the formation of metabolites and that all metabolites predicted to be formed in the liver or other organs will be excreted in the urine as such for the full 100%. In this latter case no further mathematical modeling of renal excretion is required.

5 Step 4: Solving the equations: software for PBK modeling

When the mathematical model has been defined, the next steps include solving the mass-balance differential equa-

tions and making predictions. Different software packages are available with which the ordinary differential equations of the PBK model can be numerically integrated. Commonly used software includes Berkeley Madonna (Macey and Oster, UC Berkeley, CA, USA), acslXtreme (AEgis Technologies Group), and MATLAB (The MathWorks). Integration algorithms that can be applied are, for instance, the Gear algorithm or Rosenbrock algorithm, which are both capable of handling ''stiff'' sets of differential equations, which most PBK models are [3]. The choice for a specific software package will in general depend on the required flexibility and user friendliness.

6 Step 5: Model evaluation: an important step

When the differential equations have been solved, thereby calculating the concentrations of the compounds and their relevant metabolites in the relevant compartments, the next step to perform is the evaluation of the model performance. Evaluation of a model against experimental in vivo data is an important step of the PBK modeling approach, since it supports the validity of the model and thus its subsequent predictions. Furthermore, discrepancies between the model predictions and the experimental data may be informative and may for example indicate that an important model parameter has been overlooked or its value imprecisely incorporated in the model. If such situations are encountered, the nature of the discrepancies may indicate a new hypothesis and improvements for the model.

The model evaluation step also includes a sensitivity analysis. A sensitivity analysis provides a quantitative evaluation of how the input parameters of the model influence the model output. A typical way of performing a sensitivity analysis is the central difference method, which compares the magnitude in model output due to a defined change in input parameters. In general, input parameters are changed with 1 or 5%, with each parameter being analyzed individually, keeping the other parameters to their initial values. This analysis yields sensitivity coefficients that correspond to the ratio of change in simulation output (e.g. tissue concentration or formation of metabolites) to change in parameter value. A sensitivity coefficient of, for instance, 0.8 signifies that 1% change in the numerical value of the input parameter will result in 0.8% change in model output. The greater the absolute value of the sensitivity coefficient, the more influence the parameter has on the model output. The sign of the sensitivity ratio indicates whether the model output is directly or inversely related to the parameter. In a sensitivity analysis normalized sensitivity coefficients are generally calculated for all parameters at selected dose levels, but only parameters that significantly influence the model output, having a sensitivity coefficient of, for instance, higher than 0.1 in absolute value, are generally regarded as parameters that influence the model output to a significant extent [1, 3].

7 Step 6: Model predictions: examples of PBK models

Once the performance of the model has been evaluated, simulations and predictions can be made, including the prediction of output parameters that may not be easily accessible by experiment, such as for example the concentration of an unstable reactive intermediate. This may provide new insights in the behavior of the biological system. PBK modeling is a valuable tool to evaluate tissue concentrations of parent compounds but also of relevant metabolites, including reactive unstable metabolites, under different exposure regimens and in different species. Furthermore, PBK models allow evaluation of the effects of genetic polymorphisms and lifestyle factors. In the following sections examples of PBK models are presented focusing on some important applications of PBK modeling including:

(i) PBK modeling of dose- and species-dependent effects
(ii) PBK modeling of genotype- and lifestyle-dependent interindividual variation

7.1 PBK outcomes analyzing dose- and species-dependent effects

With a PBK model physiologically relevant concentrations of a compound or, when relevant, its active metabolite(s) in any target organ of interest can be modeled for a certain dose and route of administration. Thus, PBK modeling provides a unique way to predict responses under experimentally inaccessible conditions, and realistic low-dose regimens. Furthermore, by replacing all parameters in a PBK model for rat, by the corresponding physiological and kinetic parameters, determined using relevant human in vitro models, one can build models for the human situation. As an example, Fig. 4 presents an overview of the PBK model-based predictions for the dose-dependent conversion of estragole into its major metabolites (Fig. 3) AP, HE, HEG, OE, and HES in the liver of rat and human at dose levels up to 50 mg/kg body weight (bw). The results obtained reflect significant species-dependent differences in the relative importance of *O*-demethylation, being more important in male rat than in human (Fig. 4A) as well as in the major pathway for detoxification of HE, being glucuronidation to HEG in male rat (Fig. 4C) but oxidation to OE in human (Fig. 4D). However, in spite of these marked species differences in *O*-demethylation of estragole and in glucuronidation and oxidation of HE, the resulting species differences in formation of the proximate carcinogenic metabolite HE and of the ultimate carcinogenic metabolite HES up to dose levels of 50 mg/kg bw are moderate. A daily dose of

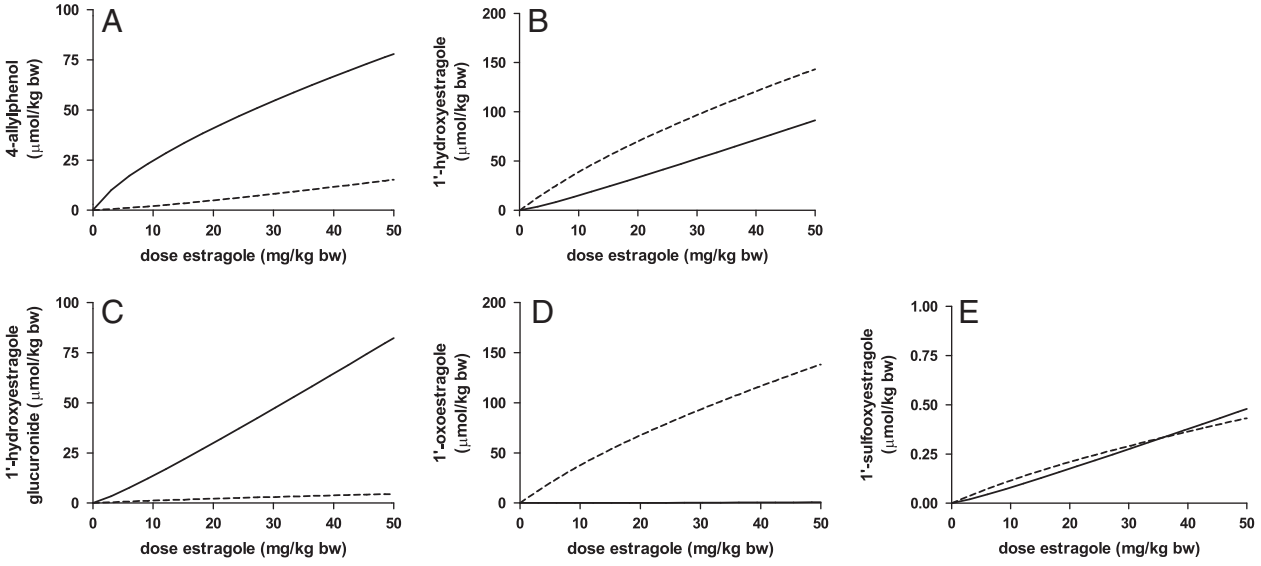

Figure 4. PBK model-based predictions for the dose-dependent formation of (A) AP, (B) HE, (C) HEG, (D) OE and (E) HES, in the liver of rat (solid line) and human (dotted line) at dose levels up to 50 mg/kg bw.

50 mg/kg bw is in the range of the $BMDL_{10}$ (the lower confidence limit of the benchmark dose causing 10% effect (BMD_{10})) for tumor formation by estragole [11, 12, 34, 35]. Considering the formation of the ultimate carcinogenic metabolite HES (Fig. 4E), predicted species differences are lower than twofold up to dose levels of 50 mg/kg bw, suggesting that the amount of reactive metabolites formed in rat and human are almost equal at these dose levels.

Some additional examples of PBK models analyzing dose- and species-dependent effects on ADME characteristics of other compounds and routes of administration are listed in Table 1.

7.2 PBK outcomes analyzing effects of genetic polymorphisms and lifestyle variation

Another characteristic of PBK models is their potential to predict not only dose- and species-dependent differences but also interindividual differences in the behavior of compounds in the body due to genotype polymorphisms and lifestyle factors. This is possible because one can define models in which equations for metabolic conversion by specific isoenzymes are included [8]. Such models allow evaluation of the effects of genetic polymorphisms and lifestyle factors, because they allow studying the effects of setting V_{max} values of specific isoenzymes to either increased values, representing enzyme induction, or to zero, representing a genetic deficiency. In addition, interindividual variability due to genetic polymorphisms and lifestyle factors can be modeled using a Monte Carlo approach where V_{max} values of specific isoenzymes are assigned a variability distribution that is representative for the population as a whole [8, 9, 51]. This latter approach also forms the basis of a commercially available PBK modeling platform Simcyp (Simcyp Limited, Sheffield, UK), thereby facilitating these types of simulations. Examples of approaches of modeling interindividual variability due to genotype polymorphisms and lifestyle factors can be found in our previous work on coumarin [52] and estragole [9].

Coumarin is a natural constituent of several flavoring ingredients added to food and personal care products. Chronic exposure to coumarin by the oral route has been reported to result in liver adenomas and carcinomas in rats and liver adenomas in mice [53–56]. Figure 5 shows an overview of coumarin metabolism. The major route of coumarin bioactivation is 3,4-epoxidation to coumarin-epoxide, which is followed by subsequent rearrangement of the epoxide to *o*-hydroxyphenylacetaldehyde (oHPA), which is considered to be the hepatotoxic intermediate [53, 57, 58]. Coumarin epoxide may also be conjugated to glutathione both chemically and enzymatically, the latter route being especially efficient in rats and mice [57]. oHPA can be detoxified by reduction to *o*-hydroxyphenylethanol, but especially by oxidation to *o*-hydroxyphenylacetic acid [53, 57]. Significant species differences between rat and man exist in coumarin bioactivation via the 3,4-epoxide pathway. In rats and mice the 3,4-epoxidation pathway appears to be the major route of coumarin biotransformation, whereas in humans the detoxifying coumarin 7-hydroxylation predominates, a reaction catalyzed by CYP2A6 [58]. Furthermore, detoxification of oHPA to *o*-hydroxyphenylacetic acid was shown to be more efficient in humans than in rats [57]. In our PBK modeling studies for coumarin we investigated the metabolic pathway(s) replacing the 7-hydroxycoumarin formation in homozygous CYP2A6-deficient subjects in order to estimate computationally the expected consequences of the CYP2A6 deficiency for oHPA

Table 1. Examples of PBK models analyzing dose- and species-dependent effects

Compound	Use	Species	Route(s) of exposure	Reference
Dichloromethane (methylene chloride)	Cross-species extrapolation, High-to low-dose extrapolation, Route-to-route extrapolation	Mouse, rat, hamster, human	Inhalation, drinking water	[36, 37]
Styrene	Cross-species extrapolation	Rat, human	Inhalation	[10]
Isopropanol	Route-to-route extrapolation, Cross-species extrapolation	Rat, human	Intravenous, intraperitoneal, oral, inhalation, dermal	[38]
Glycyrrhizic acid	Cross-species extrapolation	Rat, human	Oral	[39, 40]
1,2-Dichlorobenzene	Cross-species extrapolation	Rat, human	Oral	[41]
Acrylamide	Cross-species extrapolation	Rat, human	Oral	[42, 43]
Zearalenone	Cross-species extrapolation	Rat, human	Intravenous, oral	[44]
All-*trans*-retinoic acid	Route-to-route extrapolation, Cross-species extrapolation	Mouse, rat, monkey, human	Intravenous, oral, dermal	[45]
Hydroquinone	Route-to-route extrapolation, Cross-species extrapolation	Rat, human	Oral, intraperitoneal, intratracheal instillation, dermal	[46–48]
Manganese	Cross-species extrapolation, High-to low-dose extrapolation	Rodents, monkey, human	Oral	[49]
Cyclosporine A	High-to low-dose extrapolation	Rats	Oral	[50]

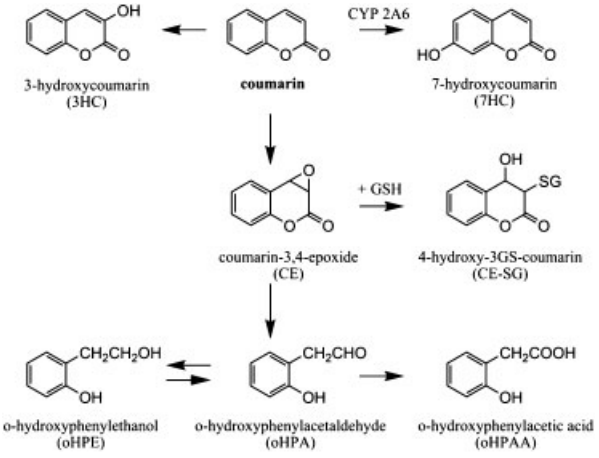

Figure 5. Metabolism of coumarin with the bioactivation pathway proceeding by formation of oHPA. Formation of the other metabolites leads to detoxification and excretion.

formation in the liver of human [52]. Since CYP2A6-deficient human samples are not easily experimentally accessible, studying the problem using a PBK modeling approach is an example for experimentally inaccessible conditions. In the PBK model the CYP2A6 genetic polymorphism can be simulated by setting the V_{max} for the CYP2A6-dependent 7-hydroxylation to zero.

The PBK model defined provided relative estimates of liver levels of oHPA, in rat and man but also in humans deficient in coumarin 7-hydroxylation due to homozygous CYP2A6 deficiency, at increasing levels of coumarin exposure (Fig. 6) [52]. For rat liver a dose-dependent increase in the C_{max} for oHPA formation is observed (Fig. 6A). For human liver of wild-type CYP2A6 subjects (Fig. 6B, dotted line) a dose-dependent increase in oHPA formation is only observed at dose levels above 15 mg/kg bw when 7-hydroxylation of coumarin becomes saturated and additional amounts of coumarin start to be metabolized through alternative biochemical pathways. For homozygous CYP2A6-deficient subjects, with V_{max} for coumarin 7-hydroxylation set to zero, there is a dose-dependent increase in the C_{max} for oHPA in the liver without an apparent threshold (Fig. 6B, solid line). Nevertheless, comparison of Fig. 6B to Fig. 6A reveals that along the whole dose range modeled, the predicted oHPA levels in liver of CYP2A6-deficient subjects remain at least ten-fold lower than the C_{max} values predicted for oHPA in rat liver at similar dose levels. This indicates that in human subjects, even when 7-hydroxylation is deficient due to the CYP2A6 polymorphism, the chances on adverse liver effects induced by oHPA will be significantly lower than those expected in the liver of rats when exposed to a similar dose on a body-weight base.

Some other examples of PBK modeling studies analyzing effects of genetic polymorphisms and lifestyle variation in humans are presented in Table 2.

7.3 Limitations and future possibilities of PBK modeling

A possible limiting factor in the application of PBK modeling is that PBK models generally apply to individual compounds. As a result, PBK-based modeling can be a labor-intensive process that requires generation of a large number of para-

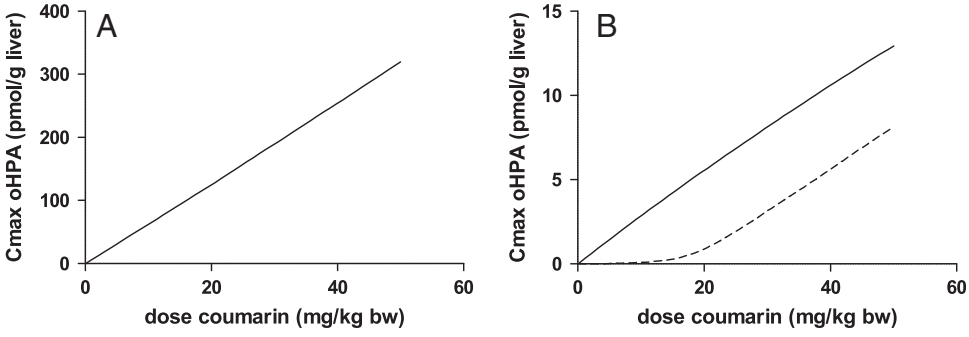

Figure 6. PBK model-based predictions for the dose-dependent concentration of oHPA in the liver of (A) rat and (B) a human wild-type CYP2A6 subject (dotted line) and a human homozygous CYP2A6-deficient subject (V_{max} for coumarin 7-hydroxylation set to zero) (solid line).

Table 2. Examples of PBK models analyzing effects of polymorphisms or lifestyle variation

Compound	Analyzing effects of	Implications on	Reference
Ethanol	Alcohol dehydrogenase polymorphisms	Blood concentrations	[59]
Acrylamide and its metabolite glycamide	Glutathione transferases, CYP2E1, and epoxide hydrolase polymorphisms	AUC acrylamide and AUC glycamide	[60]
Chlorpyrifos	CPF-oxonase polymorphisms	Detoxification chlorpyrifos	[61]
Warfarin	CYP2C9 polymorphisms	Blood concentrations	[51]
Parathion and its metabolite paraoxon	Paraoxonase polymorphisms	Blood concentrations	[51]
Acetone	Interindividual variation in various model parameters	Blood concentrations	[62]
Acetaldehyde	Aldehyde dehydrogenase polymorphisms	Nasal tissue levels	[63]
N-[2-(7-Methoxy-1-naphtyl)-ethyl]acetamide	Interindividual variation in various cytochrome P450 enzymes	Blood concentrations	[7]
Estragole	Interindividual variation in various cytochrome P450 enzymes and 7β-hydroxysteroid dehydrogenase type 2	AUC 1′-hydroxyestragole in liver	[9]

meters. Although physiological parameters such as tissue blood flows and organ volumes can be readily obtained from the literature, compound-specific parameters including tissue/blood partition coefficients and kinetic parameters describing processes contributing to absorption, distribution, metabolism and excretion need to be obtained experimentally or in silico. Presently developed PBK models often include parameters determined in in vivo studies. However, to limit the need for in vivo experiments in constructing PBK models for a specific compound, these chemical-specific parameters should ideally be obtained from in vitro and/or in silico approaches.

Another limitation may be that a PBK model only describes the kinetics of a compound of interest within an organism. However, a toxic response in vivo will be determined by kinetic and dynamic characteristics. The carcinogenic effects of estragole and coumarin will for instance also depend on toxicodynamic processes (i.e. processes of importance for the ultimate formation and development of tumors). This could be investigated in further detail by extending the PBK models to so-called physiologically based dynamic (PBD) models in which dose levels should be coupled to toxicologically relevant endpoints. Thus, integration of additional information on the subsequent reactions with target molecules or organs causing toxicity, i.e. toxicodynamic parameters, will provide a means to reliably predict the likelihood and magnitude of adverse health effects [64, 65]. The dynamic parameters required for the development of PBD models can be obtained by optimizing the fit of the model to an informative in vivo data set or from in vitro experiments. Obtaining the dynamic parameters from in vitro experiments allows extrapolation of in vitro concentration–response curves to the in vivo situation. Examples of this approach can be found in our work on estragole as well as our work on glycol ethers, both outlined below. Some other examples of PBD modeling approaches either based on in vivo or in vitro derived dynamic characteristics are listed in Table 3.

7.4 Extending PBK models to PBD models: example of DNA binding of estragole

An example of a PBD model is the extension of the PBK model developed for estragole [11, 12] to a PBD model predicting in vivo DNA adduct formation of estragole in rat liver [72]. This PBD model was developed using in vitro data on DNA adduct formation in rat primary hepatocytes exposed to HE. The model was extended by linking the area under the concentration-time curve for HE in the liver predicted by the PBK model to the area under the concentration-time curve for HE in the in vitro hepatocyte experiments. The outcome of the PBD model revealed a linear increase in DNA adduct

Table 3. Examples of PBD models

Compound	Dynamic endpoint	Dynamic parameters obtained from	Reference
Acrylamide	Impaired movement (acute) and acoustic startle response (subchronic) in rats	In vitro assays measuring basal cytotoxicity and neurite degeneration in human neuroblastoma SH-SY5Y cells	[66]
Several unspecified substances	Acute oral toxicity in rodents	In vitro assays measuring basal cytotoxicity in human lymphocytes and freshly isolated rat hepatocytes	[67]
Ethoxyacetic acid, 2-ethoxyethanol, 5-fluorouracil, methotrexate, 2-methoxyacetic acid 2-methoxyethanol, retinoic acid	Developmental toxicity in rodents	In vitro embryonic stem cell test (EST)	[30]
Lindane, acrylamide, caffeine, diazepam, phenytoin	Various in vivo effects and/ LD50 in rodents	Diverse in vitro assays using neuroblastoma SH-SY5Y cells	[68]
Acrylamide	Liver DNA adducts and hemoglobin adducts in rats mice, and humans	Optimizing model to informative in vivo data set	[69]
Formaldehyde	Tumor incidence in rat	Optimizing model to informative in vivo data set	[70]
Carbofuran	Acetylcholinesterase inhibition in rat	Optimizing model to informative in vivo data set	[71]

formation with increasing estragole doses up to 100 mg/kg bw. Comparison of the PBD model outcome to available data showed that the model adequately predicts the dose-dependent level of DNA adduct formation [72]. At a dose level of 0.01 mg estragole/kg bw/day [13] and 0.07 mg estragole/kg bw/day [14], estimated levels of regular daily human intake, the PBD model predicts amounts of the major estragole DNA adduct formed equal to respectively 2 and 12.8 in 10^8 nucleotides (nt). These levels of adducts are 1 to 2 orders of magnitude below the background level of DNA damage of 100 adducts in 10^8nt, which have been reported to be of no known consequence [73]. Furthermore, the levels are 1 to 2 orders of magnitude lower than the background levels of DNA adducts formed by low molecular weight alkylating agents, like for example *N*-nitrosamines, which are in a range of 10–100 adducts in 10^8 nt [74, 75]. Although not all DNA adducts have a mutagenic effect [76] and DNA adduct formation of genotoxic carcinogens is generally seen as a biomarker of exposure rather than a biomarker of response [77, 78], DNA adduct formation has been accepted as a hazard indication pointing at a potential cancer risk. Thus, the processes determining the level of DNA binding as possible key event in tumor induction are part of what is called the toxicodynamic phase and the PBD model is one step closer to the ultimate toxic effect than the PBK model.

7.5 Extending PBK models to PBD models: PBK outcomes converting in vitro concentration–response curves to in vivo dose–response curves

In our work on glycol ethers, we extended a PBK model for these compounds to a PBD model predicting the ultimate toxic effects of these compounds in vivo, i.e. developmental toxicity, using in vitro embryotoxicity data of the toxic metabolites of the glycol ethers. This example shows how PBD modeling can be used to convert concentration–response curves from in vitro toxicity assays to relevant in vivo dose–response scenarios. This was done by so-called reverse dosimetry in which $EC_{10,\ 20,\ 30,\ etc}$ values (effective doses causing 10, 20, 30 etc. percent response) from the in vitro concentration–response curves are set equal to plasma or tissue levels of the respective compound in the PBK model, following which the PBK model can predict the corresponding in vivo $ED_{10,\ 20,\ 30,\ etc}$ levels (effective dose levels causing 10, 20, 30, etc percent response), thereby defining the in vivo dose–response curve.

Figure 7 shows this reverse dosimetry approach using PBK modeling and in vitro toxicity data to predict dose–response curves for in vivo developmental toxicity of glycol ethers in rat and man [79, 80]. A PBK model was developed, describing the kinetics of four glycol ethers and their embryotoxic alkoxyacetic acid metabolites in rat and man. In vitro toxicity data of these metabolites derived in the embryonic stem cell test (EST) were used as input in the PBK model to extrapolate in vitro concentration–response curves to predicted in vivo dose–response curves for developmental toxicity of the parent glycol ethers in rat and man. The predicted dose–response curves for rat were found to be in concordance with the embryotoxic dose levels measured in reported in vivo rat studies. Thus, it can be foreseen that PBK modeling tools can contribute to improvement of the applicability of in vitro methods for risk assessment purposes because they enable the extrapolation of in vitro concentration–response curves to in vivo dose–response curves. Obtaining an in vivo dose–response curve based on in vitro toxicity data would allow deriving a BMD or a

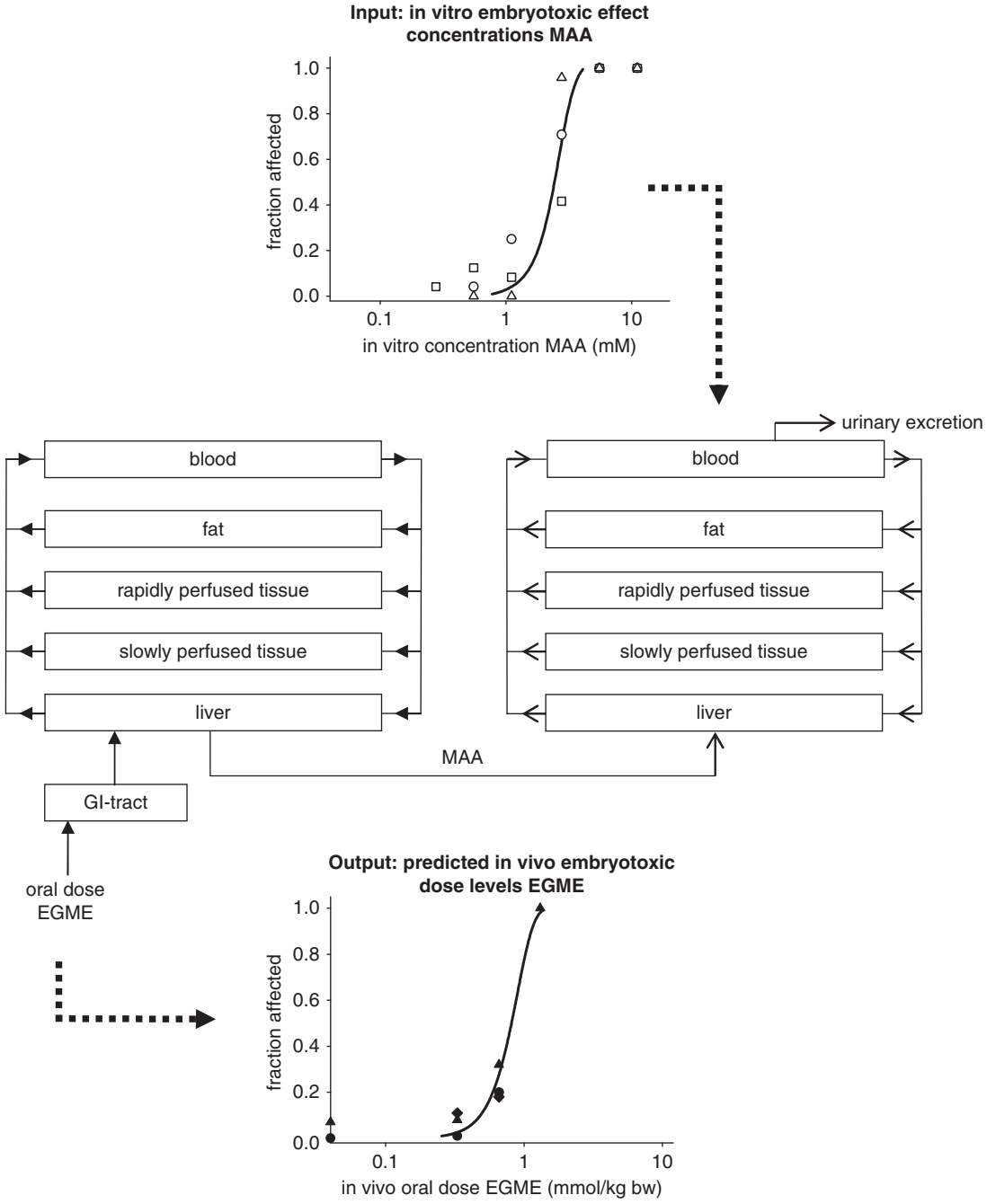

Figure 7. Concept of reverse dosimetry enabling extrapolation of an in vitro concentration–response curve for the toxicity of the embryotoxic metabolite methoxyacetic acid (MAA) of ethylene glycol monomethyl ether (EGME) in the EST to an in vivo dose–response curve for developmental toxicity of EGME using PBK modeling. Open symbols represent in vitro toxicity data of MAA obtained in the EST. The curve fitted to these in vitro data was extrapolated to an in vivo dose–response curve for developmental toxicity of EGME using the PBK model. The closed symbols represent in vivo data for developmental toxicity obtained from the literature [80]. For further details see [79].

BMDL, which are important parameters for risk assessment practice [81, 82]. This could for instance be done using the Benchmark Dose Software developed by the U.S environmental protection agency (EPA) (www.epa.gov/ncea/bmds) or using PROAST software developed by the Dutch National Institute for Public Health and the Environment (RIVM) (www.rivm.nl/proast). The BMD(L) obtained can serve as a point of departure for setting safe exposure limits for the

human situation, such as acceptable daily intakes, tolerable daily intakes, or health-based recommended occupational exposure limits.

8 Discussion

The present tutorial gives an overview of the basic concepts of PBK modeling and presents several examples that show that PBK models provide a good method to evaluate the occurrence of dose-dependent effects and species differences in bioactivation and detoxification of compounds in vivo. Using this approach, mechanisms underlying dose-dependent effects in relation to bioactivation can be studied, and insights can be obtained in the occurrence of species-, genotype- and lifestyle-dependent differences in metabolism and metabolic activation. Furthermore, route-to-route comparisons and extrapolations can be made, estimating for example what oral and inhalation dose levels would result in similar plasma or tissue concentrations.

As outlined, PBK modeling basically includes six steps, which can be summarized as follows:

(i) Define the conceptual model, which includes defining a simplified representation of the biological system containing the relevant model compartments to be included in the model. Examples have been presented that this can be done based on state-of-the art knowledge on the ADME characteristics of a compound (e.g. estragole and coumarin).
(ii) Translate the conceptual model into a mathematical model, which can be done by formulating differential equations for each compartment. These equations should describe all processes relevant for increasing (uptake from the gastrointestinal tract, by inhalation or via the skin, transport via the blood and formation by metabolism) and decreasing (transport via the venous blood, conversion to metabolites) the concentration of a compound or its relevant metabolites in a given target tissue.
(iii) Define the parameter values of the differential equations in the correct units; in this step, parameters are taken from literature or derived from experimental data and scaling factors may have to be taken into account.
(iv) Solve the differential equations, thereby calculating the concentrations of the relevant compounds and their metabolites in the relevant compartments, using software packages that are capable of numerical integration of the mass balance differential equations.
(v) Evaluate the model performance. Evaluation of a model performance may support the validity of the model or elucidate discrepancies between the model predictions and the experimental data. The latter may be informative and for example indicate that an important model parameter has been overlooked. If such situations are encountered, the nature of the discrepancies may indicate a new hypothesis and improvements for the model.
(vi) Make predictions. When the model has been evaluated, it becomes possible to perform simulations and predict relevant parameters. This may provide new insights into the behavior of the biological system.

PBK models have advantages over so-called ''classical'' pharmacokinetic models, which are not based on physiology. PBK models help interpolation and extrapolation of knowledge between:

(i) Doses: such as from the high doses typically used in laboratory experiments to those resulting from regular human dietary intakes.
(ii) Species: such as from rodents to human, prior to giving a compound for the first time to subjects in a human study, or when experiments on humans are considered unethical, such as for the safety assessment of new compounds with unknown toxicity.
(iii) Individuals: such as from males to females, from adults to children, from non-pregnant to pregnant women, between individuals with different genotype polymorphisms or different lifestyles.
(iv) Routes of administration: such as when making comparisons between inhalation exposures and oral ingestion.
(v) Exposure duration: such as from continuous to discontinuous, or single to multiple exposures.

Some of these extrapolations only require changes in input or parameter values to achieve the extrapolation (this is usually the case for dose and time extrapolations). Others may require a change in the model structure itself (for example when studying embryotoxicity, a compartment and equations for the fetus may have to be added).

The capability of predicting the target tissue exposure to toxic metabolites in people at relevant dose levels using PBK models should help reduce the uncertainty associated with the extrapolation procedures adopted in conventional risk assessment. Furthermore, using reversed dosimetry, it may become feasible to overcome a key limitation in the development and application of in vitro alternatives, namely that at present in vitro methods do not enable prediction of in vivo dose–response curves for systemic effects. PBK modeling provides a way to translate in vitro concentration–response curves to in vivo dose–response curves. This can be used for defining a point of departure for setting safe exposure limits, thereby contributing to the use of alternative testing strategies in the field of risk assessment. Although PBK modeling makes it technically possible to improve the applicability of in vitro methods in risk assessment practice, the possibilities of these methods will ultimately also depend on the validation and acceptation of such methods.

Further, improving the possibilities of deriving in vivo dose–response curves based on in vitro toxicity assays and PBK modeling would require more research on the applicability of this technique. In this respect, more research is needed on (i) the development of PBK models based on in vitro and/or in silico derived parameters and (ii) making

PBK models more applicable for larger groups of compounds by combining, for instance, PBK modeling with quantitative structure activity relationship/quantitative structure property relationship approaches and (iii) creating examples that provide proof of principle of deriving in vivo dose–response curves based on solely in vitro assays and PBK modeling techniques. Ultimately this should provide sufficient support for the incorporation of this alternative testing strategy in risk assessment practices, aiming at Replacement, Reduction and Refinement (3Rs) of animal use in testing strategies.

Altogether there are several studies in the field of molecular nutrition and food research where PBK or even PBD modeling has already been applied (Tables 1–3). From this it can be seen that there are some proofs of principle, but that there is a wide opportunity for further and future work in this field.

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