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Development of a human physiologically based pharmacokinetic (PBPK) model for phthalate (DEHP) and its metabolites: A bottom up modeling approach

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Abstract:

DEHP exposure to human comes from different sources such as food, diet, cosmetics, toys, medical products, and food wraps. Recently DEHP was categorized under non-persistent endocrine disruptor compounds (EDCs) by the world health organization (WHO). There is enough evidence from the rat experimental studies that phthalate causes hepatic, developmental and reproductive toxicity. In human, DEHP rapidly metabolizes into a toxic metabolite MEHP. This MEHP further metabolizes into the different chemical forms of 5OH-MEHP, 5oxo-MEHP, 5cx-MEPP and phthalic acid. A simple pharmacokinetics model has been developed for the DEHP with limited number of metabolites. A chemical like DEHP that extensively undergoes metabolism producing many harmful metabolites urges to develop a detail metabolic kinetics. A physiological based pharmacokinetics (PBPK) model of DEHP that considers all the major metabolites in human has not been developed yet. The objective of this study is to develop a detail human PBPK model for the DEHP and its major metabolites by a bottom-up modelling approach integrating in vitro metabolic data. This approach uses an in-vitro to in-vivo extrapolation (IVIVE) method and Quantitative structure activity relationship (QSAR) for the parameterization of the model. Monte Carlo simulations were performed to estimate the impact of parametric uncertainty onto model predictions. First the model was calibrated using control human kinetic study that represents the time course of the DEHP metabolites in blood and urine. Then the model was evaluated against the published independent data of different dosing scenarios. The results of model predictions for the DEHP metabolites in blood and urine were well within the range of experimentally observed data and it also captured the trend of time course profile similarly to the observed data, showing model good predictability. The current developed PBPK model can be used for the prediction of the time course of chemical concentrations not only in the blood and urine but also in the other compartment even for different exposure scenarios. Moreover, this model can also be used to explore different biomonitoring studies for human health risk assessment and might be useful for integrative toxicological study in improving exposure-target tissue dose–response relationship.

Keywords: DEHP; MEHP; Pharmacokinetics; PBPK; Human health Risk assessment; IVIVE; Endocrine disruptors; human biomonitoring

1. Introduction

Phthalates are ubiquitous environmental contaminants made up of dialkyl esters or alkyl and aryl esters of ortho-phthalic acid (1,2-dicarboxylic acid). Among Phthalates Di-2-ethylhexyl phthalate (DEHP) is the most important because of its large and widespread uses in industries as a plasticizer. It is found in food, cosmetics, toys, medical products and food packaging, mostly used as a plasticizer. The total dietary intake (TDI) of 50µg/kg BW/day limit has been set by the EFSA and the European chemical agency (ECHA) to assess the risk related to DEHP exposure (EFSA, 2015; ECHA, 2010). Recently reported studies on the total dietary intake mean value of DEHP in different cohort studies for several countries estimated in the range of 0.42 to 11.67 µg/kg bw/day, which is far below the threshold set by the EFSA and the ECHA (Fromme et al., 2007; Dickson-Spillmann et al., 2009; Sioenet al., 2012; Heinemeyer et al., 2013 ;Martine et al., 2013 ; Martínez et al., 2017).

DEHP has a short half-life and it does not accumulate inside the body (Krotz et al., 2012). DEHP completely metabolizes into a toxic metabolite mono-(2-ethylhexyl) phthalate (MEHP). MEHP further metabolize into different chemical forms like 5-hydroxy MEHP, 2-ethyl-5-carboxypentyl phthalate (5-Cx MEPP) and phthalic acid. 5-oxo MEHP is another metabolite result of the 5-OH MEHP metabolism. Temporal variability in phthalates exposure from the different sources and their ability to generate several forms of metabolites can lead to a stable microenvironment exposure of phthalates to internal organs. This could lead to a pseudo-steady state concentration over a long period of exposure (Meeker et al., 2009).

Currently, DEHP is of concern on its categorization as a non-persistent endocrine disruptor by the World Health Organization (WHO, 2010). Cobellis, (2003) in his epidemiological study, linked the exposure of DEHP and the prevalence of endometriosis in women. Other studies have also shown that environment relevant dose of phthalates alters estrous cycle, impaired oocyte maturation, decrease ovulation (Anas et al., 2003; Krisher, 2013; Hannon et al., 2014). DEHP and its toxic metabolite MEHP mainly alter the estrogen productions and its activity in granulosa cell, required for the development and secretion of the follicles, which might lead to infertility due to hypo-estrogenic, polycystic ovary and anovulatory cycles (Davis et al. 1994; Lovekamp-Swan & Davis 2003). Several hypotheses on phthalates effect on male reproductive toxicities was proposed based on animal studies, for more detail please refer to given references (Richburg et al., 1999; Koji et al., 2001; Sharma et al., 2017a). Increased DEHP urinary levels are associated with significant declines in the plasma testosterone concentrations were reported in several cohort studies(Duty et al., 2005; Pan et al., 2006).

To better estimate the physiological concentration of DEHP metabolites in the target tissues such as gonads, it is necessary to understand its pharmacokinetics and the factors controlling its distribution and metabolism within the quantitative framework of a physiologically based pharmacokinetic model. Reliable Physiologically based Pharmacokinetic (PBPK) model will be useful for establishment of proper dosing metrics for the target tissues (Fabrega et al., 2014), and its applicability to set upthe exposure-dose-response relationship for the systems toxicology model(Sharma et al., 2017b, 2018). . Since 1974, several pharmacokinetic analyses on the DEHP and its metabolites have been conducted both in-vitro and in-vivo (animal and humans) (Daniel and Bratt, 1974; Peck and Albro, 1982; Albro, 1986; Ito et al., 2005; Wittassek

and Angerer, 2008; Choi et al., 2013). Several pharmacokinetic (PK) models have been developed accounting its major metabolites using simple compartmental approach (Koch et al., 2003, 2004, 2005, 2006; Lorber et al., 2010). Koch et al., (2003, 2004, 2005) experimentally investigated several secondary metabolites concentration of DEHP both in the blood and urine describing their time course kinetics. A PK model developed by Lorber et al., (2010) has predicted the DEHP metabolites concentration both in the blood and urine which involves empirical fitting of the two key parameter, one is fraction of chemicals available to undergo metabolism, and, other is rate of dissipation of metabolites, against the observed blood and urine concentration data. However, It lacks the mechanistic metabolic kinetics (Michaelis-Menten reaction), considered the most important biotransformation process. Keys et al., (1999) and Cahill et al., (2003) developed a PBPK model of DEHP in both the rats and human, however, these models have not included all the metabolites and their kinetics, which might be due to insufficient data on the DEHP metabolic kinetics at that time. Recently, Choi et al., (2012) reported the *in vitro* metabolic kinetics information on DEHP and its metabolites both in the rat and human using hepatic cell line . To best of our knowledge, there is no published detailed target tissue dosimetry model (PBPK), which becomes essential for the chemical like DEHP that produces many metabolites (Daniel and Bratt, 1974; Ghosh et al., 2010). The purpose of this study is to develop a detailed PBPK model for DEHP and its major metabolites for the adult human and its evaluation against the experimental data. A bottom-up modeling approach was used for the development of model. It involves integration of *in vitro* metabolic and *in silico* data that uses IVIVE (in-vitro in-vivo extrapolation) and QSAR (Quantitative structure activity relationship) tools. These tools have led to possibly build a PBPK model with minimal or no animal experiments, supporting the 3Rs strategies of minimizing animal use. An IVIVE tool has successfully been used in connection with a PBPK to derived in-vivo kinetics from *in vitro* studies using biologically appropriate scaling (Yoon et al., 2014; Martin et al., 2015). This work is part of two major EU projects, HEALS and EuroMix, where different aspects of in silico models and its applications in human biomonitoring are investigated (Martínez et al., 2017, 2018).

This article describes a physiologically based pharmacokinetic (PBPK) model predicting the time variant concentrations of DEHP metabolites such as MEHP 5-OH MEHP, 5-cx MEPP and 5-oxo MEHP in plasma upon oral dosing of DEHP. The model was used to simulate the cumulative amount of the DEHP metabolites in urine. The *in vitro* human gut and hepatocyte DEHP metabolic kinetics data were scaled and integrated into the model (Choi et al., 2013) . The human experimental observed DEHP metabolites concentration data both in the plasma and urine are used to calibrate the PBPK model. Further model evaluation was done against the independent data on DEHP kinetics for different dosing scenarios (Anderson et al., 2011) . Prior mean parameter values were obtained from the published literature or derived from the in-vitro and in-silico experiments, whilst accounting for uncertainties in the range of ±1 to ±1.5 standard deviation. After sensitivity analysis the most uncertain parameter yet influential parameters were distributed statistically for Monte Carlo simulations.

2. Models and Methods

2.1.Overview of the modeling approach

The model was coded as a set of ordinary differential equations, written in the GNU MCSim modeling language and solved by numerical integration using the R "deSolve"

package (Bois and Maszle 1997). Model parameters value was derived from in *vitro* and *in-vivo* experiments reported in the literature or using the in-silico approach.. Sensitivity analysis of model was done using the mean value of the parameters. After sensitivity analysis the most uncertain yet influential parameters were distributed statistically for Monte Carlo simulations to estimate the impact on model predictions of uncertainty in all of the selected parameters (Bois et al., 2010; Fàbrega et al., 2016). Model equations are provided in Annex-B.

The exchange of the chemicals between blood and tissue in each organ is described by flow limited processes i.e. we implement a perfusion rate-limited PBPK model (not permeability limited). The model comprises several compartments i.e. gut, liver, blood, fat, gonad and a compartment representing rest of the body (Fig.1). The gonad compartment was included in the model for its later use in DEHP reproductive toxicity assessment. The only metabolite MEHP was distributed to the given compartments, while other metabolites were confined to the blood compartment presuming their volume of distribution is equivalent to the plasma volume. All physiological parameters such as blood flows and tissue volumes used in the model were obtained from the published literatures (Davies and Morris, 1993; Brown et al., 1997; ICRP, 2002) and are provided in Table A.1 of Annex. The partition coefficients and fractional unbound were obtained from the in-silico approach or literature are provided in Table 1. The calibration of the model was carried out against the human pharmacokinetic experimental data on both the plasma and the urine level of DEHP metabolites reported in Koch et al., (2004, 2005). This involves the plasma concentration data during the first 8 hours and the cumulative amount of metabolites in urine over 44 hours following an oral dosing of 48.5mg. Further evaluation of the developed PBPK model was done against the other independent pharmacokinetics study done by Anderson et al., (2011) for two different dosing scenarios. In this study, all major metabolites are considered namely; MEHP, 5-OH MEHP, 5-CX MEPP, 5-Oxo MEHP and phthalic acid. All the metabolic parameters were derived from *in vitro* cell line study are provided in Table 1.

2.2. Pharmacokinetics of DEHP and its Metabolite

The rate of metabolite formation is assumed to be equal to the rate of parent compound metabolism. DEHP metabolic pathway is provided in Fig.2. DEHP metabolizes to MEHP, which metabolizes into different chemical forms i.e. 5-OH MEHP, 5cx-MEPP, and 2cx-MEPP. Among them, 5-OH MEHP further metabolizes into 5-Oxo MEHP. All the metabolites excrete via urine. Absorption of DEHP from the gut to the liver was described by partition coefficient. Both DEHP and MEHP distributed to compartments such as liver, fat, plasma and gonads. However, due to insufficient data on the partition coefficients for other metabolites except MEHP, their distribution confined to the plasma compartment. Thus the volume of distribution of metabolites other than MEHP has set equal to the plasma volume.

Absorption

Koch et al., (2005) in his study reported that DEHP is completely absorbed from the gut and rapidly metabolized into the MEHP in the liver. The distribution of DEHP from the gut to the plasma is described by its partition coefficient between them. The partition coefficient (gut: plasma) was estimated using QSAR approach of Poulin and Krishnan tissue composition method (Poulin and Krishnan, 1996, 1995; Poulin and Theil, 2000).The MEHP uptake from the gut the liver was described by the first order rate constant (Adachi et al., 2015).

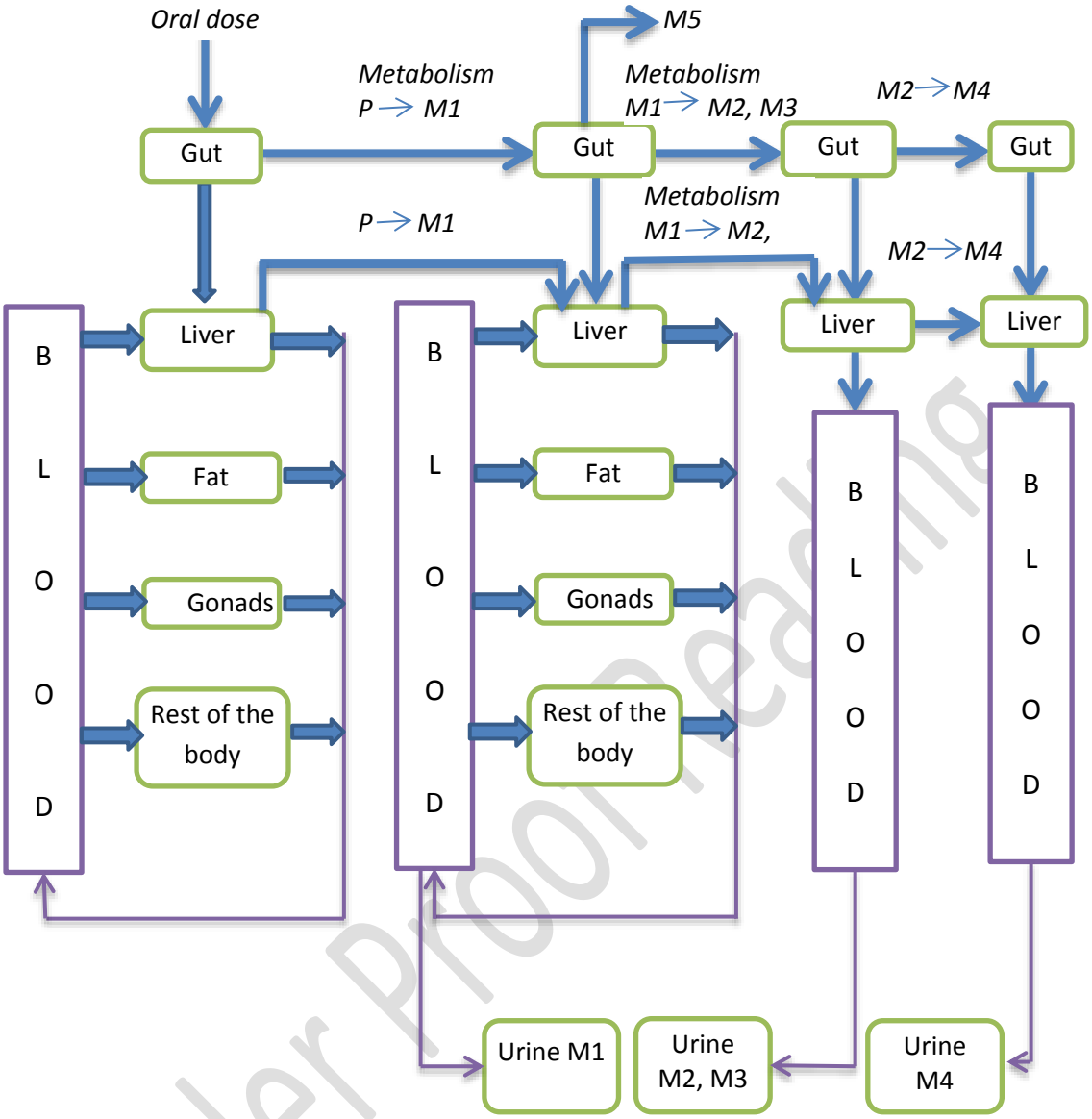

Fig. 1. The figure represents a PBPK model for the DEHP and its metabolites. It includes mainly five compartments and clearance of chemical depends on both metabolism (mainly five metabolites) and urinary elimination. Following oral administration of DEHP(P), it readily metabolizes into MEHP (M1) and MEHP further metabolizes into 5-OH MEHP (M2), 5-cx MEPP (M3) and phthalic acid (M5). 5-OH MEHP (M2) is further metabolizing into 5-oxo MEHP (M4), for detail metabolic scheme refers to Fig. 2. The DEHP and MEHP are distributed to the given compartments. However other metabolites produced in guts and liver are transferred to blood compartments assuming their distribution in a single compartment. The metabolite phthalic acid (M5) was not utilized in this model for its further distribution to blood or its elimination (except for MEHP clearance, metabolic conversion to M5), as no data are available to calibrate its concentration in urine or blood.

Distribution

Both the DEHP and the MEHP distribution to the several compartments was done using their partition coefficients estimated by in-silico or derived from the published literature and are provided in Table 2. DEHP partition coefficients were estimated using the

QSAR approach based on tissue composition method (Poulin and Krishnan, 1996, 1995; Poulin and Theil, 2000). A log ko/w of 7.6 was used to estimate the tissue: plasma partition coefficients. MEHP partition coefficient values measured experimentally via vial –equilibration method by Keys et al., (2000) was used for tissue distribution. Other metabolites distributions restricted to the blood compartment only, assuming their volume of distribution equivalent to the plasma volume. The metabolites formed in the liver transfer to the blood using first order uptake rate constants and these parameters were calibrated against the Koch et al., (2005) experimental data.

Elimination

Elimination of DEHP and its metabolites in urine was assumed to be directly proportional to its rate of clearance from the plasma. The model presumed that DEHP clearance solely depends on its metabolism into MEHP (Koch et al., 2004, 2005, 2006; Lorber et al., 2010).

The excretion rates for the MEHP and other metabolites were described by first order rate equation. These excretion rates were obtained by using the relationship of elimination rate constant and chemical's plasma half-life i.e. ratio of ln2 (0.693)/t1/2 (half-life). The mean half-lives for MEHP, 5-OH MEHP and 5-CX MEPP and 5-oxo MEHP was estimated by Lorber et al., (2010) was used for the model parameterization. . These parameters values were used for the model simulation and calibration against the reported time course concentration of chemicals in the plasma and cumulative excretion profile in the urine reported (Koch et al., 2005). . The elimination rate constant for MEHP was measured using half-life reported by Mittermeier et al., (2016).

2.3. *In vitro* intestinal and Hepatocyte metabolic studies

Metabolism of the DEHP both in the liver and gut to MEHP, 5-OH MEHP, 5oxo-MEHP, 5cx MEPP and phthalic acid was described by the Michaelis-Menten equation provided in Eq. (2). This equation includes two important parameters namely Vmax (maximum velocity of metabolic reaction) and Km (affinity i.e. concentration at which reactions occurs at half maximal rate). The *in vitro* intestinal and hepatic metabolic rates for several DEHP metabolites were reported in Choi et al., (2012)where author has described mainly five metabolites (MEHP, 5-OH MEHP, 5oxo-MEHP, 5cx MEPP and phthalic acid) kinetic both in the microsomal and cytosol fraction of the intestine and the liver. High intrinsic clearance rate i.e. ratio between Vmax and Km for the metabolic conversion of DEHP to MEHP in the cytosolic fraction of intestine and liver was observed(Choi et al., 2012). However, intrinsic clearance for other metabolites in cytosolic fraction was reported to be insignificant. The in-vitro in-vivo extrapolation (IVIVE) method, which involves scaling of *in vitro* Vmax value to *in vivo* utilizes physiological specific parameters such as tissue specific microsomal protein content or cytosol protein, specific tissue volume and, body weight (Yoon et al., 2014) was used to derive the metabolic parameters. The Eq. (1) describes the scaling approach which is used to derive the Vmax value as an input for the PBPK model. The Michaelis constant i.e. Km for the five metabolites in gut and liver were set equal to the reported in-vitro cell line study provided in Table 1. The reported Vmaxin-vitro values, maximum rate of reaction, were scaled to the whole body PBPK using Eq. (1). The reported quantity of MSP in the liver (Godin et al., 2006), and the gut is 52.5 mg/g liver and 20.6 mg/g intestine respectively (Godin et al., 2006; Cubitt et al., 2011). Mean value of 80.7 mg and 18 mg of cytosolic protein per gram of the liver and the gut respectively are used

for the IVIVE approach (Gibbs et al., 1998). In-vivo scaled Vmax values for each metabolite are provided in Table 2. The schema of metabolism is provided in Fig. 2.

$$Vmax(intestine/liver) = (Vmax_{invitro\ intestine/liver} * MPPGG/MPPGL/CytosolPGG/CytosolPGL * Vgut/Vliver)/BW^{.75} \quad \textit{Eq. (1)}$$

Where,

Vmax is the maximum rate reactions value in the unit of µg/hr/kgBW$^{.75}$; MPPGG is the microsomal protein per gram of gut; MPPGL is the microsomal protein per gram of liver; CytosolPGG is the cytosolic protein per gram of gut; CytosolPGL is the cytosolic protein per gram of liver

Vgut and Vliver is the volume of gut and liver respectively

$$\frac{dA_{mets}}{dt} = \frac{Vmax * C_t * f_u}{km + C_t * f_u} \quad \textit{Eq. (2)}$$

Where,

Ct is the corresponding concentration in tissue and fu is the fraction unbound constant.

Vmax (µg/hr/whole body weight) is the maximum rate for the corresponding reactions; Km is the affinity constant concentration at which half of the Vmax occurs.

$\frac{dA_{mets}}{dt}$ is the rate of production of metabolites

Metabolism pathway

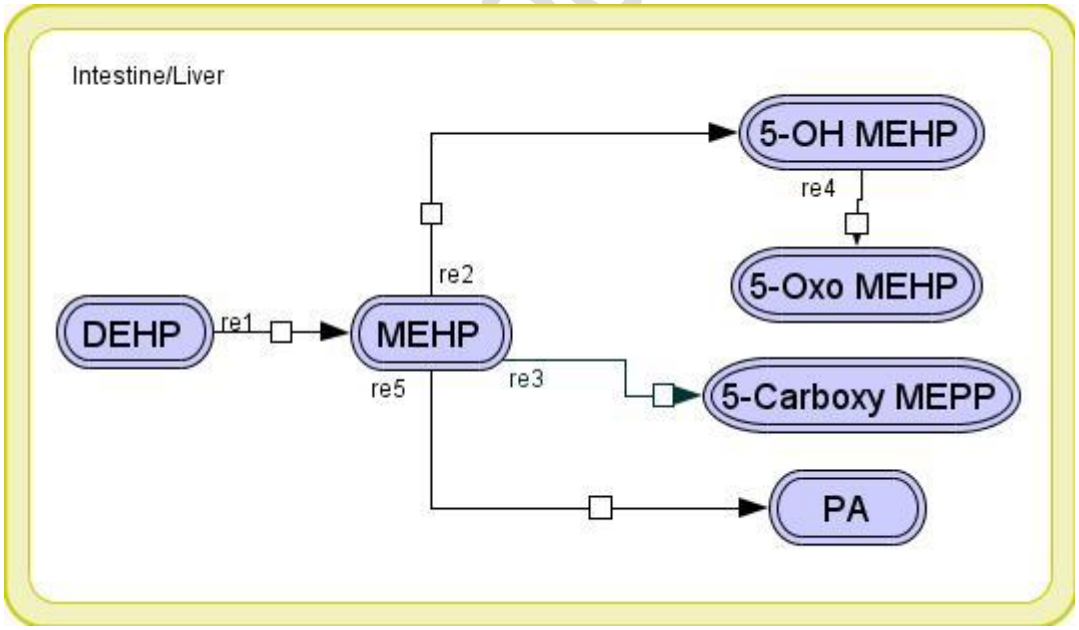

Fig. 2. Represent the schematic metabolic pathway of DEHP in the human gut and liver. The productions of metabolites follow same structure in PBPK and were described using Michaelis Menten equation. The corresponding re1, re2, re3, re4, and re5 represent the Michaelis-Menten metabolic reaction used in the model represented in the *Eq. (2)*.

2.4. In vivo Human Pharmacokinetics study

In-vivo pharmacokinetics of DEHP and its metabolites are well characterized in several studies (Koch et al., 2006, 2005, 2004; Anderson et al., 2011; Lorber et al., 2010). Koch et al., (2004, 2005) studies involved the self dosing of 48.5 mg of D4-DEHP by volunteer (n = 1) . The volunteer aged 61, 175 cm tall and weighing 75 kg. Plasma concentrations for MEHP, 5-OH MEHP, 5-oxo MEHP and 5-Cx MEPP were measured at 2,4, 6 and 8.3 hours upon DEHP self dosing. In the same study, urine samples were collected until 44hr and the cumulative amount of DEHP metabolites were reported. This study was accounted for the model calibration. koch et al., (2005) monitored two metabolites namely 5-cx MEPP and 2cx MMHP in both plasma and urine. koch et al., (2005) found 5-OH MEHP and 5-cx MEPP as major metabolites in the urine and observed no dose dependency related to the amount of metabolites. The 5-cx MEPP metabolite was not included in the current model since there is no data on its metabolic kinetics (rate of production).

Anderson et al., (2011) analyzed DEHP pharmacokinetics in urine. For this analysis two scenario were considered: one at high dose of 2.8 mg D4-DEHP and second at low dose of 0.31mg D4-DEHP. This pharmacokinetics study included 20 volunteers (10 males and 10 females) of following characteristics aged greater than 18 years, BMI between 19 and 32kg/m^2 and body weight greater than 60 kg.. The cumulative amount of DEHP metabolites concentration in urine were reported as a percentage of mole dosing were. The cumulative DEHP metabolites urine data were used for evaluation of the developed model keeping all the models parameters same except subject body characteristics such as BW and BMI.

2.5. Sensitivity analysis

A Local sensitivity analysis was carried out for the PBPK model. The R package FME was used, which measures the alteration in model output for variable of interest by changing each parameter by 1 percentage up and down whilst keeping other ones constant. Detailed information about the functions of FME can be found in Soetaert and Petzoldt, (2010).

$$Si,j = \frac{\partial yj}{\partial pi} * \frac{V_{pi}}{V_{yj}}$$

Where,

Si,j is the sensitivity of parameter i for model variable j and is normalized and dimensionless. yj is a model output variable (DEHP Metabolites time-plasma concentration profile) , pi is parameters involved in PBPK model, V_{pi} is the scaling of parameters pi and V_{yj} is the scaling of variable yj.

These sensitivity functions collapsed into a summary of sensitivity values and it includes L1 norm, L2 norm, Mean, Min and Max. The magnitude of the time-averaged sensitivity values were used to rank the parameters.

Where $L1 = \sum \frac{|Sij|}{n}$ and $L2 = \sqrt{\sum \frac{(S_{ij}^2)}{n}}$

2.6. Parameter and its distribution

Human physiological data, *in vitro* data and QSAR estimates were used for the parameterization of the model. Only Pharmacokinetic specific parameters such as partition coefficients, metabolisms and elimination rate constant are selected for uncertainty analysis. Prior mean parameter values were obtained from in-silico, in-vitro and in-vivo experiments reported in the literature. The model parameters value are provided in Table 1.The model parameters are distributed log normally in the range of ±1 to ±1.5 standard deviations accounting uncertainty on model predictions. Monte Carlo simulations were performed to estimate the uncertainty proceeded by sampling one random value (out of its assigned distribution) for each selected parameter. The model was then run and its outputs (predictions) recorded. Those two steps were iterated 20000 times, and the collected output values formed a random sample, for with we computed the mean, the SD, and any percentile of interest.

Table 1. DEHP parameter values and statistical distributions

Parameters	Symbols	Units	Values or distributions	References
Molecular weight (DEHP)	*MW*	g/mole	391	-
Molecular weight (D4-MEHP)	*MW*	g/mole	281	Anderson et al., (2011)
Molecular weight (MEHP-OH)	*MW*	g/mole	297	Anderson et al., (2011)
Molecular weight (D4-5-oxo MEHP)	*MW*	g/mole	295	Anderson et al., (2011)
Molecular weight (D4-5-cx MEPP)	*MW*	g/mole	311	Anderson et al., (2011)
Octanol:water partition coefficient	*LogKo:w*	-	7.60 [a]	-
Partition coefficients				
Gut/Plasma	k_gut_plasma		*LN* (12.86, 1.1) [b]	-
Liver /Plasma	k_liver_plasma	-	*LN* (10.16, 1.1) [b]	-
Gonads/Plasma	k_gonads_plasma	-	*LN* (6.5, 1.1) [b]	-
Fat/Plasma	k_fat_plasma	-	*LN* (188, 1.1) [b]	-
Rest of the body/Plasma	k_restbody_plasma	-	*LN* (6.24, 1.1) [b*]	-

Liver/ Plasma	k_liver_plasmaM1	-	*LN* (1.7, 1.1)	(Keys et al., 2000)
Gonads/Plasma	k_gonads_plasmaM1	-	*LN* (0.6, 1.1)	(Keys et al., 2000)
Fat/Plasma	k_fat_plasmaM1	-	*LN* (0.12, 1.1)	(Keys et al., 2000)
Rest of the body/Plasma	k_restbody_plasmaM1	-	*LN* (0.38, 1.1)	Set to slow perfused organ (muscle) (Keys et al., 1999)
Uptake rate of 5-OHMEHP to blood	K_{tM2}	1/h	*LN* (.07, 1.5)	Optimzed against data of koch et al.,(2003, 2005)
Uptake rate of 5-oxo MEHP to the blood	K_{tM4}	1/h	*LN* (0.08, 1.5)	Optimized against data koch et al.,(2003, 2005)
Absorption and elimination parameters				
Unbound fraction in plasma for MEHP	fup	-	0.007	(Adachi et al., 2015)
Oral absorption rate	kgut	1/h	*LN* (7, 1.5)	(Adachi et al., 2015)
Elimination rate constant (M1)	kurineM1	1/h	*LN* (0.35, 1.1) [c]	Calculated
Elimination rate constant (M2)	kurineM2	1/h	*LN* (0.69, 1.1) [c]	Calculated
Elimination rate constant (M3)	kurineM3	1/h	*LN* (0.69, 1.1) [c]	Calculated
Elimination rate constant (M4)	kurineM4	1/h	*LN* (3.47, 1.1) [c]	Calculated
Metabolic parameters for DEHP and its metabolites in gut				
DEHP to MEHP in intestinal MSP maximum reaction value	vmaxgutM1	µg/min/mg MSP	LN (0.11,1.1)[d]	(Choi et al., 2013)
Conc. at half maximum value	kmgutM1	µg/L	6956	(Choi et al., 2013)
DEHP to MEHP in gut cytosol maximum reaction value	vmaxgutM1cyt_invitro	µg/min/mg cytosol	LN (0.312, 1.1) [d]	(Choi et al., 2013)
Conc. at half maximum value	kmgut_cytM1	µg/L	7038	(Choi et al., 2013)

MEHP to 5-OH MEHP maximum reaction value	vmaxgutM2_invitro	µg/min/mg MSP	LN (0.0012, 1.1) [d]	(Choi et al., 2013)
Conc. at half maximum value	kmgutM2	µg/L	22508	(Choi et al., 2013)
MEHP to 5-carboxy MEPP maximum reaction value	vmaxgutM3_invitro	µg/min/mg MSP	0	(Choi et al., 2013)
Conc. at half maximum value	kmgutM3	µg/L	0	(Choi et al., 2013)
MEHP-OH to 5-oxo MEHP maximum reaction value	vmaxgutM4_invitro	µg/min/mg MSP	LN (0.0012, 1.5) [d]	(Choi et al., 2013)
Conc. at half maximum value	kmgutM4	µg/L	219076	(Choi et al., 2013)
MEHP to phthalic acid maximum reaction value	vmaxgutM5_invitro	µg/min/mg MSP	LN (0.285, 1.1) [d]	(Choi et al., 2013)
Conc. at half maximum value	kmgutM5	µg/L	187652	(Choi et al., 2013)
Metabolic parameters for DEHP and its metabolites in liver				
DEHP to MEHP in liver MSP maximum reaction value	vmaxlivM1	µg/min/mg MSP	LN (0.112, 1.1) [d]	(Choi et al., 2013)
Conc. at half maximum value	kmlivM1	µg/L	11847.3	(Choi et al., 2013)
DEHP to MEHP in liver cytosol maximum reaction value	vmaxlivM1cyt_invitro	µg/min/mg cytosol	LN (0.036, 1.1) [d]	(Choi et al., 2013)
Conc. at half maximum value	kmliv_cytM1	µg/L	2228.7	(Choi et al., 2013)
MEHP to 5-OH MEHP maximum reaction value	vmaxlivM2_invitro	µg/min/mg MSP	LN (0.172, 1.1) [d]	(Choi et al., 2013)
Conc. at half maximum value	kmlivM2	µg/L	7980.4	(Choi et al., 2013)
MEHP to 5-carboxy MEPP maximum reaction value	vmaxlivM3_invitro	µg/min/mg MSP	LN (0.0023, 1.5) [d]	(Choi et al., 2013)
Conc. at half maximum value	kmlivM3	µg/L	1124	(Choi et al., 2013)

MEHP-OH to 5-oxo MEHP maximum reaction value	vmaxlivM4_invitro	µg/min/mg MSP	LN (0.003, 1.1) [d]	(Choi et al., 2013)
Conc. at half maximum value	kmlivM4	µg/L	23,117.7	(Choi et al., 2013)
MEHP to phthalic acid maximum reaction value	vmaxlivM5_invitro	µg/min/mg MSP	LN (0.088, 1.1) [d]	(Choi et al., 2013)
Conc. at half maximum value	kmlivM5	µg/L	141315	(Choi et al., 2013)

a = value taken form PubChem
b = partition coefficient calculated based on tissue composition method using (Poulin and Krishnan, 1996, 1995; Poulin and Theil, 2000)
c = value is first estimated applying following relationship i.e. elimination rate constant = 0.693/$t_{1/2}$
d = parameters value needs to scale to whole body weight prior to use in model

3. Results and Discussions

In this study, parameters such as partition coefficient, biochemical (metabolism), absorption, elimination as an input and target variables such as DEHP metabolites concentration as a model output, were considered to conduct sensitivity analysis and uncertainty analysis. The bottom up approach was used for the development of the PBPK model and all parameters were derived from in-silico (QSAR), *in vitro* (metabolism) and published literature. The results are described and discussed in the following subsection

3.1. Sensitivity analysis results

The local sensitivity analysis was carried out for all the kinetic parameters that were used in the development of PBPK model. The human physiological parameters were not included for the Monte Carlo and the sensitivity analysis assuming their inherent variability. The sensitivity coefficient of parameters were estimated using R FME package (Soetaert and Petzoldt, 2010) (described in section 2.5) that uses the initial parameter value with allowable relative change in parameters one by one. The results are provided in Table 2. It includes L1 and L2 norm, mean, minimum, maximum, and ranking. The table summarizes the statistics of the normalized and dimensionless parameter sensitivity results. The parameters were ranked based on L1 value and a parameter with higher value signifies their higher sensitiveness towards the model output. The biochemical parameters such Vmax and Km value have very close sensitivity coefficient. The mean sensitivity coefficient of Vmax shows its negative effect and the Km has positive effect on the model output. , Hence in uncertainty analysis, only Vmax has subjected to statistical distribution not Km as sensitivity results shows that they are highly correlated with each other. The VmaxliverM2 (metabolism of MEHP to MEHP-OH) is the most sensitive parameter (Rank 1) following partition coefficient of liver:plasma (Rank 3). The partition coefficient for the rest of the body and the metabolism of DEHP in the cytosol fraction of both gut and liver are under the rank of 10, considering relatively more sensitive than other parameters. The plots for

sensitive analysis output i.e. mean sensitivity coefficient are provided in Fig. A.1 (Annex-A). The summary statistics tables of parameters' sensitivities for the output of DEHP metabolites concentration in plasma is provided in Table. A.9- A.12 (Annex-A).

Table 2. Summary statistics of parameters' sensitivities

Parameters	L1	L2	Mean	Min	Max	Rank
vmaxliverM2	0.61	0.01	-0.45	-3.40	1.00	1
kmliverM2	0.60	0.01	0.44	-1.00	3.39	2
k_liver_plasma	0.57	0.01	-0.57	-2.08	0.00	3
vmaxliverM4	0.43	0.01	-0.36	-3.63	0.99	4
kmliverM4	0.38	0.01	0.32	-0.99	3.39	5
k_restbody_plasma	0.32	0.01	0.27	-0.92	3.85	6
vmaxgut_cytM1	0.30	0.00	-0.29	-8.86	0.54	7
k_liver_plasmaM1	0.29	0.00	-0.14	-1.00	0.40	8
vmaxliver_cytM1	0.21	0.00	-0.21	-3.09	0.12	9
kmliver_cytM1	0.20	0.00	0.20	-0.12	3.04	10
vmaxliverM3	0.19	0.00	0.08	-0.32	1.00	11
kmliverM3	0.18	0.00	-0.07	-1.00	0.32	12
kurineM3	0.17	0.00	-0.15	-2.79	1.00	13
ktM2	0.17	0.00	0.05	-0.67	1.00	14
ktM4	0.15	0.00	0.15	0.00	1.00	15
kmgut_cytM1	0.15	0.00	0.15	-0.30	6.45	16
kurineM2	0.15	0.00	-0.13	-2.20	1.00	17
kurineM1	0.13	0.00	-0.03	-0.47	1.00	18
vmaxgutM1	0.12	0.00	-0.12	-3.57	0.22	19
kurineM4	0.10	0.00	-0.09	-1.13	0.98	20
k_restbody_plasmaM1	0.09	0.00	-0.08	-0.71	0.20	21
vmaxliverM1	0.08	0.00	-0.08	-1.18	0.05	22
kmliverM1	0.08	0.00	0.08	-0.05	1.17	23
kmgutM1	0.06	0.00	0.06	-0.12	2.59	24
k_gut_plasma	0.05	0.00	0.05	0.00	0.37	25
k_gonads_plasma	0.04	0.00	0.04	-0.04	1.59	26
vmaxgutM2	0.03	0.00	0.03	-0.05	1.00	27
kmgutM2	0.03	0.00	-0.03	-1.00	0.00	28
vplasmad	0.03	0.00	-0.03	-1.00	0.00	29
kmliverM5	0.02	0.00	0.02	-0.06	0.10	30
vmaxliverM5	0.02	0.00	-0.02	-0.10	0.03	31
k_fat_plasmaM1	0.02	0.00	0.00	-0.10	0.74	32
k_fat_plasma	0.01	0.00	-0.01	-0.23	0.08	33
k_gonads_plasmaM1	0.01	0.00	0.01	-0.02	0.66	34
vmaxgutM5	0.00	0.00	0.00	-0.03	0.03	35
kmgutM5	0.00	0.00	0.00	-0.01	0.03	36
vmaxgutM4	0.00	0.00	0.00	0.00	0.01	37
kmgutM4	0.00	0.00	0.00	-0.01	0.00	38

Table 2: Sensitivity results for both the rat and human PBPK model. It includes L1 and L2 norm, mean, minimum, maximum, and ranking. Ranking of parameter sensitivity coefficient was done based on L1 norm.

3.2. PBPK model calibration results and its evaluation with independent data

The time course of DEHP metabolites concentration in plasma and cumulative amount in urine were predicted at median, 2.5 and 97.5 percentiles and 20 random predictions. PBPK model has accounted the parameter statistical distribution followed by sampling one random value (out of its assigned distribution) and performing Monte Carlo simulation reflecting uncertainty in the model. The model does not include any variability factor related to physiological parameters. For the metabolic uncertainties only Vmax values were statistically distributed but not Km considering that they are highly correlated. Single oral dose of 48.5mg DEHP as an input and the observed concentration of metabolites both in the blood and urine as an output were used to calibrate the model. Most of the parameters were derived via either from in-silico (estimation of the partition coefficient) (Poulin and Krishnan, 1996, 1995; Poulin and Theil, 2000) or from *in vitro* such as, partition coefficient determined (Keys et al., 2000) and *in vitro* metabolic data (human hepatocyte and intestinal cell line) (Choi et al., 2013). The parameters such as elimination rate constants for the metabolites are derived using mathematical relationship described in models and methods section. The absorption rates of metabolites (mass transfer) from the gut to the liver were set as one (complete mass transfer) except MEHP whose absorption rate constant was derived from the literature (Adachi et al., 2015). . The mass transfer rate of metabolites from the liver to the blood was calibrated against the observed data (Koch et al., 2005). The model was developed using the parameters derived from in-silico, *in vitro* data, and previously published literature, and certain default parameter values, which needed to be calibrate. Instead of optimizing all the parameters very specifically to get a point to point prediction against the observed data rather we statistically distributed all the parameters in a range of 1-1.5 ± SD (standard deviation) providing range of predictions. Then the model was verified against the blood and urine metabolites concentration data reported by Koch et al., (2005), so that observed data for all metabolites fall within the range (2.5^{th} -97.5^{th}) of model predictions. The predictions of the DEHP metabolites concentration in blood and urine included their metabolic kinetics both in the gut and the liver described by Michaelis Menten equation. And the parameters such as Vmax and Km were estimated *in vitro* by Choi et al., (2013) were scaled to the whole body (based on organ weight) and integrated into the model.

Fig. 3 (a-d) represents the PBPK model predictions for plasma concentrations of four DEHP metabolites. It can be observed that the model predictions agree quite closely to the observed data. The cumulative excretion of DEHP metabolites is also adequately predicted by the model represented in Fig. 4 (a-d) and Table 2. The recently reported *in vitro* metabolism data shows that the production rate of MEHP from the DEHP is very

high (Choi et al., 2013). A similar trend of the kinetic profile was also reported by Koch et al., (2005) where he observed very low or undetectable DEHP blood concentration. Given the above facts, the clearance of DEHP is presumed to be completely depends on its metabolic conversion to MEHP. The Fig. 3 (a) shows that predicted Cmax (highest chemical plasma concentration) of the MEHP is slightly lower than the observed data even at 97.5 percentile simulation. However, the time course trend of chemical concentrations in plasma is similar to the observed data points. In addition to that, post Cmax, the predictability of the model are in close agreement with the observed points. The clearance of MEHP from the body includes both its metabolism and the urinary elimination.

Fig. 3 (b) represents the model predictions for MEHP-OH concentrations in blood at 2.5, 50 (median) and 97.5th percentiles including 20 random simulations, and the observed data in green dots. The blood Cmax value for 5-OH MEHP is lower than MEHP and 5-Cx MEPP and more than its metabolite 5-oxo MEHP. The observed data points at the terminal elimination are predicted at the lower boundary of the model, where almost all chemicals are eliminated. All the observed blood data points are within the range of the model prediction (2.5, 50 and 97.5th percentiles). The observed production rate of 5-OH MEHP in gut and liver i.e. *in vitro* metabolism data (Vmax) is higher than the other metabolites (Choi et al., 2013). However, reported blood concentration by Koch et al., (2005) is less than 5-Cx MEPP, another metabolite. The reason for its lower blood plasma concentration is might be due to its higher volume of distribution than the other metabolites, the similar observation was noted previously by Lorber et al., (2010) during the calibration of the model. The other reasons might be its higher clearance to the urine and its further metabolism to 5-oxo MEHP. The production of 5-OH MEHP depends on the MEHP concentration in both the liver and the gut, and then its distribution to the blood. The transfer of 5-OH MEHP from the liver to blood was done using first order rate constant and is calibrated against the observed data. 5-OH MEHP clearance was done based on both its metabolism to the 5-oxo MEHP and the urinary elimination. The urinary elimination was described using first order using first order rate constant.

Similarly, PBPK model predictions for 5-cx MEPP plasma concentrations shown in Fig. 3 (c), which is the metabolite of MEHP, appears to be in close agreement with observed data points. The volume of distribution (V_d) was confined to the plasma compartment volume since the distribution of the compound is unknown. The production of 5-cx MEPP metabolite from the MEHP in the gut was reported to be null in the *in vitro* experiment (Choi et al., 2013). So, the concentration of 5-oxo MEPP only depends on its production in the liver from the MEHP. Its clearance was described using first order rate constant from the blood to urine.

The model predictions for 5-oxo MEHP plasma concentrations shown in Fig. 3(d), results from metabolism of 5-OH MEHP in both gut and liver, are in close agreements with the observed concentrations. All the observed data points are in compliance with the predicted range of percentile. Its production in gut and liver from the 5-OH MEHP

is described using Michaelis Menten reaction. Its volume of distribution is confined to single compartment of plasma volume. The urinary elimination was described using first order elimination rate from the systemic circulation.

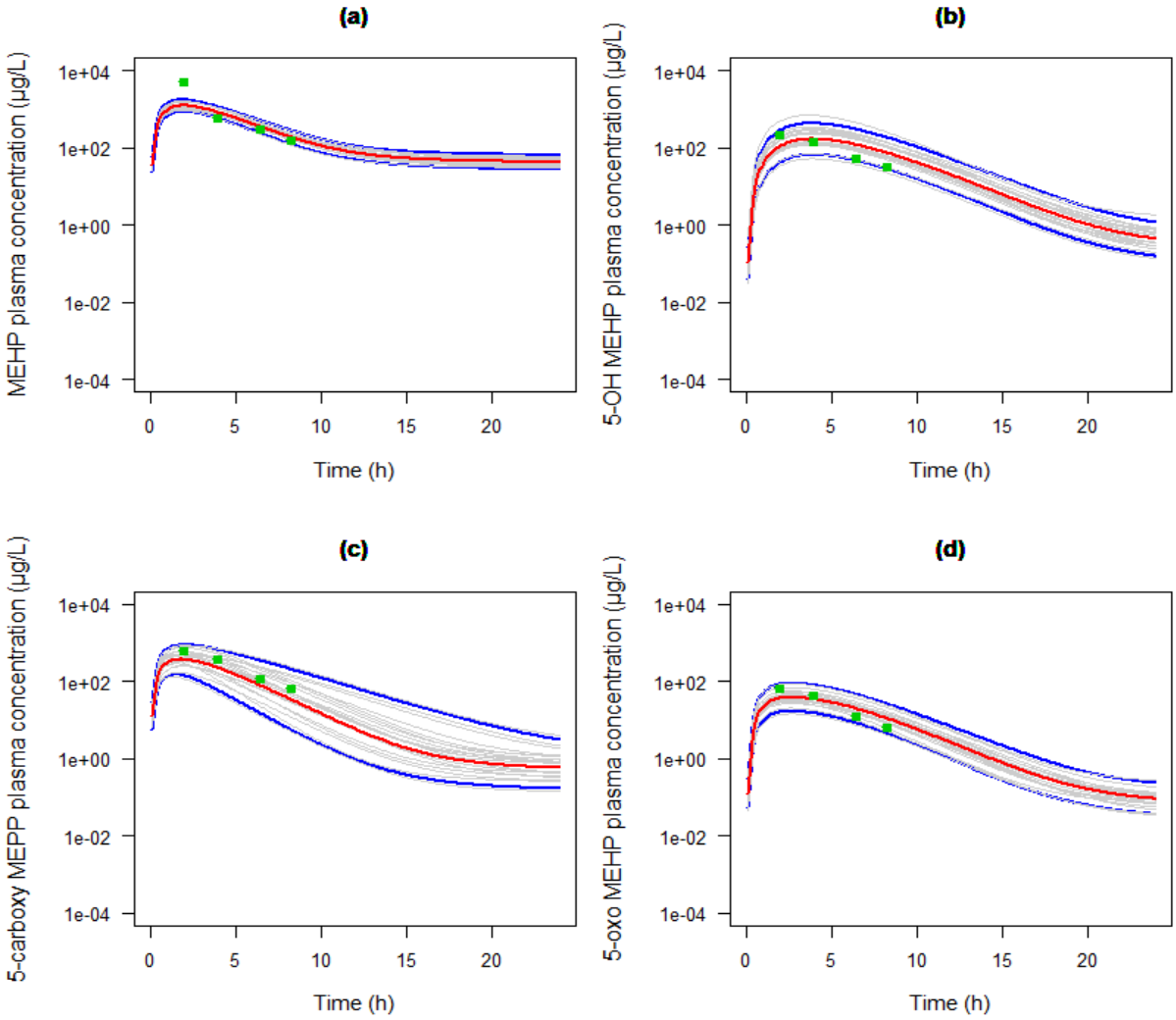

Fig. 3. PBPK model predictions of DEHP metabolites plasma concentration following 48.5 mg oral dose in human. Red lines: median predictions; blue lines: 2.5 and 97.5 percentiles; gray lines: 20 random simulations. (a) Represents MEHP plasma concentration. (b) Represents 5-hyroxy MEHP plasma concentration. (c) Represents 5-carboxy MEPP plasma concentration. (d) Represents 5-oxo MEHP plasma concentration. The green dotes indicate the observed concentrations reported in (Lorber et al., 2010). Dose unit is converted to microgram prior to use as an input for the model.

The four metabolites' blood concentrations are not only in close agreement with observed data points but also captured the time course profile. The Fig. 4 (a-d), presented PBPK prediction of the cumulative amount (µg) urinary excretion of four metabolites for 44hr at median, 2.5 and 97.5 percentiles and for 20 random simulations. The simulated urinary amount of DEHP metabolites (cumulative amount) are also in compliance with the experimentally observed cumulative amount (Koch et al., 2005), results are provided in Table 2. It also summarizes the predicted vs observed metabolites elimination as a percent of applied dose in mole for three dosing scenarios based on Koch et al., (2005) study. The observed metabolites as a percentage of mole doses are within the range of predictions of the model not only for high dose (use for calibration) but also for other two independent dosing scenarios such as medium (2.15 mg) and low dose (0.35 mg).

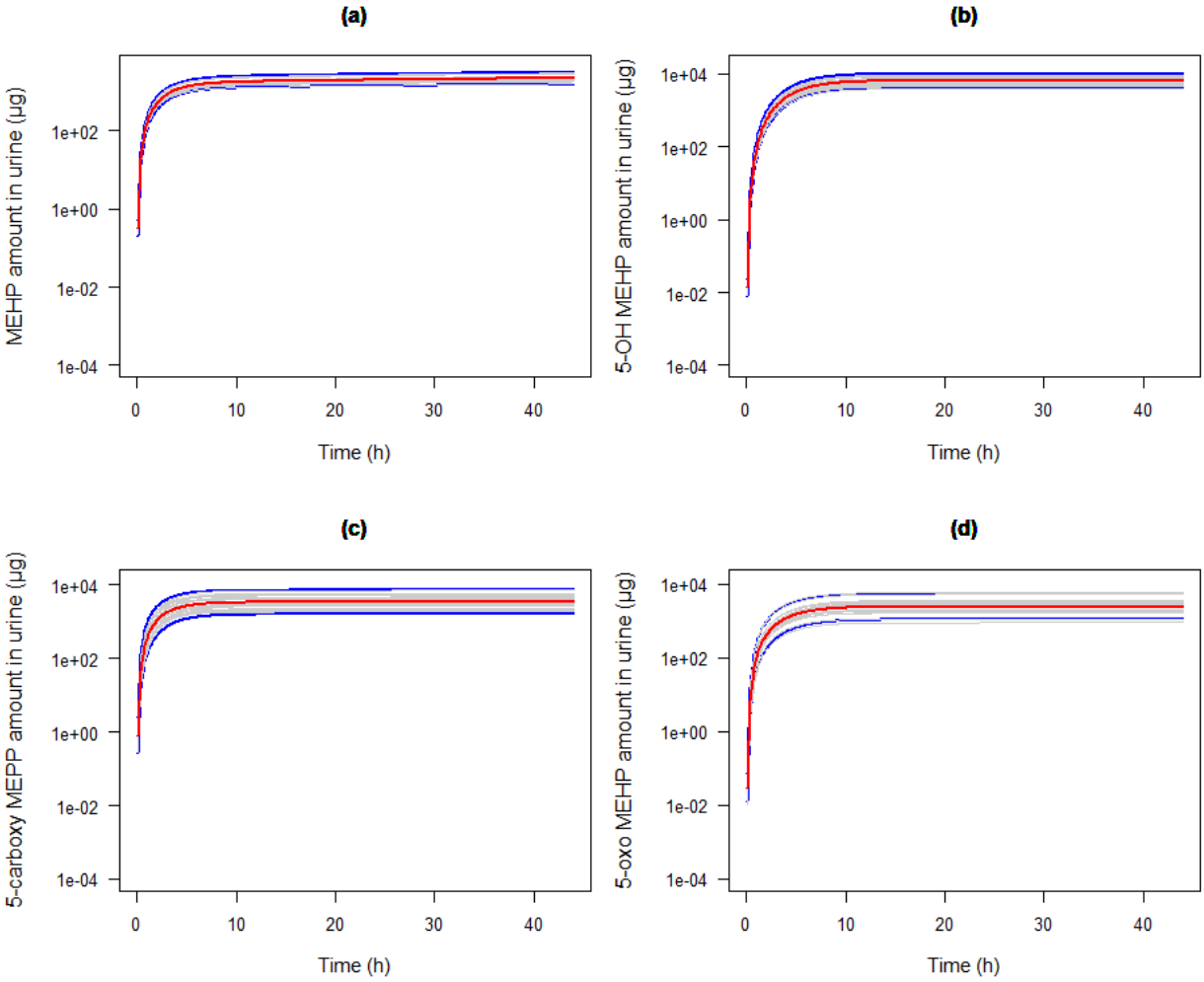

Fig. 4. PBPK model predictions of DEHP metabolites amount in urine following 48.5 mg oral dose. Red lines: median predictions; blue lines: 2.5 and 97.5 percentiles; gray lines: 20 random simulations. (a) Represents MEHP cumulative amount (µg) in urine. (b) Represents 5-hyroxy MEHP cumulative amount (µg) in urine. (c) Represents 5-carboxy MEPP cumulative amount (µg) in urine. (d) Represents 5-oxo MEHP cumulative amount (µg) in urine. Dose unit is converted to microgram prior to use as an input for the PBPK model.

Table 3. Observed and PBPK predicted amount of DEHP (µg) metabolites in urine

Cumulative amount of Metabolites (µg) of the D4-DEHP in urine						
Study involved	**Dose**	**MEHP**	**5OH-MEHP**	**5cx-MEPP**	**5oxo-MEHP**	**Total dose in µg or percent**
Koch et al., (2005) [a]	48,500µg	2500	9000	7500	5000	23500 µg
Present study 2.5th -97.5th (median)	48,500µg	1548.2-3122.7 (2230.5)	3988.6- 10148 (6511)	1585.4- 7086 (3397)	1087- 5497 (2432)	8209.2-25853.7 (14570.5) µg
Metabolites of the D4-DEHP Dose as percent of applied dose (mol)						
Koch et al., (2005)	48,500µg	7.3	24.1	20.7	14.6	66.7 %
Present study 2.5th -97.5th (median)	48,500µg	4.4-8.9 (6.4)	10.8-27.5 (17.6)	4.1-18.3 (8.8)	3.0-15.0 (6.6)	22.3-69.7 (39.44) %
Koch et al., (2005)	2,150 µg	4.3	22.7	19.4	13.0	59.4 %
Present study 2.5th -97.5th (median)	2,150 µg	4.3-8.7 (6.2)	8.9-23.3 (14.6)	4.3-19.0 (9.2)	3.02-15.3 (6.7)	20.52-66.3 (36.7) %
Koch et al., (2005)	350 µg	6.2	23.1	15.5	17.3	62.1 %
Present study 2.5th -97.5th (median)	350 µg	4.3-8.7 (6.2)	8.8-23.2 (14.5)	4.3-19.0 (9.2)	3.1-15.3 (6.8)	20.5-66.2 (36.7) %

a = values are extracted from the graph presented in manuscript by Koch et al., (2005)
Dose unit is converted to microgram prior to use as an input for the PBPK model.

Given that the model predictions fit the DEHP metabolites namely MEHP and other metabolites 5-OH MEHP, 5-cx MEPP and 5-oxo MEHP concentration in the blood and urine upon 48.5 mg of single oral dose of DEHP. The structure of the model and the model parameters remained unchanged from their calibrated values, and the predicted percentage mole elimination data for four metabolites in urine were compared with the data reported in Anderson et al., (2011) for the evaluation of model credibility. The study included 20 subjects, 10 male, and 10 female, and their overall mean body weight was 74.8 kg. The only additional change in the model is subject body weight. The present model does not include gender variability among 20 subjects, and the mean body weight was taken as an input for model simulation, as current model only accounted the parametric uncertainty, not the variability. Two dosing scenarios namely high dose; a single oral dose of 2.8 mg DEHP and low dose; a single oral dose of 0.31 mg was used for the model simulations. The subject characteristic and dosing for respective studies are provided in Table A. (1-3). The predicted urinary data were converted into moles based on their molecular weight in order to standardize the exposure unit data. Then the relation; ((predicted amounts of metabolites in urine (moles)/amounts dose (moles)) *100), is used to calculate the percentage molar eliminations on moles basis (Anderson et al., 2011; Koch et al., 2005). The detailed summarized tables are provided in Table A.5 to A.7. The PBPK predicted a range of metabolites elimination as a percentage of doses in mole reflecting the uncertainty in the

554 model. The model output was compared with the observed experimental data. Table 3
555 summarizes the predicted vs observed percentage amount elimination of metabolites.
556 The experimentally observed cumulative amount of all metabolites is well within the
557 range of PBPK simulation.

Table 4. Fraction excretion value (mole percentage) for observed and PBPK predicted of DEHP metabolites

		Metabolites of the D4-DEHP Dose (% mol elimination)				
Study involved	**Dose**	**MEHP**	**5OH-MEHP**	**5cx-MEPP**	**5oxo-MEHP**	**Total molar elimination (%)**
Anderson et al., (2011)	310µg	6.94	16.33	15.90	12.53	51.70
Present study 2.5th -97.5th (median)	310µg	4.3-8.7 (6.3)	8.8-22.9 (14.6)	4.3-18.5 (9.2)	3.0-15.2 (6.8)	20.4 -65.2 (36.9)
Anderson et al., (2011)	2800µg	5.67	14.86	11.97	10.00	42.51
Present study 2.5th -97.5th (median)	2800µg	4.4-8.7 (6.3)	9.0-23.2 (14.8)	4.3-18.9 (9.2)	3.0-15.3 (6.8)	20.7-66.1 (37.1)

558

559 ## 4. Conclusions and Future work

560 The results showed that the current developed model can able to predict the plasma and
561 the cumulative urine concentration of the DEHP metabolites for the different exposure
562 scenario. The current model included four metabolites and the generation of metabolites
563 are mechanistically described using integrated physiological parameters and Michaelis-
564 Menten (M-M) parameters such as Vmax and Km derived from a human
565 hepatic/intestine cell line. The sensitive analysis was done for all the parameters and the
566 metabolic parameters found to be more sensitive than the other parameters. Monte Carlo
567 simulation was used accounting probabilistic information about pharmacokinetics
568 parameters that estimated DEHP metabolites concentration in both the plasma and the
569 urine at three percentile considering the uncertainty into the model. Some of the major
570 strength of current predictive model over previously developed models for DEHP are:
571 1) it's a detail PBPK model that integrates the *in vitro* metabolism data with the
572 application of IVIVE to predict metabolites concentrations, instead of calibrating or
573 empirically fitting over observed data, 2) production of metabolites is described using
574 saturation kinetics (M-M equations) retaining its biological plausibility, 3) model can be
575 individualized (personalized) for different populations by understanding the
576 physiological variability, 4) it can be used to predict the target tissue internal
577 concentrations for further toxicodynamics study and human health risk assessments.
578 The current developed model did not account the 2-cx MEPP metabolite due to lack of
579 *in vitro* metabolic data, considered to be another important metabolite for the
580 biomonitoring study. The current PBPK model can be further extended for 2-cx MEPP,
581 once the metabolic data are available. Detailed rat's pharmacokinetic studies that
582 include all metabolites could be very useful for further understanding metabolites tissue

distribution. The current developed model can be applied in the biomonitoring and exposome studies for the human health risk assessment (Martínez et al., 2017, 2018). The developed model can be further extended for the development of an integrated PBPK/PD systems toxicology model (integrative systems toxicology) to establish the exposure-internal dose- response relationship (Sharma et al., 2017b).

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