Review Article

Non-Animal Approaches for Toxicokinetics in Risk Evaluations of Food Chemicals

Ans Punt [1], Ad A. C. M. Peijnenburg [1], Ron L. A. P. Hoogenboom [1] and Hans Bouwmeester [1,2]

[1]RIKILT Wageningen University and Research, Wageningen, The Netherlands; [2]Division of Toxicology, Wageningen UR, Wageningen, The Netherlands

Summary

The objective of the present work was to review the availability and predictive value of non-animal toxicokinetic approaches and to evaluate their current use in European risk evaluations of food contaminants, additives and food contact materials, as well as pesticides and medicines. Results revealed little use of quantitative animal or human kinetic data in risk evaluations of food chemicals, compared with pesticides and medicines. Risk evaluations of medicines provided sufficient *in vivo* kinetic data from different species to evaluate the predictive value of animal kinetic data for humans. These data showed a relatively poor correlation between the *in vivo* bioavailability in rats and dogs *versus* that in humans. In contrast, *in vitro* (human) kinetic data have been demonstrated to provide adequate predictions of the fate of compounds in humans, using appropriate *in vitro-in vivo* scalers and by integration of *in vitro* kinetic data with *in silico* kinetic modelling. Even though *in vitro* kinetic data were found to be occasionally included within risk evaluations of food chemicals, particularly results from Caco-2 absorption experiments and *in vitro* data on gut-microbial conversions, only minor use of *in vitro* methods for metabolism and quantitative *in vitro-in vivo* extrapolation methods was identified. Yet, such quantitative predictions are essential in the development of alternatives to animal testing as well as to increase human relevance of toxicological risk evaluations. Future research should aim at further improving and validating quantitative alternative methods for kinetics, thereby increasing regulatory acceptance of non-animal kinetic data.

Keywords: *in vitro* kinetics, alternatives to animal testing, PBPK, regulatory acceptance

1 Introduction

Toxicokinetics deals with the absorption, distribution, metabolism and excretion (ADME) of compounds in an organism. Within regulatory risk evaluations, kinetic data provide valuable insights into bioavailability, bioaccumulation potential and the formation of metabolites *inter alia*. Information on the fate of compounds allows us to better understand the toxicity and intra- and interspecies differences in toxicity of a chemical (Bessems et al., 2014; OECD, 2010). Thus, kinetic data are crucial for increasing human relevance of toxicological risk evaluations, allowing replacement of the default uncertainty factors with so-called chemical specific adjustment factors (EFSA, 2012a; Meek and Lipscomb, 2015; WHO, 2005). At present, the default factors comprise a factor of 4 for interspecies differences in toxicokinetics and a factor of 3.16 for potential differences between different humans (WHO, 2005). In addition, there is an increasing scientific interest in the use of kinetic data in the development of alternatives to animal testing, where these data provide an effective way of translating *in vitro* effect concentrations to equivalent human oral doses (Bessems et al., 2014; Coecke et al., 2013; Louisse et al., 2017; Rietjens et al., 2011; Wilk-Zasadna et al., 2015; Yoon et al., 2012).

Currently, toxicokinetic data are described within regulatory risk evaluation reports but are only used for the final assessment to a minor extent. When described, kinetic data are most often obtained from *in vivo* rodent studies measuring plasma or tissue concentrations of a chemical or its relevant metabolites over time (e.g., C_{max}, maximum concentrations in plasma; AUC, area under the concentration-time curve) (OECD, 2010). It is important to realize, however, that humans differ from animals, especially with regard to the expression of different isoforms of metabolizing enzymes and transport proteins. *In vivo* animal data are therefore increasingly criticized as deficient predictors

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of the fate and effects of chemicals in humans (Cao et al., 2006; Musther et al., 2014).

A wealth of *in vitro* approaches capturing kinetic processes in an organism has been developed. Of these assays, only the *in vitro* skin absorption test has been validated and incorporated in an OECD Test Guideline (TG 428) (OECD, 2004). Other examples of *in vitro* methods for kinetics include measurements of i) intestinal absorption and transport using intestinal epithelial cells, ii) metabolic conversion by cells or tissue fractions from different organs including liver, and iii) protein binding assays. Each of these assays captures different aspects of the biological fate of a chemical in an organism. To further improve and integrate these *in vitro* approaches, recent research efforts have focussed on developing organ-on-a-chip models, in which cells or co-cultures of cells are grown in microfluidic devices in continuously perfused chambers in order to model physiological functions of tissues and organs (Jiang et al., 2016). Other important developments in recent years have been the integration of *in vitro* kinetic data with *in silico* physiologically based pharmacokinetic (PBPK) models, with the aim to simulate the kinetics of chemicals in organisms (Bessems et al., 2014; Bois et al., 2010; Yoon et al., 2012), and the development of quantitative structure activity relationships (QSARs) to predict kinetic data based on chemical structures and their physicochemical characteristics as part of read-across approaches (Peyret and Krishnan, 2011; Peyret et al., 2010; Rodgers and Rowland, 2006).

To increase the use of alternative kinetic approaches in toxicological risk evaluations, it is crucial to identify their opportunities and understand their limitations. Various papers have reviewed available (alternative) approaches for determining kinetic parameters (Lefebvre et al., 2015; Sousa et al., 2008; Wilk-Zasadna et al., 2015). However, so far, no comparisons have been made with respect to their use in different domains of risk evaluations to define possible issues that hamper their application as alternatives to animal testing.

The objective of the present study is to review the availability of non-animal kinetic approaches and to evaluate their predictive value and current use in regulatory risk evaluations. We decided to put special emphasis on food contaminants, food additives, and food contact materials, as for many of these, particularly food contaminants, little experimental animal data is available (Alexander et al., 2012). This indicates the importance of exploiting non-animal approaches in the risk evaluations for this group of compounds. Within the context of this paper we group food contaminants, food additives, and food contact materials as "food chemicals". EFSA opinions on these types of compounds were screened for the application of *in vivo* and non-animal kinetic methods. In addition, for comparison, the use of *in vivo* and *in vitro* kinetic methods within evaluations of pesticides (EFSA Conclusions on Pesticides) and medicines (EMA Public Assessment Reports) was assessed. Overall, the overview should contribute to the identification of research activities that are needed in the future to improve the applicability of alternative kinetic approaches for regulatory risk evaluations.

2 Survey on the current requirements and use of *in vivo* and *in vitro* kinetic data within regulatory risk evaluations

2.1 Selection of EFSA opinions on food chemicals and regulatory data requirements

We examined the use of *in vitro* and *in vivo* kinetic data within EFSA opinions on food chemicals, as published in the EFSA Journal between January 2014 and June 2016. Opinions that did not contain kinetic data were excluded from the survey. The final survey contained 48 opinions, including 33 additives, 13 contaminants and 2 food contact materials.

There is only limited guidance specifying the actual kinetic data required for the risk evaluation of food chemicals. In case of contaminants the opinions are generally prepared based on data available from the public domain (Alexander et al., 2012). In the EFSA guidance for contact materials (EFSA, 2008) it is indicated that the core set of tests should comprise studies on absorption, distribution, metabolism and excretion. References are made to the IPCS (International Programme on Chemical Safety) Environmental Health Criteria documents (EHC 70 and EHC 57) for the details of such studies. The guidance document on food additives (EFSA, 2012b) provides a tiered approach for toxicokinetic testing. Demonstration of negligible conversion by gastrointestinal fluids or the gut microbiota (*in vitro*) and negligible absorption, together with absence of genotoxicity, is considered to provide a scientific justification for not undertaking higher tiered kinetic and toxicological studies. When absorption of the parent or breakdown product does occur, tier 2 studies should be carried out. This includes *in vivo* toxicokinetic studies (OECD TG 417) that provide basic toxicokinetic parameters ($T_{1/2}$, AUC, bioavailability, C_{max} and T_{max}) on systemic exposure after a single dose of the compound. Tier 3 studies, which define toxicokinetic parameters following repeated administration, are triggered when there is limited or slow excretion or when any other mechanism implies possible bioaccumulation (EFSA, 2012b).

Two of the additives within the survey are nanomaterials (i.e., gold and silver). For these types of compounds, the EFSA "Guidance on the Risk Assessment of the Application of Nanoscience and Nanotechnologies in the Food and Feed Chain" (EFSA, 2011a) applies. Within this guideline, it is indicated that ADME studies are essential for the safety evaluation of nanomaterials, as the nature of nanomaterials can result in altered and specific toxicokinetics and tissue distribution when compared to non-nanoforms.

2.2 Selection of EFSA conclusions on pesticides and background on the data requirements

EFSA conclusions on pesticides published between January 2014 and June 2016 were included in the survey. Evaluations that contained environmental risk evaluations only were omitted. In total, the survey contained 70 EFSA conclusions on pesticide evaluations.

The data requirements for pesticide active substance evaluations are laid down in Regulation (EU) No 83/2013 (EC, 2013).

Key kinetic parameters that need to be obtained from *in vivo* studies are described in Annex 5.1.1. In addition, it states that "*comparative in vitro metabolism studies shall be performed on animal species to be used in pivotal studies and on human material (microsomes or intact cell systems) in order to determine the relevance of the toxicological animal data and to guide in the interpretation of findings and in further definition of the testing strategy.*"

2.3 Selection of EMA public opinions on medicines and background on the data requirements

Risk evaluations of medicines from the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency (EMA) published between January 2014 and June 2016 were included in the survey. Only evaluations on oral medicines were selected and those on generic medicines excluded as no new kinetic data are provided in these evaluations. In total 73 EMA Public Assessment Reports were included.

According to Directive 2003/63/EC, a pharmacokinetic investigation of all pharmacologically active substances is necessary. ADME data needs to be included in the study reports on both non-clinical and clinical studies. It is also stated that *in vitro* studies can be carried out with the advantage of using human material for comparison with animal (EC, 2003). Plasma (or whole blood or serum) AUC, C_{max} and C(time) are the most commonly used parameters in assessing exposure in toxicokinetic studies (EMEA, 1995).

2.4 Exploration of the use of kinetic data within the selected opinions on food chemicals, pesticides and medicines

The contents of the pdf files of the risk evaluations were systematically analysed with Adobe Acrobat XI Pro® using the Boolean query method available within the Advanced Search option. The use of *in vivo* kinetic data (Fig. 1, 2) within the different evaluations was explored by searching for the terms "AUC or C_{max}" as well as a separate search for the terms "absorption" or "bioavailability". Evaluations that contained these terms were further analysed manually to identify whether the kinetic parameters were obtained from rodent species (i.e., mice or rats), non-rodent species (i.e., dogs, rabbits, or monkeys) or humans.

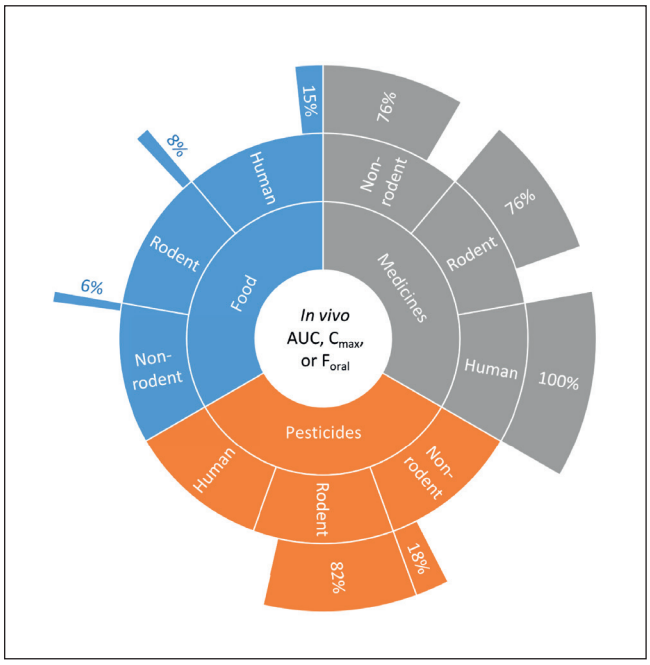

Fig. 1: Percentage evaluations containing AUC, C_{max}, or F_{oral} values derived from *in vivo* human, rodent and/or non-rodent kinetic studies

For pesticides the percentages are derived from the evaluations since January 2016 as pesticide evaluations before 2016 did not include quantitative *in vivo* kinetic data.

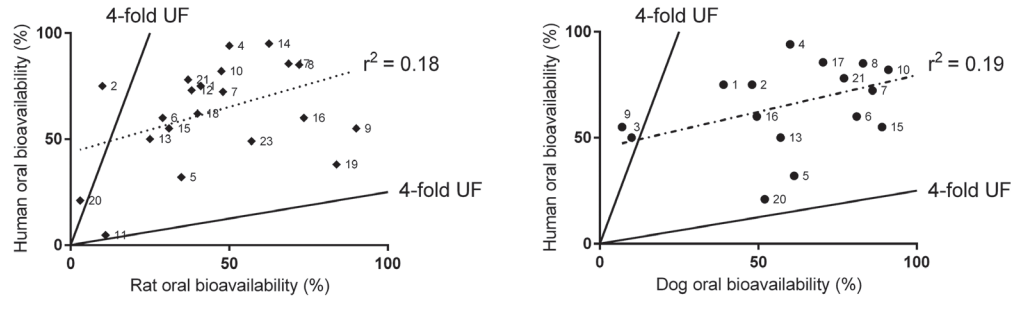

Fig. 2: Predictive value of *in vivo* kinetic data

Oral bioavailability of medicines in (A) rats and (B) dogs versus humans. Bioavailability data were obtained from EMA evaluations. Solid lines correspond to a 4-fold difference between animal and human bioavailability, representing the default uncertainty factor (UF) of 4 for interspecies kinetics differences. The dotted lines represent the fitted linear regression curves.

Tab. 1: Search terms applied to explore the use of different non-animal alternative methods for kinetics within the risk evaluations of food chemicals, pesticides and medicines
FaSSIF, fasted state simulated intestinal fluid; FeSSIF, fed state simulated intestinal fluid; PAMPA, parallel artificial membrane permeabililty assay; PBPK/PBK/PBBK, physiologically based pharmacokinetic/kinetic/biokinetic; QSAR, quantiative structure activity relationship; SAR, structure activity relationship; SGF, simulated gastric fluid; UGT, uridine diphospho-glucuronosyltransferase

Method	Search terms	Additional manual selection
In vitro methods for luminal stability (Fig. 3)	1) digestion or SGF or FaSSIF or FeSSIF 2) microbiota or microflora or flora	
In vitro, *in situ* or *ex vivo* methods for intestinal absorption (Fig. 4A)	1) Caco-2 2) "*in situ*" or perfusion 3) "*ex vivo*" or perfusion 4) PAMPA or "artificial membrane"	Manual check to identify if the *in situ*, *ex vivo* or perfusion methods reported were indeed used to measure intestinal absorption, to exclude other types of *in situ* and *ex vivo* approaches
In vitro methods for intestinal and liver metabolism (Fig. 5A)	1) supersomes or recombinant or UGT 2) S9 or microsomes or cytosol 3) hepatocytes	Manual check to exclude risk evaluations where S9 was added to *in vitro* genotoxicity tests, as well as evaluations mentioning hepatocytes in relation to toxic effects rather than metabolism
In silico methods, QSARs (Fig. 6)	1) QSAR or SAR or (Q)SAR or "OECD Toolbox"	Manual check if the match corresponded to QSAR for kinetic parameters, excluding other QSARs
In silico methods, PBPK (Fig. 6)	1) PBPK or PBK or PBBK or "physiologically based pharmacokinetic"	

Absolute bioavailability results reported within the EMA evaluations (when available) were used to derive Figure 2.

For the use of *in vitro* and *in silico* alternative methods within the risk evaluations, the search terms described in Table 1 were used. A manual check was performed on all search results to exclude non-relevant matches or for further specification of the methods used.

3 *In vivo* methods for kinetics, predictive value for humans, and current use in risk evaluations

In vivo toxicokinetic measurements include measurements of either plasma or tissue concentrations of a compound or its relevant metabolite in relevant species (OECD, 2010). The most commonly used parameters include the maximum concentration (C_{max}) or area under the concentration-time curve (AUC) within an organism and oral bioavailability (F_{oral}). F_{oral} is derived by comparing the relative difference of the AUC after intravenous dosing and oral dosing. It represents the fraction of a compound that enters the systemic blood circulation unchanged following oral administration (Equation 1) (El-Kattan and Varm, 2012). It thus includes both absorption and metabolism in the intestine and/or liver.

$$F_{oral} = AUC_{oral}/AUC_{iv} * (Dose_{iv}/Dose_{oral}) \quad (1)$$

Evaluation of the use of *in vivo* kinetic data within the investigated risk evaluations of food chemicals and pesticides (EFSA) and medicines (EMA) shows distinct differences between food chemicals as compared with pesticides and medicines (Fig. 1). This figure shows that hardly any quantitative toxicokinetic data are presented in the risk evaluations of food chemicals. Toxicokinetic data are evaluated, but not in terms of AUC, C_{max} or percentage bioavailability. In contrast, all evaluations of medicines contained AUC or C_{max} values for humans, together with quantitative data from one or more animal species (Fig. 1), as kinetic data are generally assessed during the required toxicity (and efficacy) studies. In case of pesticides, quantitative kinetic parameters from animal studies are included in all evaluations since 2016 because of the newly introduced data requirement regulation (EC, 2013).

The limited availability of quantitative *in vivo* (animal and human) kinetic data within risk evaluations of food chemicals that were surveyed in the present study hampers evaluation of the relevance of animal data for humans. Nonetheless, the two examples within the survey that contain quantitative data on bioavailability showed striking interspecies differences. Firstly, for bisphenol A, interspecies differences were particularly observed between mice and humans, with mice having 14.7-fold lower plasma levels of bisphenol A compared with humans at a similar oral exposure, suggesting a higher sensitivity of humans. This difference was taken into account in setting the tolerable daily intake (TDI) (EFSA, 2015a). Secondly, in the case of acrylamide, humans were found to have 1.4-2-fold lower blood levels of the reactive metabolite glycidamide, suggesting relatively lower sensitivity of humans (EFSA, 2015b). Nonetheless, the default safety margin of 10,000 for genotoxic carcinogens (covering a factor 4 for species differences in kinet-

ics) was not reduced based on these data (EFSA, 2015b). Apart from the examples derived from the survey, it should also be pointed out that for bioaccumulative compounds, like dioxins and brominated flame retardants, kinetic parameters are taken into account, since body burden is considered to be more relevant than the external dose (EFSA, 2005, 2011b).

For medicines, sufficient data on the bioavailability in rats and dogs were reported in the surveyed risk evaluations to allow comparison with humans. This is shown in Figure 2, which reveals a poor general correlation between either rats and humans ($r^2 = 0.18$) or dogs and humans ($r^2 = 0.19$). For most medicines, the differences are within the default interspecies uncertainty factor for kinetics of 4, but there are clearly some outliers. In the evaluation of Translarna (no. 9 in Fig. 2B), the observed species differences between dogs and humans were attributed to a relatively low urinary excretion in dogs. In case of Vargatef (number 11 in Fig. 2A), the observed species differences were attributed to a relatively high first-pass metabolism and P-glycoprotein (PgP) activity in humans. No sufficient information to explore the cause of the limited predictive values for the remaining outliers of Figure 2A and B (i.e., evaluation number 2, 3 and 20) was given in the respective evaluations. It cannot be excluded that formulation differences contributed.

The lack of correlation between animal and human bioavailability of medicines is in line with previous findings by Cao et al. (2006) and Musther et al. (2014). Both studies revealed a striking lack of correlation with r^2 of ~0.29 between rat and human bioavailability of medicines in both studies, and 0.25 between mouse and human in the study of Musther et al. (2014). The observed species differences were attributed to differences in first pass metabolism rather than species differences in absorption. For example, Cao et al. (2006) observed that both absorption by passive diffusion as well as carrier mediated absorption (by intestinal transporters such as PgP, and peptide transporter 1) correlated quite well between rats and humans ($r^2 = 0.8$) and that intestinal expression of individual transporters correlated to some extent ($r^2 = 0.41$-0.57), but that distinct differences in expression levels and patterns of metabolizing enzymes in the intestine (no correlation coefficient given) occurred.

Together, these results show that significant species differences in kinetics can occur. They stress the importance of the development of alternative methods that better reflect the human situation to increase human relevance of risk evaluations.

4 *In vitro* methods for assessing kinetics, predictive value for humans, and current use in risk evaluations

In contrast to *in vivo* kinetic studies, *in vitro* methods for kinetics capture individual aspects of kinetic processes, including, for example, absorption and metabolic rates of a compound. A specific advantage of *in vitro* methods is that human-based cell/tissue models can be used. The key processes that determine oral bioavailability (F_{oral}) of chemicals are generally described as i) the fraction that is absorbed into the enterocytes (f_{abs}), ii) the fraction that escapes intestinal metabolism or efflux (f_{gut}), and iii) the fraction that escapes first pass metabolism in the liver (f_{hep}) (Equation 2) (Peters, 2012). Though not often accounted for, the fraction that escapes luminal degradation, by, e.g., digestive enzymes or conversion by the intestinal microbiota, also affects the oral bioavailability. This fraction is generally assumed to be part of f_{gut}, as an additional source of gut metabolism (Karlsson et al., 2013).

$$F_{oral} = f_{abs} * f_{gut} * f_{hep} \quad (2)$$

To define non-animal testing strategies for predicting the total oral bioavailability, alternative methods that capture each of these individual processes are required.

4.1 *In vitro* methods for luminal degradation (by digestive enzymes or gut microbiota)

In vitro models that simulate digestion in the gastrointestinal (GI) tract are based on the sequential incubation of a chemical with various digestive fluids that represent the different parts of the GI-tract under physiologically relevant conditions, with or without a food matrix, thus representing a fasted or fed state (Klein, 2010; Lefebvre et al., 2015). Both dynamic and static methods have been developed; dynamic systems include peristaltic movements to mimic transfer along the artificial GI tract. In general, digestion models have been developed to predict i) the release of chemicals from a formulation (dissolution of medicines) (Cascone et al., 2016; Klein, 2010), ii) the release of chemicals from a complex matrix (i.e., bioaccessibility) (Oomen et al., 2003; Versantvoort et al., 2005) or iii) predicting digestion of macronutrients (e.g., Kopf-Bolanz et al., 2012). However, digestion methods are also increasingly used to study the stability of chemicals in the presence of digestive enzymes and the gastro-intestinal pH (e.g., Islam et al., 2014; Peters et al., 2012; Walczak et al., 2015a, 2012) and in some cases the breakdown by microbiota (Verwei et al., 2016). New types of dynamic models that are gaining increasing attention are microfluidic gut-on-a-chip models, which provide the potential to develop protocols where *in vitro* cellular models for absorption harbour intestinal microbiota. Microfluidic techniques have already been shown to allow long-term co-culturing of Caco-2 epithelial cells with microflora without compromising membrane integrity (Kim et al., 2012; Shah et al., 2016).

The current use of *in vitro* methods for luminal degradation shows that these methods are occasionally included in the assessment of food chemicals (Fig. 3) and medicines, but not in evaluations of pesticides. In case of the food chemicals, various evaluations concern compounds that occur in the diet as poorly absorbable hydrophilic plant conjugates (e.g., steviol glycosides used as sweetener, various masked mycotoxins and plant toxins). These could potentially be hydrolysed either at low pH in the stomach or by bacteria further on in the GI-tract, and as such become bioavailable (EFSA, 2014a). These results may explain the importance of *in vitro* methods for luminal degradadation within evaluations of food chemicals. The GI tract may also play an important role in the biotransformation of metabolites of all types of compounds excreted via the bile that are subse-

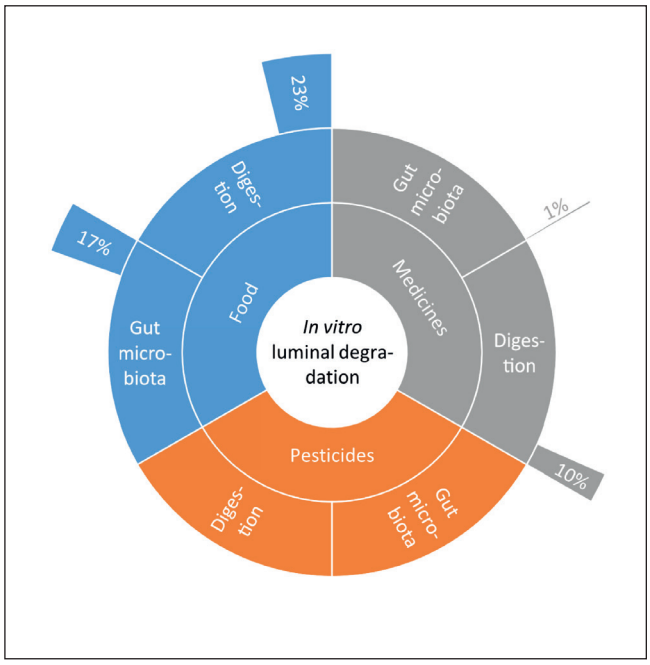

Fig. 3: Percentage of risk evaluations in which data from *in vitro* digestion and gut microbial models were described

quently reabsorbed (enterohepatic cycling), with clear species differences (Malik et al., 2016).

A challenge within current digestion methods is that they do not yet allow for quantitative *in vivo* predictions of the luminal breakdown of chemicals. No comparison can therefore be made to evaluate the predictive value of *in vitro* digestion methods. In case of conversions by the gut microbiota, a key challenge is to develop quantitative methods that reflect the bacterial numbers and diversity of the human intestine (Sousa et al., 2008). In addition, current methods for luminal degradation do not include the activity of brush border enzymes such as lactase phlorizin hydrolase (LPH) (Day et al., 2003). This requires the development of methods that integrate *in vitro* digestion methods with other *in vitro* methods, like cell cultures that contain brush border enzymes or incubations with tissue fractions (Islam et al., 2014). Recent advances with microfluidic gut-on-a-chip models could provide new opportunities in this respect.

4.2 *In vitro* methods for intestinal absorption

Models that capture the absorption of chemicals across the gut wall range from *in situ* and *ex vivo* methods (using sections of the intact intestine, e.g., everted sac model or the diffusion chamber technique) to *in vitro* cell cultures (e.g., Caco-2, MDCK cell lines) and artificial membranes that consist of hydrophobic filter material coated with a mixture of lecithin/phospholipids (e.g., PAMPA) (Volpe, 2011; Lefebvre et al., 2015).

Particularly, absorption of compounds by passive transcellular diffusion is adequately captured within the Caco-2 cell model with good *in vitro-in vivo* correlations (Cascone et al., 2016). Figure 4A summarizes the results obtained from different studies, showing an empirical sigmoidal relationship against *in vivo* human absorption values with r^2 values ranging from 0.61 to 0.81 (Marino et al., 2005; Matsson et al., 2005; Miret et al., 2004; Turco et al., 2011). Current use of *in vitro* absorption methods shows that only Caco-2 absorption experiments are occasionally included in risk evaluations of food chemicals as well as medicines (Fig. 4B). Within the risk evaluations of food chemicals, Caco-2 absorption experiments were found to be already used to reduce animal experimentation. Based on the principle that a negligible uptake of compounds (or their luminal degradation products) cannot lead to systemic effects (EFSA, 2012b), no further systemic toxicity evaluations were required in the evaluation of potassium polyaspartate using Caco-2 experiments for absorption (EFSA, 2016).

Quantitative predictions are more difficult with Caco-2 experiments when enzyme or transporter mediated processes are involved in the absorption or metabolism. Though Caco-2 cells are proficient in the main transporters, including P-glycoprotein (PgP), multidrug resistance protein 2 (MRP2), and breast cancer resistance proteins (BCRP), expression levels of these transporters are generally quite variable (Larregieu and Benet, 2013; Harwood et al., 2013, 2016). In addition, the under-expression of transporters such as peptide transporter 1 (PEPT1), organic cation transporters (OCTs), and organic anion transporters (OATs), makes the model less suitable for compounds that use these transporters (Larregieu and Benet, 2013). Under-expression of metabolic enzymes (e.g., CYP3A4) and different sulfotransferase and uridine diphospho-glucuronosyltransferase (UGT) enzymes, as compared to the human small intestine, also makes Caco-2 cells a poor model for studying intestinal metabolism (Gregory et al., 2004; Meinl et al., 2008; Peters et al., 2016; Schmiedlin-Ren et al., 1997). Additional limitations of Caco-2 absorption experiments include the smaller tight junctions and a 10-fold thicker unstirred water layer compared with the *in vivo* situation (Hubatsch et al., 2007; Stenberg et al., 2001). Likewise, the absence of a mucus layer and so-called M-cells, which are involved in the uptake of particle matter, may result in inadequate transport measurements for some chemicals like nanoparticles (Fröhlich and Roblegg, 2016; Lefebvre et al., 2015; Walczak et al., 2015b). Nonspecific binding of highly lipophilic compounds to the plastic surfaces may result in a poor predictive value of highly lipophilic compounds (Hubatsch et al., 2007; Krishna et al., 2001; Neuhoff et al., 2006). Finally, variability between laboratories occurs as a result of often minor differences in cell culture conditions (e.g., seeding density, composition of the media) and test conditions (Peters, 2012). This means that despite the gold standard use of Caco-2 cells, their use also has its boundaries.

Many protocol adjustments have been proposed to diminish the differences between the Caco-2 model and the human small intestine. For example, a reduction in unstirred water layer can be obtained by stirring (Hidalgo et al., 1991; Hubatsch et al., 2007; Stenberg et al., 2001) or performance of the assay in a microfluidics system (Kim et al., 2012). Addition of bovine

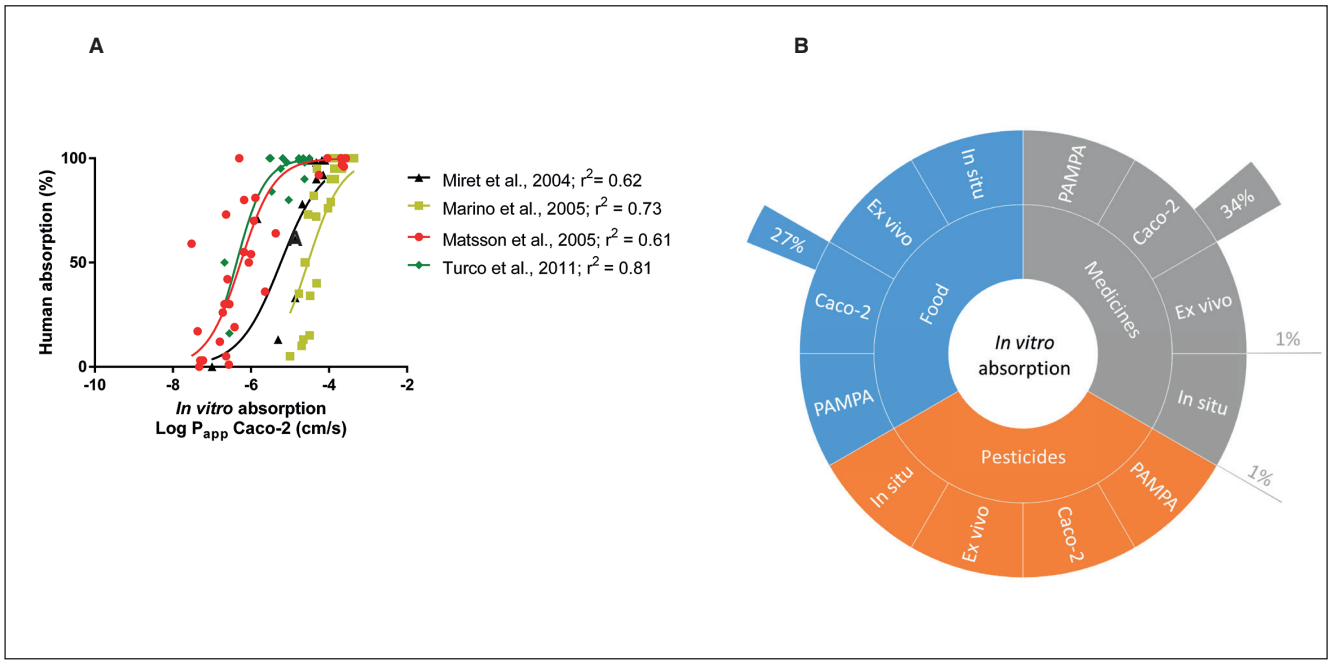

Fig. 4: Current use and predictive value of *in vitro* absorption models

(A) Reported sigmoidal correlations between *in vitro* Caco-2 permeability with *in vivo* human absorption. (B) Percentage of risk evaluations in which *in vitro* absorption models were used.

serum albumin (BSA) to the receiving compartment reduces non-specific binding of lipophilic compounds and enhances sink conditions (Neuhoff et al., 2006; Hubatsch et al., 2007). Co-culturing with HT29 cells provides a mucus layer and has been suggested to reduce the "tightness" of the tight junctions to better represent the physiology of the small intestine (Pan et al., 2015; Hilgendorf et al., 2000). Variability in expression levels of transporters can be accounted for by applying a correction factor representing the relative expression level of transporters within Caco-2 cells compared with the *in vivo* situation (Harwood et al., 2016). Addition of vitamin D3 to the culture medium results in increased expression of CYP3A4 (Schmieldlin-Ren et al., 1997). Sources of intestinal epithelial cells other than Caco-2 cells are also increasingly sought. Directed differentiation of human embryonic stem cells and induced pluripotent stem cells (iPSC) to intestine-like organoids with crypt-villus physiology and long-term culturing capacity has been achieved (Sato et al., 2011). However, current schemes for human intestine stem cells frequently rely on 3D culture conditions, whereas monolayer cultures are required for absorption experiments (Kauffman et al., 2013). Protocols are currently being optimized to achieve this goal (Astashkina and Grainger, 2014).

Overall, these results indicate that protocol development for *in vitro* absorption experiments is still a priority to cover the whole chemical space. Nonetheless, these developments do not have to restrain the quantitative use of Caco-2 results for chemicals that fall into the domain for which adequate *in vivo* predictive value is already obtained. Moreover, physiologically based kinetic computer modelling to integrate different types of *in vitro* kinetic data allows to compensate certain limitations of Caco-2 cells. For example, intestinal metabolism can be accounted for by measuring metabolic conversions separately with primary intestinal cells or tissue fractions and integrating these measurements with Caco-2 absorption data in kinetic computer models (Bois et al., 2010; Jamei et al., 2009).

4.3 *In vitro* methods for intestinal and liver metabolism

The oral bioavailability (the fraction that enters the blood unchanged) is significantly determined by first-pass metabolism in the intestine and/or liver. *In vitro* methods that allow to quantitatively predict the intestinal and liver metabolism are thus essential to determining the systemic exposure. *In vitro* methods measuring metabolic conversion are divided into methods that measure i) the depletion of a chemical over time following incubation with cells or subcellular fractions or ii) the formation of metabolites, providing Michaelis-Menten constants (i.e. K_m and V_{max}) (Houston and Carlile, 1997). The results can be scaled to the *in vivo* situation by accounting for tissue fraction yields or number of cells per gram tissue to obtain the intrinsic organ clearance rates (Barter et al., 2007; Miners et al., 2006; Pelkonen and Turpeinen, 2007).

Studies are most frequently performed with (cryopreserved) primary cells or tissue fractions, such as microsomes, S9 or cytosol, derived from animal organs or human donors (Soars et al., 2002; Pelkonen and Turpeinen, 2007). Studies can also be performed with precision-cut tissue slices (Graaf et al., 2007; van Midwoud et al., 2010). However, at present these do not yield

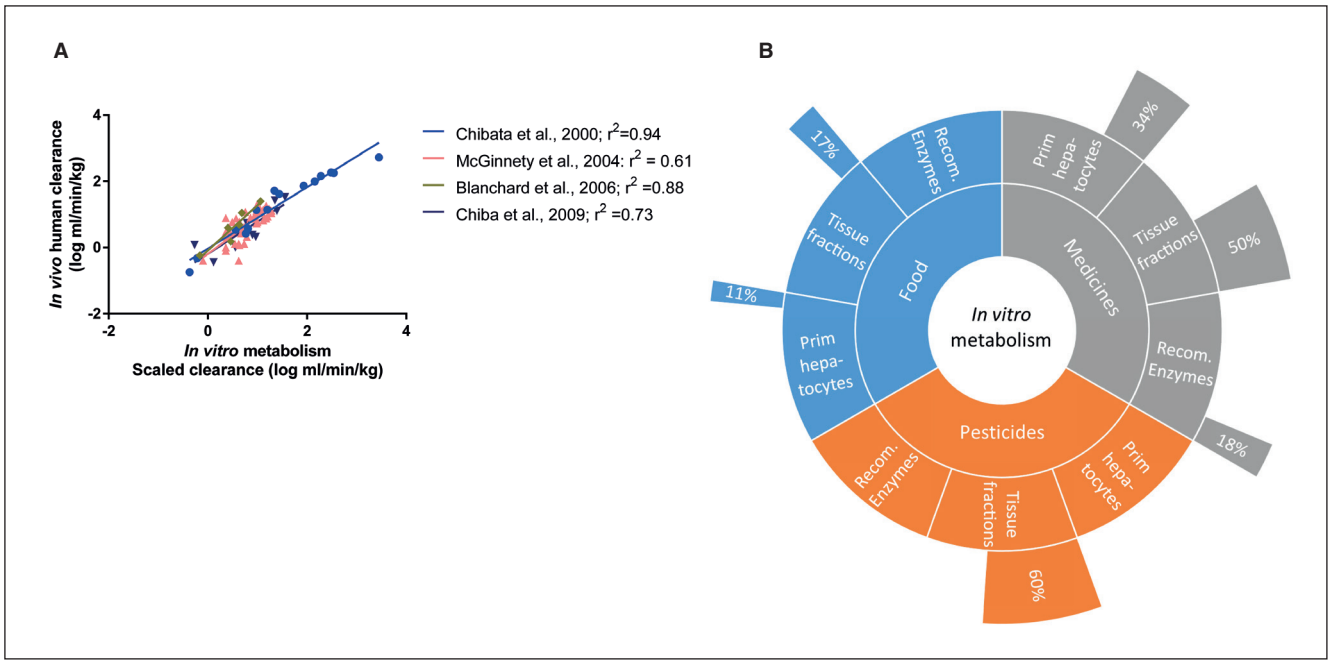

Fig. 5: Current use and predictive value of *in vitro* metabolism models

(A) Reported correlations of *in vitro* clearance with measured *in vivo* human clearance. *In vitro* measurements were obtained with primary human hepatocytes. (B) Percentage evaluations describing *in vitro* metabolic data. For pesticides the percentages are derived from the evaluations since January 2016 as evaluations before 2016 did not include *in vitro* metabolic data.

sufficient quantitative estimations of kinetic constants, as slices are heterogeneous in composition and impaired diffusion of chemicals into the cells of the slices hampers adequate measurement of the clearance, even if the slices are very thin (Houston and Carlile, 1997; van Eijkeren, 2002; Yoon et al., 2012). A final source of *in vitro* material includes recombinant enzymes, such as for example cytochrome P450 and UGT enzymes that are transfected into insect cells (Punt et al., 2016; Rostami-Hodjegan and Tucker, 2007). Measurements with these recombinant enzymes are particularly useful to explore human variation in metabolism of chemicals using information on the human variation in expression of these enzymes (Punt et al., 2016; Rostami-Hodjegan and Tucker, 2007). New sources of human metabolically competent cells that do not rely on human donor materials are being explored. Work on the human hepatoma cell line HepaRG is particularly promising. HepaRG cells express various cytochrome P450 and phase II enzymes when maintained in a differentiated state (Harwood et al., 2013; Zanelli et al., 2011).

The predictive value of *in vitro* methods for metabolism has been evaluated in various papers, predominantly for medicines and focusing on liver metabolism (Pelkonen and Turpeinen, 2007). Good correlations have been observed for *in vitro* clearance measured with human microsomes or (cryopreserved) hepatocytes and *in vivo* clearance (Blanchard et al., 2006; Chiba et al., 2009; McGinnity et al., 2004; Shibata et al., 2000). Figure 5A summarizes the reported correlations between scaled *in vitro* and *in vivo* measured clearances based on incubations with primary hepatocytes. The correlation coefficients (r^2) between the scaled *in vitro* and measured *in vivo* clearance range between 0.61 and 0.94 (McGinnity et al., 2004; Shibata et al., 2000; Zanelli et al., 2011). In addition, also the HepaRG cell line provides an adequate predictive value of *in vivo* metabolic clearance rates (r^2 = 0.53) (Zanelli et al., 2011), with a predictivity equal to that of cryopreserved primary human hepatocytes in the same study.

In comparison to *in vitro* methods for absorption or luminal digestion, there is little use of *in vitro* metabolism data within the risk evaluations of food chemicals (11-17% of the evaluations contained such data) (Fig. 5B). The relatively infrequent use becomes even more apparent when compared to risk evaluations of pesticides (60% contained *in vitro* kinetic data) or medicines (18-50% contained *in vitro* kinetic data). In case of pesticides, comparative *in vitro* metabolism studies using microsomes or intact cell systems of relevant experimental animals and human materials are a regulatory data requirement (EC, 2013) to determine the relevance of the toxicological animal data and to guide in the interpretation of findings and in further definition of the testing strategy. This shows how regulatory data requirements can increase the use of *in vitro* kinetic data.

Despite the inclusion of *in vitro* metabolism studies in regulatory risk evaluations, the use is in general restricted to a qualitative assessment of possible species differences regarding the metabolite. To enhance the use and acceptance of quantitative *in vitro* kinetic measurements, the development of uniform protocols for performing *in vitro* metabolism studies and the definition of acceptance criteria are important. In addition, more

proof of principle should be obtained on how *in vitro* kinetics can be used to improve the risk assessment and decrease the uncertainties due to, e.g., potential species differences.

5 *In silico* methods for predicting and modelling kinetics

5.1 Background on available *in silico* methods for kinetics

In silico approaches can be divided into (quantitative) structure activity relationships (Q)SAR and physiologically based pharmacokinetic (PBPK) models, each having their own goals. (Q)SARs for kinetics aim at the use of chemical descriptors of a compound to predict kinetic parameters such as rate of absorption, metabolism or the type of metabolites that might be formed (Kiwamoto et al., 2015; Pirovano et al., 2014). PBPK models mathematically describe the absorption, distribution, metabolism, and excretion of a chemical in an organism based on a series of ordinary differential equations and are used to simulate the fate of chemicals in a body (Rietjens et al., 2010).

(Q)SAR tools to predict absorption and/or metabolism are predominantly useful to explore the behaviour of chemicals when no *in vitro* or *in vivo* data are available. Various commercial tools can be used, mainly to predict potential metabolite formation. These models are developed using a training set from the literature or public databases (Ren and Lien, 2000; Bessems et al., 2014). Examples include Meteor Nexus, COMPACT, META, METabolExpert, TIMES (Bessems et al., 2014). Though these software programs are relevant for exploring new chemicals, (Q)SAR methods still have a limited quantitative predictive value (Wilk-Zasadna et al., 2015; Dressman et al., 2008).

PBPK models simulate the ADME of chemicals in an organism, allowing the prediction of blood or tissue concentrations of a chemical or relevant metabolites (Clewell and Clewell, 2008; Rietjens et al., 2010). These simulations are made with ordinary differential equations that include chemical-specific kinetic parameters (e.g., absorption and metabolic conversion rates), as well as physiological parameters (e.g., cardiac output, tissue volumes, and tissue blood flows) and physicochemical parameters (e.g., tissue:blood partition coefficients). Developed PBPK models often include kinetic parameters fitted to *in vivo* studies (Clewell and Clewell, 2008). The use of *in vitro* kinetic data (including e.g., Caco-2 absorption data and *in vitro* clearance measurements with primary hepatocytes or tissue fractions as described above) to build PBPK models has increased drastically over the last decades, thereby contributing to a reduction in animal testing (Rietjens et al., 2010; Rostami-Hodjegan and Tucker, 2007). PBPK models allow evaluation of dose-dependent effects in kinetics and can be developed for multiple species to evaluate species differences. In addition, simulations of interindividual human variation can be achieved by incorporating equations and kinetic constants for metabolic conversions by individual human samples and/or specific isoenzymes. Finally, PBPK modelling allows simulation of interactions between chemicals in transport activity as well as metabolic turnover to predict mixture effects (Rietjens et al., 2010; Rostami-Hodjegan and Tucker, 2007). PBPK models are generally evaluated on a case-by-case basis. Those using input of *in vitro* kinetic data show adequate quantitative predictions of *in vivo* kinetics, including C_{max}, AUC and bioavailability (Flanagan et al., 2016; Gobeau et al., 2016; Rietjens et al., 2010).

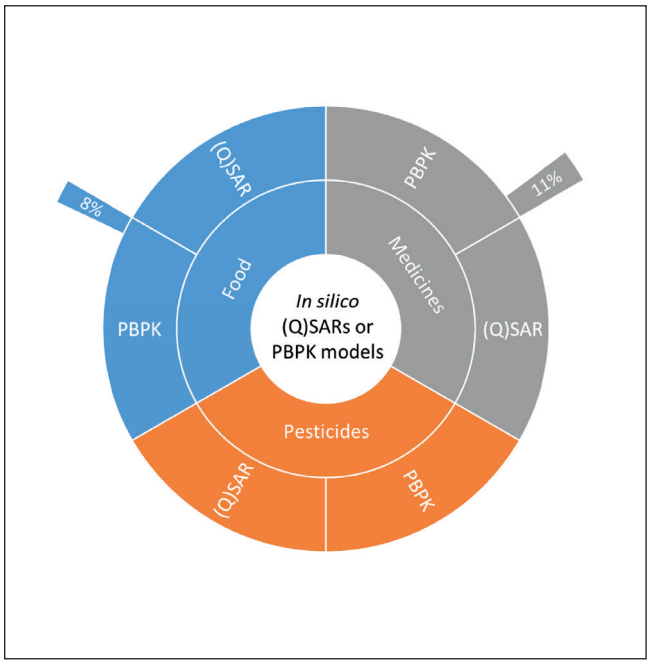

Fig. 6: Percentage evaluations containing *in silico* approaches for kinetics (i.e., (Q)SAR or PBPK modelling)

An evaluation of the various risk evaluations (Fig. 6) reveals no use of Q(SAR)s to predict kinetic data and only a very limited use of PBPK modelling approaches in risk evaluations of food chemicals and medicines. PBPK models were mainly used to support the evaluation of species differences. For example, for bisphenol A, PBK modelling was used to estimate the oral bioavailability based on the limited availability of human kinetic data (EFSA, 2015a). This model was not developed based on *in vitro* kinetic data. Within the risk evaluations of medicines, the PBPK models are primarily used to predict drug-drug interactions and not for quantitative predictions of, e.g., bioavailability or *in vivo* C_{max} values.

Particularly the limited use of PBPK models within risk evaluations is in contrast with the scientific achievements to predict *in vivo* kinetics with these models using *in vitro* input data. The use of such models would allow a better prediction of levels leading to potential effects in humans and, as such, decrease the uncertainty in the risk assessment. This may not only avoid unnecessary conservativeness but should also result in better protection in case the applied default values are not large enough. Future efforts should focus on further improve-

ment of the models and their regulatory acceptance. A similar conclusion was recently made by Flanagan et al. (2016), who revealed a gap in the use of PBPK modelling in risk evaluations of medicines compared with the use of such methods during the drug development stage. Construction of these models based on *in vitro* data can be enhanced by the development of standardized protocols as described above. In addition, user-friendly PBPK modelling platforms and model evaluation criteria are required (Bessems et al., 2014; Flanagan et al., 2016; Loizou et al., 2008).

6 Conclusions and recommendations

Here we reviewed the availability of non-animal toxicokinetic approaches for luminal degradation, absorption and metabolism, their predictive value for humans, and their current use in European regulatory risk evaluations of food chemicals. Through the comparison of the application of these toxicokinetic approaches with risk evaluation of pesticides and medicines we identified best practices. Finally, we identified future needs to maximize the exploitation of these approaches in regulatory risk assessment.

Data from *in vitro* Caco-2 absorption experiments and *in vitro* data on gut-microbial conversions were relatively frequently included in risk evaluations of different food chemicals but to a lesser extent in risk evaluations of pesticides or medicines. A less frequent use of *in vitro* data for metabolic conversions was observed in case of food chemicals. In addition, we observed only a minor use of quantitative *in vitro* kinetic data, including the use of *in silico* PBPK models within all the assessed risk evaluations on food chemicals, pesticides and medicines.

The minor use of quantitative non-animal methods for kinetics in regulatory risk evaluations is in contrast with recent scientific advances. The human *in vivo* predictive values of transcellular absorption based on Caco-2 cell experiments (r^2 = 0.61-0.81) (Marino et al., 2005; Matsson et al., 2005; Miret et al., 2004; Turco et al., 2011) and metabolism based primary hepatocyte incubations (r^2 = 0.61-0.9) (Blanchard et al., 2006; Chiba et al., 2009; McGinnity et al., 2004; Shibata et al., 2000) are strikingly better than the correlation between animal and human bioavailability (r^2 = 0.18-0.29) (Cao et al., 2006; Musther et al., 2014; this study). Integrating these *in vitro* kinetic data with PBPK modelling can provide a method that has the potential to obtain predictions of the fate of a chemical in humans that are better than those currently obtained with animal studies. The need for inclusion of quantitative non-animal kinetic methods in risk evaluations is also increasingly recognized by different European regulatory bodies (EFSA, 2014b; EMA, 2016).

We propose some specific actions to improve the use of quantitative predictions of the fate of chemicals in humans. Clearly, the recommendations are different, depending on the type of *in vitro* assay or *in silico* approach. Firstly, *in vitro* methods that adequately correlate with the *in vivo* situation will greatly benefit from i) uniform protocols and ii) the definition of acceptance criteria (i.e., definition of the application domain, time-points and concentrations that are selected). This applies to Caco-2 absorption experiments for chemicals that are passively transported by the transcellular route and *in vitro* metabolic measurements with (cryopreserved) primary hepatocytes and tissue fractions, and the development of PBPK models based on these data. Formulation of regulatory requirements and/or guidance will also facilitate the use of these models. The effectiveness of regulatory data requirements on the use of *in vitro* kinetic data is demonstrated by our survey of the recent risk evaluations of pesticides. Only since implementation of the regulation on data requirements (EC, 2013), *in vitro* metabolic measurements are included in the risk evaluations of pesticides.

For those non-animal approaches that currently do not allow quantitative *in vivo* predictions, there remain challenges to develop and implement adequate methods. This concerns *in vitro* methods for degradation by digestion enzymes, degradation by the gut microbiota, and absorption studies with Caco-2 cells for chemicals that are not transported via the transcellular route. New experimental approaches, including microfluidic devices, as well as new culturing methods (e.g., stem cells), may be a way forward to better represent human physiology in an *in vitro* system.

Overall it can be concluded that quantitative predictions of *in vivo* kinetics using non-animal data offer great opportunities to reduce uncertainty in human risk assessments and will facilitate the further development and regulatory acceptance of alternatives to animal testing.

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Conflict of interest

The authors declare no conflicts of interest.

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Correspondence to

Ans Punt, PhD
RIKILT Wageningen University and Research
Akkermaalsbos 2
6708 PD, Wageningen
The Netherlands
Phone: +31 (0)6 24921608
e-mail: ans.punt@wur.nl