Review Article

Non-animal approaches for toxicokinetics in risk evaluations of food chemicals

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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 vs 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 a 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.

Key words: 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 in e.g. bioavailability, bioaccumulation potential and the formation of metabolites. Information on the fate of compounds allows to better understand the toxicity and inter- and interspecies differences in toxicity of a chemical (Bessems et al., 2014; OECD, 2010). As such, 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 these default factors comprise a factor of 4 for interspecies differences in toxicokinetics and a factor of 3.16 for potential differences between 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 only to a minor extent used for the final assessment. 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 in time (e.g. Cmax; 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 isofoms of metabolizing enzymes and transport proteins. In vivo animal data are therefore increasingly criticized as predictor of the fate and effects of chemicals in humans (Cao et al., 2006; Mustel et al., 2014).

A wealth of in vitro approaches capturing kinetic processes in an organism have been developed. Of these assays, only the in vitro skin absorption test has been validated and incorporated in a 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 and liver, and iii) protein binding assays. Each of these assays capture 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 \textit{in vitro} kinetic data with \textit{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 boundaries. 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 animal experimental data are 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 defined food contaminants, -additives, and -contact materials as food chemicals. EFSA opinions on these types of compounds were screened for the application of \textit{in vivo} and non-animal kinetic methods. In addition, for comparison, the use of \textit{in vivo} and \textit{in vitro} kinetic methods within evaluations of pesticides (EFSA Conclusions on Pesticides) and medicines (EMA Public Assessment Reports) was assessed as well. 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 \textit{in vivo} and \textit{in vitro} kinetic data within regulatory risk evaluations

2.1 Selection of EFSA opinions on food contaminants, additives, and contact materials and regulatory data requirements.

We examined the use of \textit{in vitro} and \textit{in vivo} kinetic data within EFSA opinions on food contaminants, food additives, and food contact materials, 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 49 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 evaluations of contaminants, additives and contact materials. In case of contaminants the opinions are generally prepared based on data available from the public domain (Alexander et al., 2012). In the EFSA note for 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 documents on food additives (EFSA, 2012b) provides a tiered approach for toxicokinetic testing. Demonstration of negligible conversion by gastrointestinal fluids or the gut microbiota (\textit{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 \textit{in vivo} toxicokinetic studies (OECD TG 417) that should provide data on systemic exposure after a single dose of the compound like basic toxicokinetic parameters (\textit{T_{1/2}}, AUC, bioavailability, Cmax and T\textsubscript{max}). Tier 3 studies, defining toxicokinetic parameters following repeated administration, are triggered when there is limited or slow excretion or any other mechanism that 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 applies (EFSA, 2011a). 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-nanofoms.

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 pesticides evaluations.

The data requirements for pesticide active substance evaluations are laid down in Regulation (EU) No 283/2013 (European Commission, 2013). Key kinetic parameters that need to be obtained from \textit{in vivo} studies are described in annex 5.1.1. In addition, it states that “\textit{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 Medicine 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 (i.e. absorption, distribution, metabolism, and excretion) data needs to be included in the study reports on both non-clinical and clinical studies. It is also indicated that \textit{in vitro} studies can be carried out with the advantage of using
human material for comparison with animal (European Commission, 2003). Plasma (or whole blood or serum) AUC, Cmax 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 Advanced Search option. The use of in vivo kinetic data (Fig. 1 and 2) within the different evaluations was explored by searching for the terms “AUC or Cmax” and a separate search for the occurrence of the terms “absorption” or “bioavailability”. Evaluations that contained these terms were manually further analysed 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. Absolute bioavailability results reported within the EMA evaluations (when available) were used to derive Fig. 2.

For the use of in vitro and in silico alternative methods within the risk evaluations the search terms as described in Tab. 1 were used. A manual check was always applied on all searches to exclude non relevant matches or for further specification of the methods that were used.

Fig. 1: Percentage evaluations containing AUC, Cmax, or Foral values derived from in vivo human, rodent and/or non-rodent kinetic studies

For pesticides the percentages are derived from the evaluations since Jan 2016. Pesticide evaluations before 2016 did not include quantitative in vivo kinetic data.

Fig. 2: Predictive value in vivo kinetic data

(A) Oral bioavailability in rats and (B) dogs versus humans. Bioavailability data were obtained from EMA evaluations. Solid lines correspond to a 4-fold differences between animal and human bioavailability, representing the default uncertainty factor (UF) of 4 for interspecies differences kinetics. The dotted lines represent the fitted linear regression curves.
The observed species differences between dogs and humans were attributed to a relatively low urinary excretion in dogs. In humans (to allow comparison with humans relatively of acrylamide, humans were found to have 1.4 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 TDI (EFSA, 2015a).

Nonetheless, the two examples within the survey that contain quantitative data on bioavailability showed strikingly different results. Firstly, for bisphenol A, mice having 14.7 fold lower plasma levels of bisphenol A compared with humans at a similar oral exposure, suggesting a higher sensitivity of mice (EFSA, 2015b). Nonetheless, the default safety margin of 10,000 for genotoxicity endpoints (EFSA, 2015a) is shown in Figure 2.

In the evaluation of Translarna (number 9 in Figure 2), some outliers were observed, possibly because of formulation differences that have contributed. In the newly introduced data requirement regulation (European Commission, 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 strikingly different results. 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 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 glycammide, 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 kinetics) 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 Figures 2 and 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 kinetic of 4, but there are clearly some outliers. In the evaluation of Translarna (number 9 in Figure 2B) the observed species differences between dogs and humans were attributed to a relatively low urinary excretion in dogs.

In the case of Vargatel (number 11 in Figure 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 have contributed.

### 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). Most commonly used parameters include the maximum concentration (Cmax) 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 and represents the fraction of a compound that enters the 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.

$$F_{oral} = \frac{AUC_{oral}}{AUC_{iv}} \times \frac{(Dose_{oral} / Dose_{iv})}{eq. 1}$$

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, Cmax or percentage bioavailability. In contrast, all evaluations of medicines contained AUC or Cmax 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 (European Commission, 2013).

### 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

<table>
<thead>
<tr>
<th>Method</th>
<th>Search terms</th>
<th>Additional manual selection</th>
</tr>
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<tbody>
<tr>
<td>In vitro methods for luminal stability (Fig. 3)</td>
<td>1) digestion or SGF or FaSSIF or FeSSIF or microbiota or microflora or flora</td>
<td>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</td>
</tr>
<tr>
<td>In vitro, in situ, or ex vivo methods for intestinal absorption (Fig. 4A)</td>
<td>1) Caco-2 2) &quot;in situ&quot; or perfusion 3) &quot;ex vivo&quot; or perfusion 4) PAMPA or &quot;artificial membrane&quot;</td>
<td>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</td>
</tr>
<tr>
<td>In vitro methods for intestinal and liver metabolism (Fig. 5A)</td>
<td>1) supersomes or recombinant or UGT 2) S9 or microsomes or cytosol 3) hepatocytes</td>
<td>Manual check to exclude other types of in situ and ex vivo approaches</td>
</tr>
<tr>
<td>In silico methods, QSARs (Fig. 6)</td>
<td>1) QSAR or SAR or (Q)SAR or &quot;OECD Toolbox&quot;</td>
<td>Manual check if the match corresponded to QSAR for kinetic parameters, excluding other QSARs</td>
</tr>
<tr>
<td>In silico methods, PBPK (Fig. 6)</td>
<td>1) PBPK or PBK or PBPK or &quot;physiologically based pharmacokinetic&quot;</td>
<td></td>
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Abbreviations: SG, simulated gastric fluid; FaSSIF, fasted state simulated intestinal fluid; FeSSIF, fed state simulated intestinal fluid; PAMPA, parallel artificial membrane permeability assay; UGT, uridine diphospho-glucuronosyltransferase; QSAR, quantitative structure activity relationship; SAR, structure activity relationship; OECD, organization for economic co-operation and development; PBPK/PB/K/PBBK, physiologically based pharmacokinetic/biokinetic.

### 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). Most commonly used parameters include the maximum concentration (Cmax) 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 and represents the fraction of a compound that enters the 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.

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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 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 glycammide, 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 kinetics) 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 Figures 2 and 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 kinetic of 4, but there are clearly some outliers. In the evaluation of Translarna (number 9 in Figure 2B) the observed species differences between dogs and humans were attributed to a relatively low urinary excretion in dogs. In the case of Vargatel (number 11 in Figure 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 have contributed.
The lack of correlation between animal and human bioavailability of medicines is line with the previous findings by Cao et al. (2006) and Musterer 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 Musterer 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 transporter 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 and 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 (\( F_{\text{oral}} \)) of chemicals are generally described as i) the fraction that is absorbed into the enterocytes (\( F_{ab} \)), ii) the fraction that escapes intestinal metabolism or efflux (\( F_{\text{gut}} \)), and iii) the fraction that escapes first pass metabolism in the liver (\( F_{\text{hep}} \))(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 \( F_{\text{gut}} \) as an additional source of gut metabolism (Karlsson et al., 2013).

\[
F_{\text{oral}} = F_{ab} \times F_{\text{gut}} \times F_{\text{hep}}
\]

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 at physiological 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, in which 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 macro nutrients (e.g. Kopf-Bolanz et al., 2012). However, digestion methods are also increasingly used to study the stability of chemicals themselves 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 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 of the 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 degrading 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 which are subsequently reabsorbed (enterohepatic cycling), with clear species differences (Malik et al., 2016).

A challenge within current digestion methods is that they do not allow yet 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-II 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 (Casone et al., 2016). Fig. 4A summarizes the results obtained from different studies, showing an empirical sigmoidal relationship against in vivo human absorption values with r² 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 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 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 are transported by 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 also 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 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, the application also has its boundaries.

Many protocol adjustments have been proposed to diminish the gaps 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 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 allows to obtain 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 2016). Addition of vitamin D3 to the culture medium results in increased expression of
CYP3A4 (Schmiedlin-Ren et al., 1997). Other sources of intestinal epithelial cells than Caco-2 cells are also increasingly sought. Directed differentiation of human embryonic stem cell and induced pluripotent stem cells (iPSC) 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 a 3D culture conditions, whereas for absorption experiments monolayer cultures are required (Kaufman et al., 2013). Protocols are currently being optimized to achieve this goal (Astakhkina and Grainger, 2014).

Overall these results indicate, that to cover the whole chemical space, protocol development for in vitro absorption experiments is still a priority. 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, given the possibilities of physiologically based kinetic computer modelling to integrate different types 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).

Fig. 4: Current use and predictive value 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.

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 in methods that i) measure 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. Km and Vmax) (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; Miner 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 sufficient quantitative estimations of kinetic constants, as slices are heterogeneous in composition and impaired diffusion of chemicals into the cells of the slices hampers the 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 based using information on the human variation in expression of these
enzymes (Punt et al., 2016; Rostami-Hodjegan and Tucker, 2007). New sources of human metabolic 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 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 data 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). Fig. 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 0.93 (McGinnity et al., 2004; Shibata et al., 2000; Zanelli et al., 2011). In addition to these data, also the HepaRG cell line provides an adequate predictive value of in vivo metabolic clearance rates ($r^2 = 0.53$) (Zanelli et al., 2011), equally predictive as 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 containing such data (Fig. 5B). The relative low use becomes even more apparent when compared to risk evaluations of pesticides (60% containing in vitro kinetic data) or medicines (18-50%). In case of pesticides, comparative in vitro metabolism studies using microsomes or intact cell systems from relevant experimental animals and human materials are a regulatory data requirement (European Commission, 2013), 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. This shows the impact of regulatory data requirements to 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 qualitative comparison of possible species differences in the type of metabolites. 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 in (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 yet. 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 to predict 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 to evaluate 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. Focussing on those using input of in vitro kinetic data, show adequate quantitative predictions of in vivo kinetics, including Cmax, AUC and bioavailability (Flanagan et al., 2016; Gobeau et al., 2016; Rietjens et al., 2010).

An evaluation of the various risk evaluations (Fig. 6), reveals no use of (Q)SARs 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 Cmax values.

![Fig. 6: Percentage evaluations containing in silico approaches for kinetics (i.e. (Q)SAR or PBPK modelling)](image-url)

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 conservatism but theoretically also in better protection, in case the applied default values are not large enough. Future efforts should focus on further improvement of the models and increasing regulatory acceptance of these models. A similar conclusion was recently obtained 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 in drug development. Development 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 the current use in European regulatory risk evaluations of food contaminants, additives and food contact materials. Through the comparison of the application of these toxicokinetic approaches in risk evaluation of pesticides and medicines we identified best practices. Finally we identified future needs to maximize the use 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 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 the 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 that the application 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, allows to 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 to 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 model 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 after implementation of the regulation on data requirements (European Commission, 2013), in vitro metabolic measurements are included in the risk evaluations of pesticides.

For those non-animal approaches that currently do not allow to provide 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 by the transcellular route. New experimental approaches, including microfluidic devices, as well as new culturing methods (e.g. stem cells) may provide a significant way forward to better represent human physiologically in an in vitro system. Overall it can be concluded that quantitative predictions of in vivo kinetics by using non-animal data has great possibilities to reduce the uncertainty in human risk assessments, and will facilitate the further development and regulatory acceptance of alternatives to animal testing.

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