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Rationalization and prediction of *in vivo* metabolite exposures: The role of metabolite kinetics, clearance predictions and *in vitro* parameters

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Abstract

Importance of the field—Due to growing concerns over toxic or active metabolites, significant efforts have been focused on qualitative identification of potential *in vivo* metabolites from *in vitro* data. However, limited tools are available to quantitatively predict their human exposures.

Areas covered in this review—Theory of clearance predictions and metabolite kinetics is reviewed together with supporting experimental data. I*n vitro* and *in vivo* data of known circulating metabolites and their parent drugs was collected and the predictions of *in vivo* exposures of the metabolites were evaluated.

What the reader will gain—The theory and data reviewed will be useful in early identification of human metabolites that will circulate at significant levels *in vivo* and help in designing *in vivo* studies that focus on characterization of metabolites. It will also assist in rationalization of metabolite-to-parent ratios used as markers of specific enzyme activity.

Take home message—The relative importance of a metabolite in comparison to the parent compound as well as other metabolites *in vivo* can only be predicted using the metabolites *in vitro* formation and elimination clearances, and the *in vivo* disposition of a metabolite can only be rationalized when the elimination pathways of that metabolite are known.

Keywords

metabolite pharmacokinetics; *in vitro*-to-*in vivo* prediction; prediction of circulating metabolites; metabolic clearance

1. Introduction

A metabolite can be formed from any enzymatic transformation of a parent drug after the parent is administered *in vivo* or is incubated as substrate *in vitro*. Often, these metabolites prove to have *in vivo* pharmacologic activity. Classic examples of metabolites that have pharmacologic activity are metabolites of tricyclic antidepressants and benzodiazepine anxiolytics, where many of the metabolites are also marketed drugs [1,2]. Metabolites can also possess toxicological activity. Examples of *in vivo* toxic metabolites have been well established for many parent drugs, such as carbamazepine, valproic acid and nefazodone [3–5]. Additionally, it has been demonstrated that some *in vivo* inhibitors have inhibitory

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metabolites of similar potencies, such as fluoxetine, itraconazole and atomoxetine [6–8]. Due to the realization that metabolites can oftentimes have *in vivo* activity, it is important to understand the disposition of a metabolite after the administration of a parent drug.

A recent FDA guidance on metabolites in safety testing (MIST) has drawn more attention to identifying and predicting human metabolites [9]. This guidance states that a metabolite found to circulate at equivalent or greater concentrations in at least one pre-clinical animal species when compared to in human has been adequately evaluated for safety and no further non-clinical testing is warranted. If this cannot be demonstrated, any metabolite with exposure > 10% of the parent at steady-state in humans warrants separate non-clinical toxicological and pharmacokinetic studies. In contrast to the MIST guidance, the European guidance states that separate studies are only warranted when a metabolite exposure is > 10 % of the total drug-related material exposure [10]. These guidance pose two important dilemmas in new drug development: 1) how to identify and reliably predict potentially important circulating metabolites sufficiently early in new drug development to allow timely synthesis of reference material, development of validated assays and toxicological evaluation, and 2) how to determine the steady-state area under the plasma concentration versus time curve for the metabolite (AUC_m) relative to the parent (AUC_p), or total drug related material, for relevant metabolites without performing elaborate multiple dose studies with radiolabeled drug.

In vitro metabolism and pre-clinical animal data as well as single dose pharmacokinetic data are often used to predict the *in vivo* steady-state disposition of new drug candidates, as well as the *in vivo* metabolite profile of a candidate drug. However, attempts to predict important circulating metabolites in humans from pre-clinical data are qualitative and have met with variable success [11]. Direct translation of the metabolite profile from animal species to humans may be confounded by species differences in enzyme activity and expression, whereas *in vitro* HLM and hepatocyte studies qualitatively identify the primary metabolites that are likely to be formed *in vivo* but detection of secondary metabolites remains challenging. In this review, established *in vivo* metabolite kinetic theory will be discussed and a method for predicting *in vivo* metabolite disposition from *in vitro* data will be presented and evaluated for its usefulness in preclinical prediction of metabolite exposure as well as in rationalization of *in vivo* metabolite exposures.

2. Metabolite Kinetic Theory: *In vivo* Aspects

During the late 1960's through to the early 1980's, much interest was paid to the development of pharmacokinetic theory that describes the *in vivo* disposition of a metabolite formed after administration of a parent drug. The metabolite plasma concentration (C_m) versus time (t) curve for a metabolite formed after intravenous (IV) administration will exhibit biphasic kinetics and depend on the dose of parent (D), the fraction of parent that is converted to metabolite (f_m), the metabolite volume of distribution ($V_{d,m}$) and the formation (k_f) and elimination (k_m) rate constants for the metabolite [12]:

$$C_m = \frac{k_f * f_m * D}{V_{d,m} * \left(k_m - k_f\right)} * \left(e^{-k_f * t} - e^{-k_m * t}\right) \quad (1)$$

This expression dictates that the slope of the linear terminal portion of a metabolite concentration versus time profile will either be equal to that of the parent, i.e. formation rate limited (FRL, $k_f < k_m$) kinetics, or less than that of the parent, i.e. elimination rate limited (ERL, $k_f < k_m$) kinetics [13]. After oral (PO) administration, the metabolite concentration versus time profile will further depend on the fraction of drug absorbed into the body (F_a)

and will be either biphasic or triphasic, depending on the efficiency of metabolite formation during first pass metabolism [14].

The AUC_m after either IV or PO administration of the parent was demonstrated to be determined by D, F_a (only after PO administration), f_m and the clearance of the metabolite (Cl_m) [15,16]. Additionally, the *in vivo* metabolite-to-parent AUC ratio (AUC_m/AUC_p) was determined to be dependent only on the *in vivo* formation and elimination clearances for the metabolite [17,18]. Patel et al [18], demonstrated that after IV administration of a drug the ratio of AUC_m to the AUC_p is:

$$\frac{AUC_m}{AUC_p}=\frac{f_m*Cl_p}{Cl_m}=\frac{Cl_f}{Cl_m} \quad (2)$$

where Cl_p and Cl_m are the total *in vivo* clearances of the parent and the metabolite, respectively, and Cl_f is the formation clearance of the metabolite. The assumptions that are made in this model are that 1) the kinetics of the parent and metabolite are linear, 2) only one metabolite is formed from the parent (i.e. f_m equals the fraction of drug excreted unchanged subtracted from unity) and 3) all metabolite formed is available to the systemic circulation. It should be noted, that the above expression can be adapted to PO administration if metabolism occurs only in the liver and Cl_p is the apparent oral clearance of parent, i.e. the quotient of the true clearance of parent and the fraction of parent that escapes first pass in the liver (F_h):

$$\frac{AUC_m}{AUC_p}=\frac{f_m*Cl_p}{F_h*Cl_m} \quad (3)$$

The AUC_m/AUC_p is a primary measure utilized in the MIST guidance and hence, this theory was reexamined to address the first dilemma posed by the guidance. Although the *in vivo* expression for AUC_m/AUC_p has been available for 25 years, it has only been tested in the norclobazam/clobazam metabolite/parent (M/P) pair [17]. In order to further validate the above kinetic theory, literature data for three M/P pairs, morphine/codeine, morphine-6-glucuronide/morphine and theophylline/caffeine, were reviewed with Equations 2 and 3 to test the existing metabolite kinetic theory in an *in vivo*-to-*in vivo* extrapolation of the AUC_m/AUC_p. For these three M/P pairs, the clearance values for both the parents and metabolites were available following IV administration. The literature clearance values utilized for codeine, morphine, morphine-6-glucuronide, caffeine and theophylline were 63, 120, 7.5, 6.7 and 0.59 L/hr, respectively [19–22]. The F_h values utilized for the parents codeine, morphine and caffeine were 0.5, 0.2 and 1, respectively [23,24]. The f_m values utilized for the formation of the metabolites morphine, morphine-6-glucuronide and theophylline were 0.1, 0.1 and 0.03, respectively [25–27]. Since codeine, morphine and caffeine are highly water soluble and readily absorbed from the gastrointestinal tract, F_a is expected to be unity for these drugs [24,26,27]; hence, the bioavailability values utilized for the predictions are considered to exclusively represent the fraction of drug eliminated during first pass hepatic metabolism (F_h). Table 1 summarizes the observed and predicted AUC_m/AUC_p values for these M/P pairs. Using the known *in vivo* clearance values, the predictions were within a 3-fold error in comparison to the average observed AUC_m/AUC_p for all 3 M/P pairs after both IV and PO administration.

The second dilemma posed by the MIST guidance is how to determine the steady-state AUC_m/AUC_p for relevant, potentially yet unidentified, metabolites without performing elaborate multiple dose studies with radiolabeled drug. Based on established

pharmacokinetic theory [28] single dose data could be used as a measure of steady-state AUC_m/AUC_p as long as clearance is constant (linear kinetics). A classic study demonstrated in nonhuman primates that the $AUC_{0-\infty}$ ratios of carbamazepine epoxide/carbamazepine and 3-bromocinnamamide/cinromide P/M pairs after single dose are identical to the $AUC_{0-\tau}$ ratios at steady-state [29]. To further examine this relationship, the AUC_m/AUC_p for all M/P pairs with both single dose and steady-state *in vivo* data were collected. In total, 25 M/P pairs were examined (Figure 1 and Table 2). There was no statistically significant difference between single dose and steady-state AUC_m/AUC_p (Wilcoxon Signed Rank p-value > 0.47) and 84% of the M/P pairs included in the analysis had a single dose AUC_m/AUC_p within 2-fold of the steady-state AUC_m/AUC_p. 56% of the M/P pairs analyzed contained a metabolite that displayed ERL kinetics and hence the metabolite accumulation to steady-state is determined by its elimination half-life rather than the half-life of the parent (Table 2). Therefore parent drug will reach steady state before the metabolite. This presents a fundamental dilemma in study design since it is impossible to know whether a metabolite has reached steady-state, before relevant *in vivo* metabolites are identified and characterized. The ambiguity of when metabolite steady-state is reached is emphasized by the fact that over half of the metabolites examined between single dose and steady-state, displayed elimination rate limited kinetics. This analysis suggests that single dose AUC_m/AUC_p data can be used as a surrogate for steady-state AUC_m/AUC_p and may be more reliable. However, it is critical to confirm that after single dose administration, samples are collected for 4 to 6 parent or metabolite half-lives, whichever is longer, to capture $AUC_{0-\infty}$.

3. Metabolite Kinetic Theory: *In vitro*-to-*In vivo* Extrapolations

3.1 Qualitative Predictions of *In vivo* Metabolite Exposure

There has been a considerable amount of discussion on how to identify important circulating metabolites during pre-clinical phases of development of a new drug candidate [11,30]. Advances in and increased access to analytical technologies have made metabolite identification a routine part of new drug development. Most techniques focus on metabolic incubations of either a radiolabeled (if available) or nonradiolabeled new drug candidate to generate potential metabolites. The products of these incubations are then subjected to ultra performance liquid chromatography, to separate closely chemically related species, and coupled to either accurate mass spectrometry or NMR spectroscopy for structural determination [31–33]. This procedure results in the identification and quantification of potential metabolites formed from the new drug candidate, but the relative abundance of each metabolite formed *in vitro* often does not agree with its relative abundance *in vivo* [11].

Intuitively, one would expect that a major metabolite in HLMs or hepatocytes would also be a major metabolite in plasma. However, the clearances of primary metabolites vary, even within closely chemically related species such as two primary metabolites of the same parent. The relative exposure to different metabolites formed from the same parent drug will depend on the rank order of the ratio of formation clearance to elimination clearance for each metabolite (Equation 2). Hence, a major metabolite observed in HLMs or hepatocytes will not be dominant *in vivo* unless it has sufficiently low elimination clearance in comparison to other metabolites formed and in comparison to the parent drug. Whether this is clinically important was tested using published literature data of all M/P pairs for which both *in vivo* AUC_m/AUC_p and *in vitro* metabolite intrinsic formation clearance ($Cl_{i,f}$) data for parent drug with at least two metabolites was available. In total, 31 M/P pairs from 14 parent drugs were examined. Table 3 summarizes the *in vitro* $Cl_{i,f}$ and the observed *in vivo* AUC_m/AUC_p ratios for each M/P pair examined for a given parent drug. AUC_m/AUC_p ratios for each M/P pair, for a given parent, were considered to rank correctly with respect to *in vitro* $Cl_{i,f}$ if the M/P pair with a > 15% higher $Cl_{i,f}$ also had a > 15% AUC_m/AUC_p. Only 7 parent drugs (50%) had AUC_m/AUC_p ratios for their respective metabolites that rank

ordered correctly. One of the parent drugs examined in the rank order analysis was clomipramine. Clomipramine has three important metabolites: 8-hydroxyclomipramine, N-desmethylclomipramine and 2-hydroxyclomipramine. Based solely on *in vitro* $Cl_{i,f}$, N-desmethylclomipramine would be predicted to be the major metabolite, followed by equal exposures to 8-hydroxy and 2-hydroxyclomipramine. However, 8-hydroxyclomipramine can be detected *in vivo* in plasma at 40% of the parent whereas 2-hydroxyclomipramine is undetectable in plasma. This can be explained by the greater intrinsic elimination clearance of 2-hydroxyclomipramine when compared to 8-hydroxyclomipramine, 6.5 versus 1.5 μL/min/mg microsomal protein [34]. Incorrect rank ordering of metabolites for a given parent drug for half of the cases examined demonstrates that the consideration of only *in vitro* metabolite formation clearance is not sufficient for predicting the relative importance of a given metabolite *in vivo*.

3.2 Quantitative Predictions of *In vivo* Metabolite Exposure

The *in vivo* metabolite kinetic theory developed by Pang et al, Houston et al and others laid the foundation of *in vivo* metabolite pharmacokinetics but these theories have not yet been applied to *in vitro*-to-*in vivo* extrapolation, a useful tool in anticipating the *in vivo* pharmacokinetics of a parent drug during new drug development. The prediction of *in vivo* clearance of drugs based on *in vitro* metabolism data is well established, although predictions have varying degrees of accuracy [35,36]. In an extensive analysis of scaling *in vitro* HLM clearance values to *in vivo* clearance using multiple hepatic clearance models and plasma protein binding considerations, 29 drugs with varying physicochemical properties were predicted with a 2.14 to 4.39 average fold error [37]. Another study of scaling *in vitro* Cl_i values from human hepatocyte data for 50 drugs, obtained a 2.5 average fold error to the observed *in vivo* clearance, with outliers having up to 15-fold error [38]. Although quantitative *in vitro*-to-*in vivo* clearance prediction for a parent drug is now commonplace, little attention has been paid to the prediction of the *in vivo* disposition of a metabolite from *in vitro* metabolism data.

The MIST guidance requires the evaluation of absolute steady-state AUC_m between pre-clinical animal species and human for major metabolites. If similar exposure is not obtained in animals, additional safety testing of the metabolite may be required. This requirement generates a need to predict, prior to clinical studies, what metabolites will be quantitatively important in humans. Predicting absolute AUC_m values in humans poses a significant challenge because the result will depend on the dose of the parent, the fraction of the dose absorbed after PO administration, and the overall clearance of the parent drug. In addition, the AUC_m will depend on the metabolite specific parameters, such as the fraction of the dose converted to the metabolite of interest and the metabolite clearance. Within the MIST guidance, the secondary qualification of the *relative* exposure to the metabolite in human, i.e. $AUC_m/AUC_p > 0.1$, appears more conducive to prediction. The AUC_m/AUC_p is independent of the parent dose and fraction absorbed after PO administration and the predicted AUC_m/AUC_p can be utilized as a proportionality constant for anticipating the absolute levels of the metabolite of interest when a desired AUC or steady-state concentration of parent is ascertained. This can be illustrated via a review of the existing data on desipramine as a metabolite of imipramine, which demonstrates that within a 4-fold range of *in vivo* doses of imipramine, the AUC_m/AUC_p remains constant (Table 4) in cytochrome P450 (CYP) 2D6 extensive metabolizers (EMs). The absolute steady-state concentration of desipramine could be predicted by use of the steady-state concentration of imipramine and the AUC_m/AUC_p ratio. Normalizing the predicted metabolite exposure to that of the parent also provides valuable insight into whether the metabolite will be quantitatively important *in vivo*, regardless of the parent dose.

The prediction of *in vivo* AUC_m/AUC_p from *in vitro* parameters relies on methods for clearance predictions of both parent and metabolite. This is because an important principle of metabolite kinetics is that the *in vivo* disposition of a metabolite is dependent not only on its formation clearance, but also its elimination. Based on this principle, predicting relative exposure to human metabolites can only be done if the formation and elimination clearances for the metabolite are predicted.

To adapt Equation 2 to *in vitro*-to-*in vivo* extrapolation of AUC_m/AUC_p after intravenous (IV) or oral (PO) administration, four assumptions were made: 1) the kinetics of both parent and metabolite are linear, 2) all metabolite formed is available to the systemic circulation, 3) parent and metabolite elimination is via metabolism only and 4) metabolism occurs only in the liver which can be represented by the well-stirred model [39]:

$$Cl_h = \frac{Q * f_u * Cl_i}{Q + f_u * Cl_i} \quad (4)$$

where CL_h is the hepatic clearance, Q is the hepatic blood flow, $f_{u,}$ is the plasma fraction unbound and Cl_i is the hepatic intrinsic metabolic clearance. This clearance model can be applied to either the metabolite or the parent drug. In addition to this well-stirred model of the liver, two other prominent hepatic clearance models, the parallel tube and dispersion model, could be used for predictions [40]. At present the potential advantages of the alternative hepatic clearance models for predicting metabolite formation and elimination clearances are unknown and require further study. The well-stirred model was chosen for this analysis because it is the most commonly utilized hepatic clearance model and easiest to adapt for predictions. Furthermore, a general metabolic scheme based on the scheme of Houston and Taylor [14] can be considered for both IV and PO administration (Figure 2). In this scheme, D_g, D_h and D_s are the amounts of drug in the gut lumen, liver during first pass and systemic circulation, respectively. F_a and F_h are the fraction of drug absorbed from the gut lumen into the enterocytes and fraction of drug that escapes first pass elimination in the liver, respectively, and M_h and M_s refer to the amount of metabolite formed from first pass in the liver and from systemic elimination, respectively. The *in vivo* fraction of parent converted to the metabolite of interest, when the parent is cleared only through hepatic metabolism was previously defined as the fraction of hepatic parent drug clearance that results in the metabolite of interest [41]. This definition was adapted to *in vitro* parameters and defined as $f_{m,h}$:

$$f_{m,h} = \frac{Cl_{i,f}}{Cl_{i,p}} \quad (5)$$

where $Cl_{i,f}$ and $Cl_{i,f}$ are the intrinsic formation clearance of the metabolite *in vitro* and intrinsic elimination clearance of the parent *in vitro*, respectively. When the parent is cleared entirely via hepatic metabolism, the *in vivo* f_m for the metabolite of interest is equal to the *in vitro* $f_{m,h}$. Utilizing this metabolic scheme, the AUC_m after PO administration can be defined as:

$$AUC_m = \frac{f_{m,h} * F_a * (1 - F_h) * D}{Cl_m} + \frac{f_{m,h} * F_a * F_h * D}{Cl_m} \quad (6)$$

and the AUC_p after PO administration can be defined as:

$$AUC_p = \frac{F_a * F_h * D}{Cl_p} \tag{7}$$

By definition [42], the hepatic bioavailability (F_h) is a function of the extraction ratio of the parent (ER_p):

$$F_h = 1 - ER_p = \frac{Q}{Q + f_{u,p} * Cl_{i,p}} \tag{8}$$

Substituting for F_h and the well-stirred model for Cl_p and Cl_m (as defined by Equations 9 and 5, respectively) into the quotient of Equations 7 and 8 yields:

$$\frac{AUC_m}{AUC_p} = \frac{f_{u,p} * Cl_{i,f}}{f_{u,m} * Cl_{i,m}} * \frac{Q + f_{u,m} * Cl_{i,m}}{Q} \tag{9}$$

After intravenous administration, by substituting $f_{m,h}$ for f_m (Equation 6) and the well-stirred model for Cl_m and Cl_p (Equation 5), Equation 2 can be defined as:

$$\frac{AUC_m}{AUC_p} = \frac{f_{u,p} * Cl_{i,f}}{f_{u,m} * Cl_{i,m}} * \frac{Q + f_{u,m} * Cl_{i,m}}{Q + f_{u,p} * Cl_{i,p}} \tag{10}$$

Utilizing the common technique of evaluating the limits of pharmacokinetic models with respect to high ER ($Q \ll f_u * Cl_i$) or low ER ($Q \gg f_u * Cl_i$), the above two models presented result in three pharmacokinetic outcomes: 1) the relative exposure to the metabolite (AUC_m/AUC_p) will be different after IV and PO administration when the parent drug has a high ER, but the AUC_m/AUC_p is independent of route of administration when ER_p is low, 2) changes in the intrinsic clearance of a metabolite with a low ER (ER_m) will alter the exposure to the metabolite resulting in changes in the AUC_m/AUC_p and 3) the relative exposure to the metabolite depends on the ratio between its formation and elimination clearances, not on the absolute value of either of these two terms. To determine whether the relative exposure to a metabolite is dependent on route of administration when the parent drug has a high ER, the exposure to morphine after PO or IV administration of codeine and the exposure to morphine-6-glucuronide after PO or IV administration of morphine was revisited (Table 1). Based on literature *in vivo* clearance values, formation of morphine from codeine is classified as high ER_p, high ER_m, while formation of morphine-6-glucuronide from morphine is classified as high ER_p, low ER_m. The observed AUC_m/AUC_p values are shown in Table 1. Indeed, as the model suggests, the relative exposure to the metabolite was dependent on route of administration for morphine-6-glucuronide, where it was always greater after PO administration than IV administration. The relative exposure to morphine after IV administration of codeine ranged from 3.5-fold less to 2.4-fold greater than after PO administration. Given the variability in AUC_m/AUC_p for the morphine/codeine pair after PO administration, it was not possible to define a clear relationship between route of administration and AUC_m/AUC_p.

It has been theoretically demonstrated that changes in the elimination clearance of a low ER metabolite will alter the relative exposure to that metabolite [13]. This can be illustrated by considering the exposure to desipramine as a metabolite of imipramine. If the clearance pathway of the metabolite is subject to genetic polymorphisms, the AUC_m/AUC_p will

depend on the individual's genotype for that elimination pathway. The formation of desipramine from imipramine is mediated primarily by CYP2C19, whereas desipramine elimination is mediated by CYP2D6. The desipramine/imipramine AUC_m/AUC_p was 5.4-fold higher in CYP2D6 poor metabolizers (PMs) in comparison to EMs making desipramine the major circulating species in CYP2D6 PMs (Table 4). This increase in AUC_m/AUC_p was due to a 1.7- and 9.1-fold increase in the AUC of imipramine and desipramine, respectively.

To test the developed *in vitro*-to-*in vivo* extrapolation model (Equations 10 and 11 for PO and IV administration, respectively), the AUC_m/AUC_p of seven M/P pairs: morphine/codeine, theophylline/caffeine, desipramine/imipramine, nortriptyline/amitriptyline, N-desmethylclomipramine/clomipramine, 8-hydroxyclomipramine/clomipramine and 2-hydroxyclomipramine/clomipramine were predicted. The predictions were accomplished using literature *in vitro* metabolite $Cl_{i,f}$ and $Cl_{i,m}$ obtained in human liver microsomes (HLM), literature plasma fraction unbound values for both parent and metabolite and common methods of clearance scaling. The Cl_i values (in μL/min/mg microsomal protein) were first scaled to grams of liver, using the value of 54.7 mg microsomal protein per g liver, and then to kg body weight, using the value of 21.43 g liver per kg body weight [43,44]. An average body weight of 70 kg was considered. Summary of the obtained predicted and observed AUC_m/AUC_p values are shown in Table 5. Four of the seven M/P pairs were accurately predicted (< 2-fold error in comparison to the average observed AUC_m/AUC_p) and two of the seven were predicted within < 5-fold error (Table 5). 2-hydroxyclomipramine had undetectable metabolite levels after clomipramine administration and hence the prediction of very low AUC_m/AUC_p for this pair is considered in agreement with the *in vivo* finding.

It is unlikely that the major inaccuracies in the above AUC_m/AUC_p predictions are a result of *in vitro* to *in vivo* scaling, since the use of *in vivo* parameters did not yield more accurate predictions (Tables 1 and 5). The morphine/codeine AUC_m/AUC_p could be predicted accurately from both *in vitro* and *in vivo* parameters (< 2-fold error in comparison to the average observed AUC_m/AUC_p) after both IV and PO administration and the theophylline/caffeine M/P pair yielded similar prediction accuracies when using either *in vitro* values or *in vivo* (4-fold versus 3-fold error). Additionally, by utilizing a ratio as the primary predicted measure, any systematic error made in the scaling of *in vitro*-to-*in vivo* clearances is negated although random error in the prediction will propagate in the AUC ratio.

The model is designed to predict the nonparametric outcome of AUC_m/AUC_p. Oftentimes, the pharmacologic or toxicologic effect is a function of the maximum concentration (C_{max}) and not the total body exposure. Predictions of metabolite C_{max} would require additional parametric information about the input and disposition rates of both the parent and the metabolite only obtainable after *in vivo* administration of the compounds.

One obvious limitation to this model is that it can only address the disposition of primary metabolites formed after parent administration. Theoretically, the same necessity of predicting both formation and elimination are relevant for subsequent downstream metabolites making quantitative predictions of downstream metabolites very complicated. However, if primary metabolites are used in *in vitro* incubation experiments, the likelihood of qualitatively identifying downstream metabolites is greatly increased.

Renal clearance as well as biliary excretion and gut metabolism are often important elimination pathways for xenobiotics. These pathways were not considered for either the parent or metabolite in this review and it is likely that to be fully applicable in new drug development, prediction of the total clearance of the metabolite (sum of predicted hepatic and renal clearances) will be necessary. Unfortunately *in vitro*-to-*in vivo* extrapolation

models for prediction of renal clearance, transport and gut metabolism are currently not as well established as hepatic clearance predictions. Nonetheless, the preliminary success of the predictions indicates that *in vitro*-to-*in vivo* extrapolation of AUC_m/AUC_p, after further development and validation, could prove to be a useful tool in addressing metabolite-related concerns in new drug development.

4. Conclusions

This review of the available data on metabolite disposition shows that as predicted by original metabolite kinetic theory, for any quantitative or semi-quantitative prediction of metabolite abundance or relative importance *in vivo*, the *in vivo* elimination clearance of the metabolite has to be predicted or rationalized in addition to basic metabolite profiling *in vitro*. This is shown by the fact that the *in vivo* abundance of metabolites of a given drug are no more likely to rank order correctly based on *in vitro* formation clearances than when left to random probability. An interesting outcome of the literature review is that in comparison to parent clearance predictions, the AUC_m/AUC_p predicts with similar accuracy suggesting that important human metabolites can be quantitatively predicted using *in vitro* data. This accuracy was achieved despite the fact that *in vitro* $Cl_{i,f}$ and $Cl_{i,m}$ were usually not determined in the same study. Finally, the fact that AUC_m/AUC_p ratios measured after single dose administration were not significantly different from multiple dose AUC_m/AUC_p ratios, but ERL kinetics of metabolites were common suggests that early single dose studies for metabolite identification may be justified.

5. Expert Opinion

Based on available pharmacokinetic theory and literature data, the AUC_m/AUC_p is most appropriate value to be used as the relevant outcome measure of metabolite exposure in *in vitro*-to-*in vivo* extrapolation. Since this value does not depend on dose or the F_a of the parent drug, it is a more robust and generally applicable measure of metabolite exposure than the dose-dependent measure of absolute AUC_m. Predicting absolute AUC_m would require knowledge of the clinical dose of the parent drug and prediction of its F_a. Additionally, the predicted AUC_m/AUC_p, when multiplied by the expected clinically effective average steady state concentration, can be used to determine prior to human studies how likely the need for additional safety evaluation is.

The results of this review clearly show that the formation clearance of a metabolite is not sufficient for understanding and predicting its *in vivo* relative exposure (AUC_m/AUC_p) or importance in comparison to other metabolites. Although the equally important role of metabolite elimination and metabolite formation in metabolite disposition *in vivo* is generally known, this concept has yet to be applied to *in vitro*-to-*in vivo* extrapolation in preclinical new drug development. Inclusion of metabolite formation and elimination clearances in predictions allows prediction of *in vivo* AUC_m/AUC_p or, if AUC_p is known, the absolute AUC_m from *in vitro* data. It is interesting that after development of the early metabolite kinetic theory it has not been thoroughly reexamined in light of modern experimental approaches. It is likely that better understanding of metabolite disposition can be obtained by further testing *in vitro*-to-*in vivo* extrapolation models applied to metabolites even by using a relatively simplistic model detailed in this review. It would also be beneficial if such models would be further developed and validated. This is important not only to address the MIST guidance, but to improve our current understanding of the *in vivo* pharmacokinetics of probe metabolic ratios that are used in drug-drug interaction studies as well as in pharmacogenetic studies.

The developed model and future models addressing the same primary outcome may be useful in lead compound selection and toxicology stages of new drug development, allowing early attention on potential quantitatively important metabolites. Since chemical synthesis of metabolites can be time consuming, expensive and difficult, it is likely that predictions of metabolite clearance need to be obtained from minimal amounts of primary metabolites generated in *in vitro* systems and isolated using chromatographic techniques to justify investment of resources to synthesis of reference materials. However, the obtained predictions can be used to guide prioritization of synthetic efforts of metabolite standards. It is noteworthy, that intrinsic metabolic elimination clearance of a metabolite can be predicted from a substrate depletion experiment conducted below K_m (Michaelis-Menten affinity constant) concentrations of the metabolite [45,46]. Additionally, pre-clinical animal studies can be utilized not only to determine the absolute abundance of a metabolite after parent administration, but also to examine the overall pharmacokinetics of the metabolite after administration of the metabolite or the parent. This data can be leveraged for in vivo human metabolite kinetic prediction and may provide further confidence in the *in vivo* predictions of metabolite clearance and AUC_m/AUC_p from *in vitro* data.

The *in vivo* AUC_m/AUC_p of only seven M/P pairs were predicted from *in vitro* data in this review. The unfortunate limitation was the dearth of literature on *in vitro* metabolite Cl_i values, and this review suggests that there is a great need to generate more metabolite relevant *in vitro* kinetic data. Even M/P pairs that are commonly utilized as *in vivo* CYP probes possess metabolite elimination pathways that are not kinetically characterized. For example, the urinary or plasma ratio of dextrorphan and dextromethorphan is a common probe for phenotyping CYP2D6, yet the major elimination pathway of dextrorphan is via glucuronidation, a pathway that has never been kinetically characterized *in vitro* [47,48]. Additionally, the plasma ratios of 5-hydroxyomeprazole and omeprazole or omeprazole sulfone and omeprazole are common probes for CYP2C19 and CYP3A4 drug interactions, respectively, yet neither the kinetics of 5-hydroxyomeprazole nor omeprazole sulfone metabolism are characterized *in vitro* [49,50]. This raises some concerns of the validity of these ratios, as genetic factors or drug-drug interactions affecting the unknown elimination pathways of the metabolite could result in skewed data.

Increasingly, M/P ratios are being utilized as specific *in vivo* CYP markers [51]. When a metabolite is considered pharmacokinetically relevant, the enzymes responsible for the elimination of that metabolite should be identified for proper interpretation of drug-drug interaction and genetic polymorphism studies. When a metabolite is considered pharmacologically relevant, again, these secondary metabolic pathways should be identified in order to understand the therapeutic impact of said drug-drug interaction or genetic polymorphism.

ARTICLE HIGHLIGHTS

- There is an increased interest in qualitative and quantitative prediction of *in vivo* circulating metabolites and in rationalization of metabolite exposures.
- The relative importance of metabolites *in vivo* is measured as the ratio between the metabolite's and parent drug's area under the plasma concentration versus time curve (AUC_m/AUC_p). This ratio can be predicted using *in vitro*-to-*in vivo* scaling of formation and elimination clearances of the metabolite of interest.
- Single dose AUC_m/AUC_p data can be utilized as a surrogate for steady-state AUC_m/AUC_p when the compounds have linear kinetics. 56% of reviewed metabolites underwent elimination rate limited kinetics and hence time to reach

steady-state for the metabolites is governed by the half-life of the metabolite, not the half-life of the parent drug.

- When multiple metabolites are formed from the same parent drug, the rank order of importance observed in *in vitro* systems correctly predicts the rank order importance *in vivo* for only half of the drugs. This discrepancy is most likely due to great variability in the clearances of the metabolites. Prediction of the identity of major metabolites in contrast to minor metabolites in circulation is likely to require determination of the clearance of the metabolites as well. There is no significant correlation between the formation clearance of a given metabolite *in vitro* and the relative abundance (AUC_m/AUC_p) of that metabolite *in vivo*.
- If a metabolite is used as a probe of specific enzyme activity, the elimination pathways of that metabolite should be characterized.

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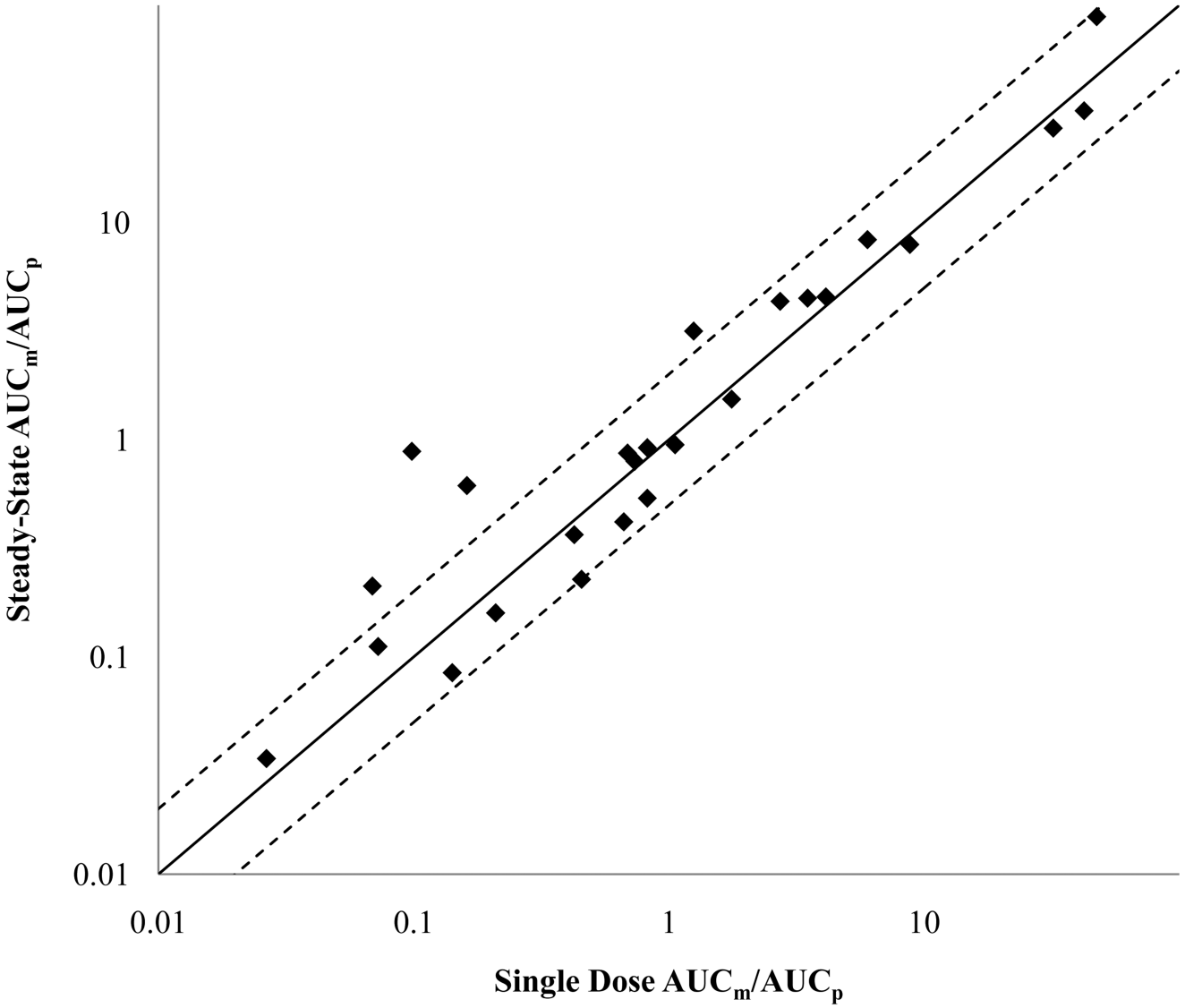

Figure 1. Relationship between the single dose and steady-state *in vivo* AUC_m/AUC_p for 25 M/P pairs

The solid line indicates no difference and the dashed line indicates a 2-fold difference between single dose and steady-state dosage regimens.

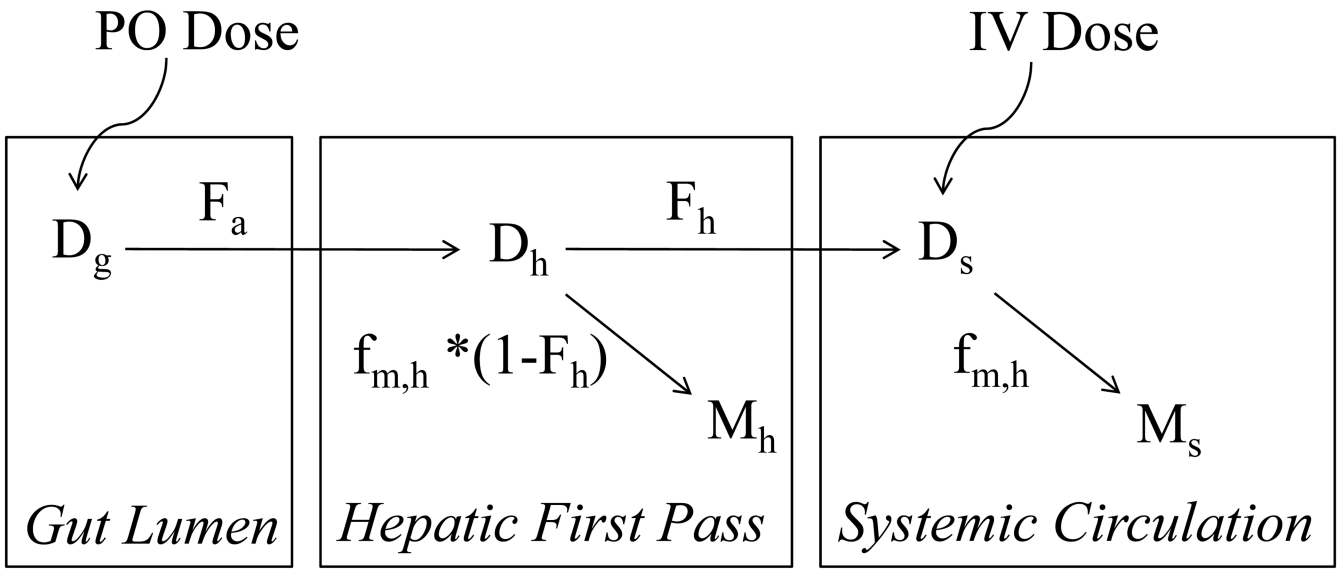

Figure 2. Schematic representation of the metabolic fate of a parent drug after PO or IV administration

In this scheme, D_g, D_h and D_s are the amounts of drug in the gut lumen, liver during first pass and systemic circulation, respectively. F_a and F_h are the fractions of drug that are absorbed into the enterocytes and that escape first pass elimination in the liver, respectively. The $f_{m,h}$ term is the fraction of hepatic metabolism that results in the metabolite of interest and M_h and M_s refer to the amount of metabolite of interest, formed from first pass in the liver and systemic elimination, respectively.

Table 1

The AUC_m/AUC_p observed *in vivo* and predicted using observed Cl_p, Cl_m, f_m, and bioavailability (F) values for three M/P pairs for which *in vivo* clearance data of the metabolite were available.

Parent	Metabolite	Route of Administration	Observed AUC_m/AUC_p (Range)	Predicted AUC_m/AUC_p	Predicted/ Observed (Range)	References
Codeine	Morphine	IV	0.068[a]	0.053	0.71	1,2,3
		PO	0.071 (0.019 – 0.17)	0.11	1.5 (0.64 – 5.8)	
Morphine	Morphine-6-glucuronide	IV	1.4[a]	1.6	1.1	4,5,6
		PO	2.8 (2.8 – 2.8)	7.9	2.8 (2.8 – 2.8)	
Caffeine	Theophylline	PO	0.12 (0.11 – 0.14)	0.34	2.8 (2.4 – 3.1)	7,8

Notes: Where multiple data sets exist, the ranges of AUC_m/AUC_p values are provided in parentheses. All observed AUC_m/AUC_p values were determined after single dose administration.

Superscript 'a' indicates that only one *in vivo* study was available. Reference numbers coincide with the below provided references and not with the bibliography references.

References: [1]Osborne et al, 1990; [2]Yue et al, 1991a; [3]Yue et al, 1991b; [4]Du et al, 1999; [5]Eliot et al, 2002; [6]Guay et al, 1988; [7]Akinyinka et al, 2000 and [8]Rodopoulos et al, 1996.

Expert Opin Drug Metab Toxicol. Author manuscript; available in PMC 2011 September 1.

Table 2

The 25 M/P pairs utilized to examine the difference between single dose and steady-state AUC_m/AUC_p. Included is the AUC_m/AUC_p after a single dose and at steady-state and whether or not the metabolite is elimination rate limited.

Parent	Metabolite	Elimination Rate Limited?	Single Dose AUC_m/AUC_p	Steady-State AUC_m/AUC_p	References
Allopurinol	Oxypurinol	Yes	42	33	1,2
Alprazolam	4-hydroxyalprazolam	No	0.073	0.11	3,4,5
	1-hydroxyalprazolam	Yes	0.027	0.034	
Atorvastatin	2-hydroxyatorvastatin	Yes	0.69	0.87	6,7
	4-hydroxyatorvastatin	Yes	0.098	0.88	
Clarithromycin	14-hydroxyclarithromycin	Yes	0.67	0.42	8,9
Ezetimibe	Ezetimibe-O-glucuronide	No	8.8	7.9	10,11
Hydralazine	Hydralazine pyruvic acid hydrazone	Yes	47	89	12
Imatinib	N-desmethylimatinib	Yes	0.21	0.16	13,14
Lansoprazole	5-hydroxylansoprazole	No	0.14	0.085	15,16
	Lansoprazole sulfone	No	0.16	0.61	
Modafinil	Modafinil acid	No	0.43	0.37	17,18
Nelfinavir	Nelfinavir hydroxy-t-butylamide	No	0.45	0.23	19
Nortriptyline	10-hydroxynortriptyline	No	1.3	3.2	20,21
Omeprazole	5-hydroxyomeprazole	No	0.82	0.92	22,23
	Omeprazole sulfone	Yes	0.82	0.54	
Oxcarbazepine	10-hydroxycarbazepine	Yes	32	27	24,25

Parent	Metabolite	Elimination Rate Limited?	Single Dose AUC_m/AUC_p	Steady-State AUC_m/AUC_p	References
Pentoxifylline	1-(3-carboxypropyl)-3,7-dimethylxanthine	Yes	6.0	8.3	26,27
	1-(4-carboxybutyl)-3,7-dimethylxanthine	Yes	0.74	0.80	
	1-(5-hydroxyhexyl)-3,7-dimethylxanthine	Yes	4.1	4.5	
Risperidone	Paliperidone	No	3.5	4.5	28,29
Sertraline	N-desmethylsertraline	Yes	1.8	1.5	30,31
Sulfinpyrazone	Sulfinpyrazone sulfone	No	0.069	0.21	32,33
Terbinafine	N-desmethylterbinafine	No	1.1	0.95	34,35
Venlafaxine	Desvenlafaxine	Yes	2.7	4.3	36,37

Notes: The AUC for both parent and metabolite after single dose was assured to represent the $AUC_{0-\infty}$, and the $AUC_{0-\tau}$ for both parent and metabolite at steady-state were assured to be truly under steady-state conditions. In the studies of hydralazine and nelfinavir, the AUC_m/AUC_p was determined after pairing single dose and multiple doses in the same subjects. Reference numbers coincide with the below provided references and not with the bibliography references.

References: [1]Ayalasomayajula et al, 2008; [2]Guerra et al, 2001; [3]Buch et al, 1993; [4]Greene et al, 1995; [5]Wennerholm et al, 2005; [6]Lau et al, 2007; [7]Tekturna New Drug Application, 2007; [8]Cheng et al, 1998; [9]Hassan-Alin et al, 2006; [10]Bergman et al, 2006; [11]Reyderman, 2005; [12]Shepherd et al, 1980; [13]Egorin et al, 2009; [14]Van Erp et al, 2007; [15]Kim et al, 2002; [16]Ieiri et al, 2001; [17]Wong et al, 1998; [18]Wong et al, 1999; [19]Damle et al, 2009; [20]Laine et al, 2001; [21]Yue et al, 1998; [22]He et al, 2003; [23]Shirai et al, 2001; [24]Keranen et al, 1992; [25]Theis et al, 2005; [26]Mauro et al, 1992; [27]Smith et al, 1986; [28]Bialer et al, 2004; [29]Bondolfi et al, 2002; [30]Allard et al, 1999; [31]Hamelin et al, 1996; [32]Bradbrook et al, 1982; [33]Rosenkranz et al, 1983; [34]Kovarik et al, 1992; [35]Robbins et al, 1996; [36]Lindh et al, 2003 and [37]Troy et al, 1995.

Table 3

Rank order analysis of the *in vitro* $Cl_{i,f}$ and the *in vivo* AUC_m/AUC_p for M/P pairs of a given parent drug.

Parent	Metabolite	*In vitro* $Cl_{i,f}$ (μL/min/mg)	*In vivo* AUC_m/AUC_p	Rank Order Correctly?	References
Alprazolam	1-hydroxyalprazolam	0.51	0.11	No	1,2,3
	4-hydroxyalprazolam	4.1	0.034		
Atorvastatin	2-hydroxyatorvastatin	20	0.87	No[b]	4,5
	4-hydroxyatorvastatin	9.1	0.88		
Caffeine*	Theophylline	0.072	0.14	Yes	6,7
	Paraxanthine	1.4	0.48		
	Theobromine	0.019	0.080		
Clomipramine	2-hydroxyclomipramine	0.98	ND[a]	No[b]	8,9
	8-hydroxyclomipramine	0.94	0.36		
	N-desmethylclomipramine	16	2.2		
Codeine*	Morphine	0.23	0.029	No	10,11,12
	Norcodeine	1.1	0.25		
	Codeine-6-glucuronide	0.45	9.2		
Imipramine*	Desipramine	8.3	0.54	Yes	13,14
	2-hydroxyimipramine	2.8	0.37		
Lansoprazole	5-hydroxylansoprazole	19	0.085	No	15,16
	Lansoprazole sulfone	10	0.61		
Morphine*	Morphine-3-glucuronide	8.4	23	Yes	17,18
	Morphine-6-glucuronide	1.8	2.8		
Nefazodone	Hydroxynefazodone	239	0.36	Yes	2,19
	Meta-chlorophenylpiperazine	190	0.090		
Omeprazole	5-hydroxyomeprazole	24	0.92	Yes	20,21
	Omeprazole sulfone	20	0.54		

Parent	Metabolite	*In vitro* $Cl_{i,f}$ (μL/min/mg)	*In vivo* AUC_m/AUC_p	Rank Order Correctly?	References
Propafenone	5-hydroxypropafenone	530	0.50	Yes	22,23
	N-desalkylpropafenone	3.5	0.064		
Quinidine	3-hydroxyquinidine	15	0.24	No	24,25
	Quinidine N-oxide	2.9	0.37		
Tamoxifen	4-hydroxytamoxifen	2.3	0.034	Yes	26,27
	N-desmethyltamoxifen	31	2.1		
Theophylline*	3-methylxanthine	0.012	0.035	No[b]	28,29
	1,3-dimethyluric acid	0.16	0.032		

Notes: The *in vitro* $Cl_{i,f}$ and *in vivo* AUC_m/AUC_p values are provided. Additionally, whether or not the metabolites of a given parent rank ordered correctly with respect to these two values is provided. All *in vivo* AUC_m/AUC_p values were determined under steady-state conditions except for those parent drugs marked with an asterisk, which were determined after single dose administration.

Superscript 'a' indicates a value not determined because the observed *in vivo* metabolite concentrations were not detectable.

Superscript 'b' indicates a set of M/P pairs for a given parent drug that were considered to not rank order correctly because the *in vitro* $Cl_{i,f}$ values exhibited a > 15% difference but the AUC_m/AUC_p values did not or the AUC_m/AUC_p values exhibited a > 15% difference but the *in vitro* $Cl_{i,f}$ values did not. Reference numbers coincide with the below provided references and not with the bibliography references.

References: [1]Buch et al, 1993; [2]Greene et al, 1995; [3]Hirota et al, 2001; [4]Park et al, 2008; [5]Tekturna New Drug Application, 2007; [6]Labedzki et al, 2002; [7]Akinyinka et al, 2000; [8]Kramer-Nielsen et al, 1996; [9]Linnoila et al, 1982; [10]Soars et al, 2001; [11]Yue et al, 1997; [12]Yue et al, 1991b; [13]Skelbo et al, 1992; [14]Koyama et al, 1994; [15]Ieira et al, 2001; [16]Kim et al, 2003; [17]Morrish et al, 2006; [18]Eliot et al, 2002; [19]VonMoltke et al, 1999; [20]Shirai et al, 2001; [21]Shu et al, 2000; [22]Dilger et al, 1999; [23]Hemeryck et al, 2000; [24]Nielsen et al, 1999; [25]Schellens et al, 1991; [26]Desta et al, 2004; [27]Lien et al, 1990; [28]Tjia et al, 1996 and [29]Rodopoulos et al, 1996.

Table 4

The effect of dose and CYP2D6 phenotype on the imipramine to desipramine AUC_m/AUC_p ratio after PO administration of imipramine.

Imipramine Dose (mg)	CYP2D6 Phenotype	Imipramine AUC (nmol*h/mL)	Desipramine AUC (nmol*h/mL)	Observed AUC_m/AUC_p	References
25	EM	0.68	0.37	0.54	1,2,3
25	PM	1.2	3.4	2.9	
50	EM	1.4	0.79	0.58	
100	EM	3.1	1.6	0.50	

Notes: All AUC values were determined after single dose administration. Reference numbers coincide with the below provided references and not with the bibliography references.

References: [1]Albers et al, 2000; [2]Koyama et al, 1994 and [3]Kurtz et al, 1997.

Table 5

The *in vivo* observed and *in vitro* predicted AUC_m/AUC_p values for seven M/P pairs.

Parent	Metabolite	Predicted AUC_m/AUC_p	Observed AUC_m/AUC_p (Range)	Predicted/ Observed (Range)	References
Codeine	Morphine	0.051[a]	0.068[a,b]	0.75	1,2,3
		0.042	0.071 (0.019 – 0.17)	0.60 (0.26 – 2.2)	
Caffeine	Theophylline	0.50	0.12 (0.11 – 0.14)	4.0 (3.6 – 4.6)	4,5
Imipramine	Desipramine	1.43	0.89 (0.50 – 1.6)	1.6 (0.89 – 2.9)	6,7,8,9, 10,11,12
	N-desmethylclomipramine	7.49	1.7 (1.2 – 2.2)	4.3 (3.4 – 6.1)	
Clomipramine	8-hydroxyclomipramine	0.22	0.40 (0.36 – 0.44)	0.55 (0.49 – 0.61)	13,14
	2-hydroxyclomipramine	0.05	ND[c]	ND	
Amitriptyline	Nortriptyline	0.45	0.47 (0.43 – 0.53)	0.91 (0.81 – 1.0)	15,16,17

Notes: All values are for after PO administration unless otherwise noted. All observed AUC_m/AUC_p ratios were determined after single dose administration, save for the two *in vivo* studies of clomipramine. ND indicates a value not determined.

Superscript 'a' indicates a predicted or observed AUC_m/AUC_p after IV administration.

Superscript 'b' indicates that only one *in vivo* study was available.

Superscript 'c' indicates a value not determined because the metabolite was not detected *in vivo*. Reference numbers coincide with the below provided references and not with the bibliography references.

References: [1]Guay et al, 1988; [2]Yue et al, 1991a; [3]Yue et al, 1991b; [4]Akinyinka et al, 2000; [5]Rodopoulos et al, 1996; [6]Abernethy et al, 1984; [7]Albers et al, 1995; [8]Albers et al, 2000; [9]Bergstrom et al, 1992; [10]Koyama et al, 1994; [11]Kurtz et al, 1997; [12]Wells et al, 1986; [13]Linnoila et al, 1982; [14]Shimoda et al, 1995; [15]Jiang et al, 2002; [16]Liedholm et al, 1998 and [17]Venkatakrishnan et al, 2001.