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Permalink
https://escholarship.org/uc/item/4s53v3fz

Journal
The AAPS Journal, 22(5)

ISSN
1550-7416

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Publication Date
2020-09-01

DOI
10.1208/s12248-020-00501-9

Peer reviewed

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Author manuscript

AAPS J. Author manuscript; available in PMC 2021 September 10.

Published in final edited form as:

AAPS J. ; 22(5): 120. doi:10.1208/s12248-020-00501-9.

Investigating the Theoretical Basis for In Vitro-In Vivo Extrapolation (IVIVE) in Predicting Drug Metabolic Clearance and Proposing Future Experimental Pathways

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Abstract

Introduction: Extensive studies have been conducted to predict in vivo metabolic clearance from in vitro human liver metabolism parameters (i.e., in vitro-in vivo-extrapolation; IVIVE) with little success.

Methods: Here, deriving IVIVE from first principles, we show that the product of fraction unbound in blood and the predicted in vivo intrinsic clearance determined from hepatocyte or microsomal incubations is the lower boundary condition for in vivo hepatic clearance and the prerequisite for IVIVE predictions to be valid, regardless of extraction ratio.

Results and Discussion: For 60-80% of drugs evaluated here, this product is markedly less than the in vivo measured clearance, a result that violates the lower boundary of the predictive relationship. This can only be explained by (a) suboptimal in vitro metabolic stability assay conditions, (b) significant error in the assumption that in vitro intrinsic clearance determinations will predict in vivo intrinsic clearance simply by scaling-up the amount of enzyme (in vitro incubation to in vivo liver) and/or (c) the methods of determining fraction unbound are incorrect. We further suggest that widely-employed organ blood flow values underpredict the effective blood flow within the organ by approximately 2.5-fold, thus impacting IVIVE of high clearance compounds.

Conclusion: We propose future pathways that should be investigated in terms of the relationship to experimentally measured clearance values, rather than model dependent intrinsic clearance. IVIVE outcome can be improved by estimating the ratio of unbound drug concentration in the liver tissue to the liver plasma, examining the assumption of the free drug theory (i.e., there are no transporter effects at the blood cell membrane) and the finding that the upper limit of organ clearance may be greater than blood flow entering the organ.

Keywords

IVIVE; Hepatic Clearance; Organ Blood Flow; Fraction Unbound

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Introduction

Drug development is an extremely expensive, time-consuming process with an unacceptable, very poor success rate. It is well recognized that if we could predict an NME's (new molecular entity's) human ADME (absorption, distribution, metabolism, excretion) pharmacokinetic characteristics prior to dosing the drug to humans, or even animals, this could markedly speed the drug development process (1, 2). It seemed logical that measurements of in vitro elimination in human hepatocytes or subcellular fractions such as microsomes should be a useful pathway to predict in vivo drug clearance for metabolized drugs (3-6). Obach et al. (7) detailed two methods to estimate in vivo human metabolic clearance from in vitro metabolism measurements, designated IVIVE, in vitro to in vivo extrapolation. The first followed that of Rané et al. (3) where in vitro measures of maximum rate of drug elimination and the concentration giving half of the maximum rate were determined and translated into values of intrinsic clearance (CL_{int}). In the second method, designated as the "in vitro T½ method", CL_{int} was determined by measuring the first order rate constant for drug elimination in a hepatocyte or microsome in vitro incubation and scaling this value up using relevant amounts of human liver metabolic enzymes. It is this second method that is primarily used today, with Obach (8) initially predicting human clearance of 29 drugs from hepatic microsomal intrinsic clearance data. Many further studies have attempted to use this methodology. However, in vitro measures of drug metabolic clearance in human liver tissue cannot adequately predict the in vivo human drug metabolic liver clearance across the panoply of metabolized substrates (9, 10), nor can in vitro measures of drug metabolic clearance in rat liver tissue adequately predict in vivo rat drug metabolic clearance (10). We reviewed multiple human hepatic IVIVE (in vitro-in vivo extrapolation) prediction studies where it is assumed that only unbound drug in blood/plasma is available for metabolism; the weighted average of studies indicates that 66.5% of predictions fell more than 2-fold outside of measured in vivo values with both microsomes and hepatocytes yielding approximately equivalent poor predictions (9). In the great majority of cases IVIVE under predicts human metabolic clearance. Wood et al. (10) also showed comparable poor predictions for hepatocytes and microsomes with a somewhat higher percentage of predictions (~75%) falling more than 2-fold outside of measured in vivo human metabolic clearance values.

Compounding the difficulty is that the field does not understand why the IVIVE predictions are inaccurate, even for purely metabolized drugs. In 2016, Takano et al. (11) wrote, "Theoretically, in vivo functions of enzymes/transporters should be predicted from in vitro metabolic/transporter activities simply by scaling up in vitro parameters with the use of the ratio of expression levels of molecules in whole tissue to that in the expression system." That is:

$$CL_{int,\,in\,vivo} = \frac{mg\,enzyme\,in\,the\,whole\,liver}{mg\,enzyme\,in\,the\,in\,vitro\,incubation} \cdot CL_{int,\,in\,vitro} \quad (1)$$

where CL_{int} terms represent the clearance capacity of the in vitro metabolic incubation or the in vivo whole liver to clear unbound drug (with units of volume per time per mg of metabolic enzymes) independent of flow and protein binding considerations. Chiba et al.

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(12) enumerate potential reasons for these CL_{int} inaccuracies “Extrinsic factors such as preparation process and/or storage conditions of liver samples from human are likely responsible for the potential loss of metabolic activity, resulting in the systematic under predictions by biological scaling factors for human samples.” Wood et al. (10, 13) have comprehensively reviewed these reasons and many others in the literature (e.g., protein binding issues, cofactor depletion, unstirred water layers) suggesting that “ultimately, the in vitro causes of under prediction are likely to be multifactorial” and “more investigation is required to resolve the quantitative capabilities of in vitro systems”. We concur with the statement of Hallifax and Houston (14) concerning the need for further “mechanistic elucidation to improve prediction methodology rather than empirical correction of bias”, believing that the marked poor predictability may be a result of unappreciated conceptual understanding rather than poor experimental results. A recent investigation by Riccardi et al. (15) reported that correcting Eq. 1 for the partition of unbound drug between whole liver tissue and liver plasma for 16 out of 19 Extended Clearance Classification System (ECCS) class 1 and 2 drugs yielded predictions within 3-fold of observed values. For ECCS class 3 and 4 drugs, predictions were only improved to within 3-fold for 5 of 13 drugs.

Here we analyze the theoretical basis for IVIVE with focus on each of the individual contributors to total clearance, intrinsic clearance (CL_{int}), protein binding ($f_{u,B}$), and hepatic blood flow (Q_H), to illustrate the reasons for the present inadequacies of the methods used and the potential methodologies that can improve IVIVE predictions. We further suggest a major flaw in IVIVE theory which results from utilizing a “Chemistry” approach to predict a “Pharmacokinetic” parameter. In this context, the term “Chemistry” refers to the in vitro measurement of drug concentration loss (i.e., rate of reaction) in a fixed measurable incubation volume that is drug independent. All pharmacokinetic derivations are based on mass balance (amount) considerations. These amounts are converted to concentrations incorporating a volume of distribution that does not have physiological relevance, that is not independently measurable, and is drug dependent. The term “Pharmacokinetics” refers to the in vivo scenario, where observed drug elimination (based on total drug amounts rather than concentrations) can also be influenced by drug distribution within the liver, can occur to a different degree for each drug, and is an aspect not currently captured by traditional “chemistry”-based incubations.

Materials and Methods

Pharmacokinetics vs. Chemistry

The most critical pharmacokinetic parameter is clearance, CL, a measure of the body’s ability to eliminate drug, since at steady-state during multiple dosing of a drug, the rate into the body will equal the rate out.

$$F \cdot Dosing\ Rate = CL \cdot Therapeutic\ Concentration \tag{2}$$

where a proper Dosing Rate (dose, D, divided by the dosing interval) can be determined knowing the desired therapeutic concentration, the fractional availability of dosed drug that will reach the systemic circulation intact (F) and CL. Equation 2 is a definition of clearance, a term exclusive to pharmacokinetics, as the amount of drug lost from systemic circulation

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over a given time period divided by the exposure driving that elimination. More frequently clearance is defined in vivo as

$$CL = \frac{F \cdot D}{AUC} \quad (3)$$

where the amount of drug lost from the systemic circulation following a single dose (available dose, $F \cdot D$) is divided by the exposure driving that elimination, measured as the total area under the systemic concentration time curve over all time (AUC). Thus, giving an intravenous bolus dose of drug (F=1) and measuring the total drug exposure will allow CL to be determined. Clearance will change in predictable ways as a function of physiology, pharmacogenomics and pathology, as well as a result of drug-drug interactions.

To help understand why in vivo clearance is commonly underpredicted by IVIVE approaches, it would be helpful to review the development of IVIVE practices by the field of Drug Metabolism. Drug metabolism can be considered analogous to a chemical reaction; however, clearance is not a measurement used in chemistry. A chemical reaction can be considered analogous to drug elimination in humans or animals where metabolism is the major route of elimination, and thus for the current discussion we focus only on the simple case of metabolism with no involvement of xenobiotic transporters. In chemistry under conditions of linear processes, the rate of elimination in an incubation mixture is most frequently characterized by the Michaelis-Menten ratio of V_{max}, the maximum rate of the decrease in the concentration of a drug in units of concentration/time, to K_m, the concentration of drug at half V_{max}. This ratio V_{max}/K_m thus has units of time^{-1} since in chemistry reaction rates are the measure of relevance, where V_{max} is a function of the concentration of enzyme present to carry out metabolism. For linear processes (when drug concentrations are much less than K_m), this ratio can be simplified into a single half-life, and such an approach has been adopted for substrate-depletion drug metabolism incubations where drug concentrations are measured i.e., the “in vitro T½ method” of Obach et al. (7). Note that although V_{max} was originally and continues to be defined in chemistry in terms of a concentration change, in pharmacokinetics V_{max} has been defined as an amount change that results in the ratio V_{max}/K_m as a clearance parameter (with the units of volume per unit time) rather than as a rate constant. Pharmacokinetic scientists have approached this discrepancy by converting the units of V_{max}, as discussed subsequently, rather than derive the classic Michaelis-Menten relationship based on amounts (so that the units of V_{max} will be amount per unit time).

So how is the rate constant from the in vitro incubation mixture (with the units of time^{-1}) converted into a clearance (with the units of volume per unit time)? By multiplying this rate constant by the volume of fluid in the incubation mixture. This detail has not been widely-recognized by the field, since the volume term is typically introduced by dividing the measured rate of elimination (units of time^{-1}) by the concentration of enzymes in the incubation (units of amount per volume).

$$CL_{int,\,in\,vivo} = \frac{mg\,enzyme\,in\,the\,whole\,liver}{1} \cdot k_{in\,vitro} \cdot \frac{volume\,of\,incubation}{mg\,enzyme\,in\,the\,in\,vitro\,incubation} \tag{1a}$$

Comparison of Eq. 1a to Eq. 1 clearly depicts how the fixed volume of incubation is incorporated in the conversion of in vitro rate of drug loss to what the field considers a prediction of in vivo intrinsic clearance. However, this approach does not account for the pharmacokinetic volume of distribution of drug within the liver that cannot be recapitulated nor scaled-up from traditional IVIVE incubations.

Since this parameter is determined for unbound drug (by accounting for $f_{u,inc}$, the unbound fraction of drug within the incubation) and independent of blood flow to the liver, this clearance is defined as an intrinsic clearance, a concept introduced in the early 1970s (16, 17), which had a revolutionary effect on our ability to select and modify drug dosing regimens as opposed to prior dependence on measures of drug half-lives. This is because drugs can exhibit multiple half-lives in the systemic circulation as concentrations decrease and upon multiple dosing, and require a model of drug elimination incorporating multiple exponential terms, even for drugs exhibiting linear kinetics. In contrast CL is compartment model independent and is defined (and can be determined) as the ratio of the amount of drug lost divided by the drug exposure in the systemic circulation driving that elimination (Eq. 3). There is a second difference between chemistry and pharmacokinetics of relevance to this analysis. In chemistry, the volume of the incubation mixture is fixed and independent of the drug for which the elimination is being measured. While in pharmacokinetics, volume terms are drug dependent and are non-physiologic apparent volumes that relate the concentration of drug measured in the systemic circulation to the amount of drug in the body. In pharmacokinetics, it is well recognized that drug clearance and drug volume of distribution are independent parameters whereas half-life is the dependent variable that can change as a result of change in clearance or in volume of distribution (18). A physiologic condition such as aging can change the volume of distribution of a drug such as diazepam, changing the half-life but not changing the drug clearance (19, 20). It is also well recognized that the volume of distribution is drug dependent and does not represent actual physiologic volumes in the body (18). Pharmacokinetic volume terms do not represent a definable space, such as the volume of fluid in a beaker in a chemistry reaction. Thus, the two major differences between chemistry and pharmacokinetics are in the definition of V_{max} and the possibility that volume of distribution may vary depending on the drug evaluated in pharmacokinetics but remains a fixed, drug independent value in chemistry.

The Relationship between Clearance and Intrinsic Clearance for In Vivo Hepatic Elimination

Rowland et al. (16) and Wilkinson and Shand (17) defined the relationship between the in vivo measured clearance of a drug, presented in terms of Eqs. 2 and 3, and a hypothetical in vivo intrinsic clearance based on the well-stirred model of liver elimination as given in Eq. 4:

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$$CL_{H,\,in\,vivo} = \frac{Q_H \cdot f_{u,\,B} \cdot CL_{int,\,in\,vivo}}{Q_H + f_{u,\,B} \cdot CL_{int,\,in\,vivo}} \quad (4)$$

where $f_{u,B}$ is the ratio of the unbound concentration of drug in the plasma to the whole blood concentration and Q_H is the hepatic blood flow rate. Since Q_H and $f_{u,B}$ can be experimentally measured or estimated, it is this $CL_{int,in\ vivo}$ value that one tries to determine using Eq. 1.

Let us now look at the relationship between $f_{u,B} \cdot CL_{int}$ and $CL_{H,in,vivo}$ to understand how such in vitro measures of drug metabolism (in tandem with predictions of unbound drug in the blood) are directly related to total clearance in vivo. When the product $f_{u,B} \cdot CL_{int}$ is a small value, for instance for low extraction ratio drugs for which $Q_H >> f_{u,B} \cdot CL_{int}$, the predictive clearance relationship (Eq. 4) simplifies to $f_{u,B} \cdot CL_{int,in\ vivo}$ that is:

$$\text{when}\, Q_H \gg f_{u,\,B} \cdot CL_{int}\,,\text{then}\, CL_H = f_{u,\,B} \cdot CL_{int}\ \text{(low extraction ratio drugs)} \quad (5)$$

When the product $f_{u,B} \cdot CL_{int}$ is a large value, for instance for high extraction ratio drugs for which $Q_H << f_{u,B} \cdot CL_{int}$, the Eq. 4 clearance relationship simplifies to $CL_H = Q_H$, that is:

$$\text{when}\, f_{u,\,B} \cdot CL_{int} \gg Q_H\,,\text{then}\, CL_H = Q_H\ \text{(high extraction ratio drugs)} \quad (6)$$

consistent with the belief that an organ cannot eliminate a drug faster than its delivery to that organ, clearance can never exceed the effective blood flow to that organ. Here it is important to note that although the values of CL_H range from $f_{u,B} \cdot CL_{int}$ to Q_H, for high clearance compounds (where CL_H approaches Q_H) the value of $f_{u,B} \cdot CL_{int}$ is significantly larger than Q_H. Therefore, when comparing the values of $f_{u,B} \cdot CL_{int}$ to CL_H, the value of CL_H can never be greater than the product $f_{u,B} \cdot CL_{int}$, that is:

$$\text{for all durgs}\, CL_H \le f_{u,\,B} \cdot CL_{int} \quad (7)$$

Conceptually, this can be rationalized by considering that for very high clearance drugs (those with high CL_{int} values), the large product $f_{u,B} \cdot CL_{int}$ will result in total clearance values that are limited by the rate at which drug is presented to the organ (Q_H). Thus, the value of $f_{u,B} \cdot CL_{int}$ will always be equal to or larger than total CL_H, and can never be smaller. This conclusion is based on theoretical considerations, and in practice experimental determinations of each parameter may result in under- or over-predictions, as we will evaluate in detail subsequently. Thus, comparison of the product $f_{u,B} \cdot CL_{int}$ to total CL_H will provide enhanced insight into the overall predictability of in vitro determinations, as opposed to comparison of CL_{int} values alone.

In the evaluation of IVIVE predictability, we emphasize that the contribution of both experimental and theoretical error in each of the terms CL_{int}, $f_{u,B}$ and Q_H can have impact on the resulting CL_H predictions. Many IVIVE investigations in the literature are based on evaluation of error between $CL_{H,in\ vivo,predicted}$ and $CL_{int,in\ vivo}$ where the $CL_{int,in\ vivo}$ is back-calculated from total CL_H measurements under the assumption that 1) measured in

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vivo CL_H is accurate, 2) determinations of $f_{u,B}$ are accurate, and that 3) the value of Q_H is accurate. These assumptions may be considered reasonable; however, any resulting errors in IVIVE are thus primarily attributed to issues with in vitro determination of CL_{int}. Further, CL_H is a quantifiable in vivo parameter, whereas CL_{int} can only be estimated indirectly by assuming a model of hepatic disposition. Since IVIVE unpredictability continues to challenge the field, here we evaluate IVIVE success by comparing CL_H values in order to better understand the potential contribution of errors in each term. This was achieved by examining notable IVIVE studies to determine $CL_{H,in\ vivo,predicted}$ by combining Eqs. 1 and 4.

We specifically analyze the 29 drugs first compiled by Obach (8) for human microsome measurements and the very large data set of Wood et al. (10), which included data for 101 drugs where in vitro measures of $CL_{int,on\ vitro}$ were determined in human hepatocytes, 83 drugs with human microsome incubations, 127 compounds with rat hepatocyte incubations and 71 compounds with rat microsome incubations. The Obach (8) dataset reported $CL_{H,in\ vivo,predicted}$, however the $CL_{int,in\ vivo}$ values reported by the Wood et al. (10) analysis were utilized to predict $CL_{H,in\ vivo,predicted}$ and were compared with measured $CL_{H,in\ vivo}$ values listed in those papers, resulting in the poor predictability detailed above. We also analyzed the results for the 11 ECCS class 1 and 2 drugs of Riccardi et al. (15) where in vitro hepatocyte data were also available in the Wood et al. (10) data base. The $f_{u,B}$ and Q_H values used by the authors of these three investigations were also utilized in the current analysis.

Results

We first determined how many of the tabulated measurements met the Eq. 7 lower boundary requirement in each of the Wood et al. (10) and Obach (8) data sets, i.e. drugs for which $CL_{H,in\ vivo} \quad f_{u,B} \cdot CL_{int,in\ vitro}$, as presented in Table I. As can be seen for the Wood et al. (10) data set, 74.3% of the human hepatocyte predictions were used to predict $CL_{H,in\ vivo}$ from $f_{u,B} \cdot CL_{int,\ in\ vivo}$ measures that violate the boundary conditions of the clearance prediction equation. In other words, for 74.3% of the human hepatocyte predictions, it would not be possible to obtain a correct prediction of human metabolic clearance. For human microsomes 52 of 83 predictions (62.7%) used $f_{u,B} \cdot CL_{int,in\ vitro}$ measures that violate the Eq. 7 boundary condition. For the analyses in rats the values are 100 of 127 (78.7%) for hepatocytes and 44 of 71 (62.0%) for microsomes. For the 29 drugs assessed by Obach (8) using human microsomes, the percentage violating the Eq. 7 boundary condition is 69.0%, somewhat higher than the 62.7% for the Wood et al. (10) data using human microsomes. Table II presents the predictability within 2-fold for each of the Wood et al. (10) data sets when the Eq. 7 boundary condition is met (i.e. $CL_{H,in\ vivo} \quad f_{u,B} \cdot CL_{int,in\ vitro}$). When the boundary condition is not violated, 73.1-77.8% of the predictions are within 2-fold for the Wood et al. (10) data sets, while all 9 (100%) of the Obach (8) predictions are correct within 2-fold. The predictability for data violating the boundary conditions ($CL_{H,in\ vivo} > f_{u,B} \cdot CL_{int,in\ vitro}$) is much poorer, 11.0-22.7% in Table II and 35% in the Obach (8) data set. Note in Fig. I that all of the human predictions that meet the boundary condition but that are not within 2-fold are overpredictions. There are no overpredictions greater than 2-fold for the data violating the Eq. 7 boundary condition. To determine if the observed difference could

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be improved if drugs where transporters affect clearance are excluded, we also evaluated the subset of Class 1 BDDCS drugs (drugs that exhibit high permeability rate, are extensively metabolized and highly soluble) where transporters are believed to not have a clinically significant effect on systemic clearance (21). As seen in Table I, no significant differences were observed between the BDDCS Class 1 drugs and the entire data set with regard to the distribution of drugs between the two groups for humans. However, from Table II it can be seen that successful IVIVE predictability increases by 10 to 20% when only BDDCS Class 1 marketed drugs not violating the Eq. 7 boundary condition are evaluated.

Figure I presents the comparison of the accuracy of the human IVIVE prediction for the Wood et al. (10) data set versus the measured in vivo hepatic clearance for drugs where the product $f_{u,B} \cdot CL_{int,in\ vitro}$ violates the Eq. 7 boundary condition (red dots) and where it does not (green dots). The results for the rat data are presented in Fig. II. It is obvious that the predictions for drugs that do not violate the Eq. 7 boundary condition give a better IVIVE outcome. We also investigated the charge distribution of drugs relative to violation of the Eq. 7 boundary condition as presented in Fig. III and Table III. Acids are over represented in drugs violating the Eq. 7 boundary condition both for hepatocytes and microsomes. The distribution in the 29 drug Obach (8) data set is similar. Only 1 of 9 acids did not violate the Eq. 7 boundary condition, 6 of 12 bases and 2 of 8 neutral compounds.

For the 19 ECCS class 1 and 2 drugs analyzed by Riccardi et al. (15), 11 were in the data base of Wood et al. (10). Ten of the 11 violated the Eq. 7 boundary condition. Only theophylline did not, but theophylline was one of three drugs analyzed by Riccardi et al. (15) that was not improved by correcting Eq. 1 for the partition of unbound drug between whole liver tissue and liver plasma. Since Riccardi et al. (15) do not actually provide the in vitro experimental $CL_{int,\ in\ vitro}$ measures in their paper, we do not know if their values would lead to the same Eq. 7 boundary condition violations for the 11 drugs. But at this point the Riccardi method improves the prediction for the drugs violating the Eq. 7 boundary condition, but does not for the one drug not in violation, theophylline.

More recently, Hallifax and Houston (22) presented analyses to attempt to explain the increasing degree of underprediction of CL_{int} observed with increasing values of in vivo CL_{int}, calculated from measures of in vivo clearance by rearrangement of Eq. 4. These studies evaluate IVIVE prediction success by comparing the calculated in vivo CL_{int} (from Eq. 4) to the prediction of in vivo CL_{int} derived from hepatocyte and microsome in vitro CL_{int} measurements (Eq. 1). We prefer rather to evaluate the total $CL_{in\ vivo,\ predicted}$ determined using Eqs. 1 and 4 with $CL_{in\ vivo,\ measured}$, since ultimately total clearance is the most relevant parameter, and because IVIVE errors based on CL_{int} for high clearance compounds may not translate to significant error in total clearance predictions. In Figs. I and II, IVIVE underprediction also becomes greater with increasing observed measured clearance, as observed by Hallifax and Houston (22). This trend is observed for all data, and separately for the predictions where Eq. 7 is violated (red dots) and where it is not (green dots). The underprediction error becomes greater and greater as the measured in vivo clearance approaches the upper boundary Eq. 6 condition, where $CL_{in\ vivo,\ predicted}$ approaches hepatic blood flow. The potential reasons for such an outcome have recently been discussed (10) and have primarily been attributed to inadequacies of in vitro

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methodologies. However, this outcome could also be explained by proposing that the measured hepatic blood flow in both humans and rats is not the relevant value to use in Eq. 4. In Table IV we have taken the human hepatocyte and microsome data of Wood et al. (10) that do not violate the Eq. 7 boundary condition and examined the effect of changing hepatic blood flow. As can be seen, increasing Q_H from 15 ml/min to 20 [the value used by Wood et al. (10) was 20.7] to 25, 30, 40 and 50 ml/min brings the slope very close to 1.0 for both hepatocytes and microsomes, eliminating the trend of high clearance drugs giving a greater underprediction. As the change in Q_H will have little effect on low clearance drugs, this is reflected in only minimal change in the Y-intercept values. Thus, we suggest that widely-employed organ blood flow values could underpredict the effective blood flow within the organ by approximately 2.5-fold, thus impacting IVIVE of high clearance compounds.

This increasing error with measured $CL_{in\ vivo}$ values raises the question as to whether the errors involved with the violation of the boundary condition of Eq. 7 are greater for high clearance compounds versus low clearance compounds. To address this, we evaluated the relative error observed in Figs. 1 and 2 with increasing measured $CL_{in\ vivo}$ values and found no relationship (analysis not shown).

Discussion

In the latter half of the 2010s our laboratory began investigating the reasons that IVIVE predictions were so poor. We first confirmed this finding and showed that it was probably not a function of confounding elimination of metabolized drugs with drugs that were also substrates for transporters (9) and these results were further confirmed by Wood et al. (10). We followed by examining interlaboratory variability in human hepatocyte intrinsic clearance values and trends with physicochemical properties (23), confirming the hepatic clearance-dependent underprediction (24) and identifying an apparent microsomal IVIVE anomaly for CYP3A4 substrates for which microsomes give markedly higher IVIVE success in humans than for other enzymes, but the same trend was not observed in hepatocytes (25). We also addressed the measurement of $f_{u,B}$ and protein-facilitated uptake relating to IVIVE (26) and proposed the presence of a transporter-induced protein binding shift as a new explanation for protein-facilitated uptake and to improve IVIVE (27). During the course of these studies we found that all theoretical aspects related to IVIVE may not be fully recognized and specifically that although CL_{int} may be relevant for all models of hepatic elimination, we have recently suggested that it can only be determined for the well-stirred model via Eq. 4 (28). However, it is important to recognize for the present analysis that the total clearance boundary conditions expressed in Eqs. 5 and 6 hold for all models of hepatic elimination. Thus, the violation of the Eq. 7 boundary condition by underprediction (Tables I and II, Figs. I and II) cannot be explained by examining different models of hepatic elimination.

What then are the possible explanations for the poor IVIVE predictability? It could be that all or some of the published measurements of $CL_{H,in\ vivo}$, $CL_{int,\ in\ vitro,}$, $f_{u,B}$ and Q_H are invalid. This could be, but since many of these parameters have been investigated by many scientists, with a reasonable degree of concordance, this is probably not the answer. Let us look now at the individual parameters.

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Correctly measuring in vivo blood clearance.

We believe that most investigators would agree that measurements of $CL_{H,in\ vivo}$ determined from clinical studies in humans using versions of Eqs. 2 and 3 would be reasonably accurate. However, it is important to recognize that this assumption depends on a number of factors, including that total clearance values must be determined following IV dosing or with an accurate estimate of F. In addition, the influence of inter-individual variability and potential of saturable processes such as absorption or metabolism, adequate early sampling for high clearance compounds (to accurately capture initial concentrations) as well as adequate terminal-phase sampling to minimize errors associated with AUC extrapolation. In general, many of these aspects are given consideration in clearance determinations conducted in the drug approval process. Of concern, however, is the recognition that the total clearance value must be in terms of blood concentrations, not plasma. For example, the largest compilation of 1352 drug compound IV clearance values are all presented as plasma clearances (29). To convert these plasma values to blood clearances one must have an accurate measurement of the blood to plasma concentration ratio ($(\frac{B}{P})$). When such measurements are unavailable investigators often assume the value to be equal to 1 for a basic or neutral compound and 0.55 for an acidic compound, as was done by Wood et al. (10). Riccardi et al. (15) measured $\frac{B}{P}$ for each of the compounds studied and Obach (8) measured this value for the 11 drugs for which literature values were not available at the time.

Protein binding determinations.

Most investigators may also believe that $f_{u,B}$ measurements are relatively error free and reproducible. However, there is a theoretical issue that needs to be recognized. The experimental $f_{u,B}$ values are not the fraction unbound in blood, but rather the ratio of the unbound concentration of drug in the plasma to the whole blood concentration as given in Eq. 8.

$$f_{u,B} = \frac{f_{u,P}}{\frac{B}{P}} \tag{8}$$

where $f_{u,P}$ is the fraction unbound in plasma. Typically, measurements of $f_{u,P}$ and $\frac{B}{P}$ are conducted in separate in vitro incubations and $f_{u,B}$ is calculated via Eq. 8. For $f_{u,B}$ to be the fraction unbound in blood, which is what should be required in Eq. 4, the free drug hypothesis must hold. That is, free drug concentrations must be equal in the plasma and the blood cell, or simply that there must be no transporter effects in blood cell to plasma distribution. Transporters have certainly been identified in blood cell membranes and one might suggest that the poor predictability of acids, as demonstrated in Fig. III and Table III, could be explained by transporter effects in blood cells, since acids are very frequently substrates for transporters. The great majority of the compounds studied by Riccardi et al. (15) were acids. Further investigations into the implications of the free drug theory assumption in calculating $f_{u,B}$ are currently underway in our laboratory, allowing further insight into when in vitro measurement of $f_{u,P}$ and $\frac{B}{P}$ can provide reliable estimates of $f_{u,B}$, and the impact this will have on IVIVE predictability. Further consideration of the potential

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for red blood cells to support the energetics of active transport processes is warranted, as these cells lack mitochondria.

Another protein binding issue relates to determining the fraction unbound in the in vitro incubation mixture ($f_{u,inc}$) to obtain the $CL_{int,\ in\ vitro}$ parameter in Eq. 1. Here fraction unbound in the microsomal or hepatocyte incubation is measured or when unavailable calculated from predictive regression equations as reported by Wood et al. (10).

Correctly determining in vivo intrinsic clearance.

The most probable source of error is in the determination of $CL_{int,\ in\ vivo}$ in Eq. 1. From the in vitro incubation, utilizing the "in vitro T½ method", we calculate a "chemistry" clearance as the product of the rate constant for drug elimination multiplied by the volume of fluid in the incubation mixture, a volume term that is drug independent. But in vivo, clearances are the product of the rate constant for elimination multiplied by a pharmacokinetic drug dependent volume of distribution. That is, the volume of distribution for each drug is different and not reflective of an actual fluid space (18). We are suggesting that the volume of distribution within the liver itself, for instance into lipophilic regions of the liver, is currently unaccounted for in IVIVE practices. Thus, it is possible that in vivo the extent of distribution into lipophilic regions of the liver (away from the intracellular hepatocyte water where metabolic enzymes exist) will be different for each drug, depending on the unique physicochemical properties of each drug. This is an area of research that needs to be further investigated, and significant efforts towards understanding the impact of liver volume of distribution on IVIVE are currently being pursued by our laboratory. We point out that the suggested liver volume of distribution should not be confused with the total-body volume of distribution at steady state (V_{ss}). It is possible that the in vivo the volume of distribution of drug in the whole liver may be different than the volume of distribution of drug in contact with metabolic enzymes, versus the in vitro condition where the volume of distribution of drug and enzymes will be the same. A similar difference between the in vitro and in vivo conditions was proposed by Riccardi et al. (15) who utilize a measurement of the partition of unbound drug between whole liver tissue and liver plasma to improve IVIVE predictions primarily for acid drugs where transporter effects may be anticipated. We propose that the in vitro experimental measure of drug elimination in a hepatocyte incubation may not reflect the difference found in the in vivo liver where the unbound concentration of drug directly in contact with the liver enzymes and throughout the whole liver may not be the same, possibly due to drug distribution into lipophilic regions of the liver. It appears that for the drugs that are consistent with Eq. 7 in Figs. I and II (green dots) this correction is not needed, but for drugs not consistent with Eq. 7 (red dots), the correction is required. There are some red dots that fall within the 2-fold estimation bands. But the estimation, of course, also depends on correctly determining $f_{u,B}$, as we have discussed above. At present, we believe that a correction for an NME using the Riccardi et al. (15) methodology, or other to-be-developed methodologies, will be necessary when the NME violate the Eq. 7 boundary condition. But at this point, there is no way to determine if the correction is needed or not *a priori*.

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Organ flow characterization.

Probably the most surprising finding in the analysis presented here is that actual organ blood flow may be underestimating the parameter to be utilized in Eq. 4. It is universally agreed in pharmacokinetics that organ blood clearance cannot exceed organ blood flow, but here we are suggesting that relevant organ blood flow for metabolism may be greater than the blood flow into the organ. Perhaps this could be the result of blood flow circling and becoming available to the hepatocyte two or three times for each ml/min entering the liver, however, we currently do not have a theory based on liver architecture to explain this potential phenomenon. This only becomes evident when $CL_{in\ vivo,\ predicted}$ continues to decrease from $CL_{in\ vivo\ measured}$ for higher and higher extraction ratio drugs, and of course is consistent with Eq. 4. The data in Table IV shows the decrease in slope for the predicted vs measured $CL_{in\ vivo}$ values, with little change in intercept. However, we also note that r^2 values for the regression also become poorer with increasing flow, and perhaps this may reflect the influence of $f_{u,B}$ errors that become more obvious as slope decreases. These simulations are by no means direct evidence of this phenomenon, but this is a starting point and a potentially fruitful area requiring further investigation.

Conclusions

Future experimental pathways.

The present manuscript was written to examine the theoretical basis for IVIVE, to identify the assumptions that have been made, in some cases unknowingly, and provide direction for future experimental pathways for the field to pursue to improve IVIVE practices. We critically re-evaluate the experimental data for the recently published large data set of Wood et al. (10), the initial 29 drug data set of Obach (8) and the drugs investigated by Riccardi et al. (15), which includes a number of transporter substrates. We point out that from two-thirds to three-fourths of the data examined to predict IVIVE (supposedly for drugs where transporter effects are minimal) violate the fundamental assumption of all models of hepatic elimination, i.e., that $f_{u,B} \cdot CL_{int}$ must be greater than $CL_{H,in\ vivo}$, again confirming the significant IVIVE-underprediction trend that the field has struggled with for decades. This outcome depends on the assumption that measured in vivo clearance values are reasonably accurate and noting that accurate blood clearance values depend on blood concentration to plasma concentration ratios when plasma clearance is reported, which may be inaccurate for transporter substrates due to the presence of transporters in the red blood cell membranes. The assumption of the free drug theory in calculating $f_{u,B}$ may result in significant errors that not only applies to total clearance values, but also in scaling up in vitro CL_{int} for prediction of $CL_{H,in\ vivo}$, and should be further investigated to elucidate its impact on IVIVE success for transporter substrates. We derived the relationship between the in vitro and in vivo intrinsic clearances, showing that calculating the in vitro intrinsic clearance employs a chemistry approach where the volume multiplied by the rate constant of elimination is fixed. In contrast, for the in vivo pharmacokinetic approach, clearance may be considered the product of the rate constant of elimination multiplied by a drug dependent (and physiologically unidentifiable) volume of distribution. We propose that the relationship between the in vitro and in vivo intrinsic clearances should consider the physiologic difference of drug distribution in the whole liver versus the uniform distribution in vitro. The

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methodology of Riccardi et al. (15) provides a good starting point in examining when the correction is necessary and when it is not. Finally, the analysis here suggests that for high clearance compounds, the value for blood flow entering the liver may be underpredicting the blood flow that sets the upper limit (Eq. 6) for in vivo clearance. Future experimental studies should investigate this possibility and the other issues raised in this manuscript

Acknowledgements

The authors thank members of our laboratory Dr. Jin Dong (present address: Simulations Plus), Alan R. Wolfe, Shufang Liu (present address: SUNY Buffalo) and Dr. Giovani Bocci (present address: University of New Mexico) for very helpful discussions.

This work was supported in part by a Mary Anne Koda-Kimble Seed Award for Innovation. Dr. Benet is a member of the UCSF Liver Center supported by NIH Grant P30 DK026743. Ms. Sodhi was supported in part by an American Foundation for Pharmaceutical Education Pre-Doctoral Fellowship, NIGMS grant R25 GM56847 and a Louis Zeh Fellowship.

Abbreviations

ADME	absorption, distribution, metabolism, excretion
AUC	area under the concentration time curve
BDDCS	biopharmaceutics drug disposition classification system
$\frac{B}{P}$	blood to plasma partitioning ratio
CL	clearance
CL_{int}	intrinsic clearance
CYP3A	cytochrome P450 3A
D	dose
ECCS	Extended Clearance Classification System
F	bioavailability
$f_{u,B}$	fraction of unbound drug in blood
$f_{u,inc}$	fraction of unbound drug in an *in vitro* incubation
$f_{u,P}$	fraction of unbound drug in plasma
IVIVE	in vitro-in vivo extrapolation
NME	new molecular entity
Q_H	hepatic blood flow

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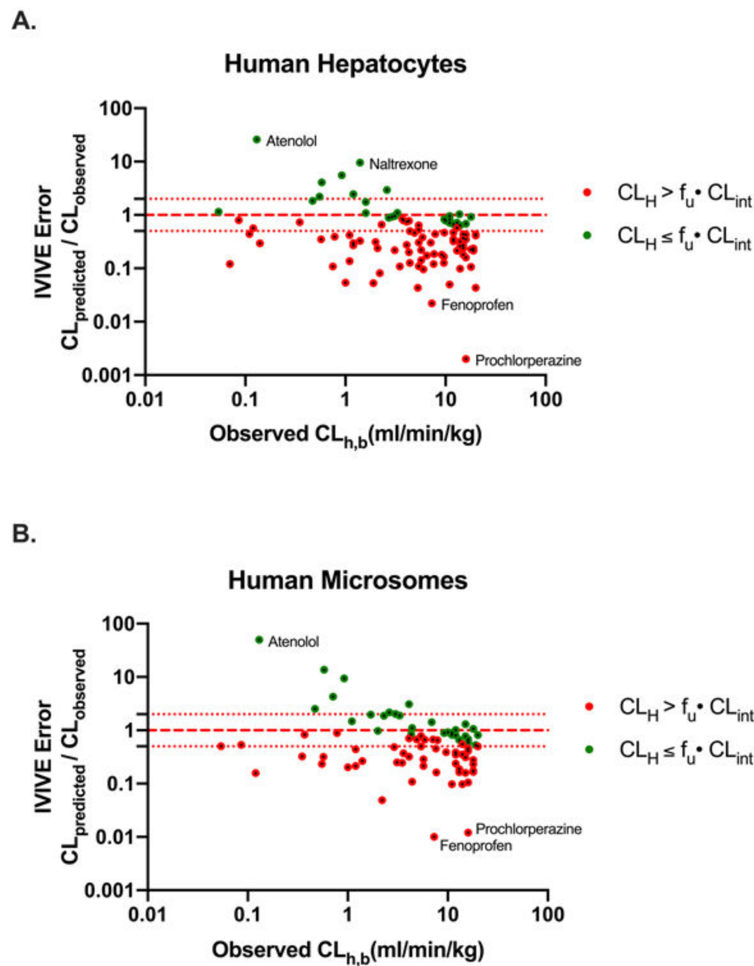

Figure 1.

Comparison of IVIVE error (predicted in vivo clearance/observed in vivo clearance) versus observed in vivo clearance using A. human hepatocytes and B. human microsomes from the Wood et al. (10) data set for analyses where the product of the in vitro measured CL_{int} and $f_{u,B}$ violates hepatic model boundary conditions (red dots) and where it does not (green dots).

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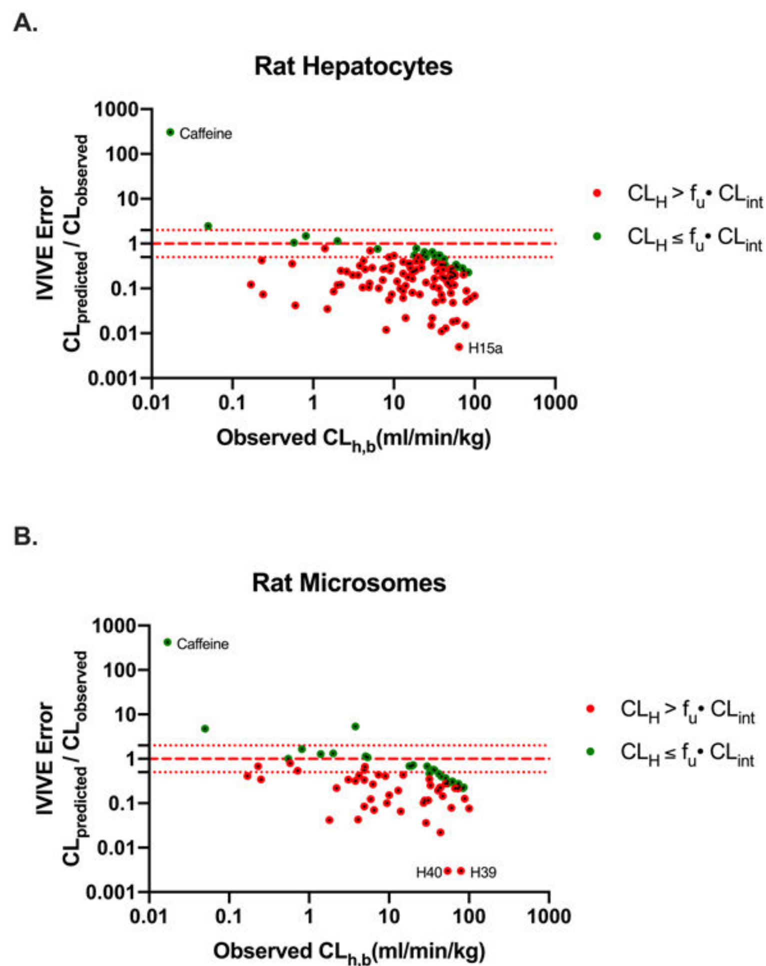

Figure 2.

Comparison of IVIVE error (predicted in vivo clearance/observed in vivo clearance) versus observed in vivo clearance using A. rat hepatocytes and B. rat microsomes from the Wood et al. (10) data set for analyses where the product of the in vitro measured CL_{int} and $f_{u,B}$ violates hepatic model boundary conditions (red dots) and where it does not (green dots).

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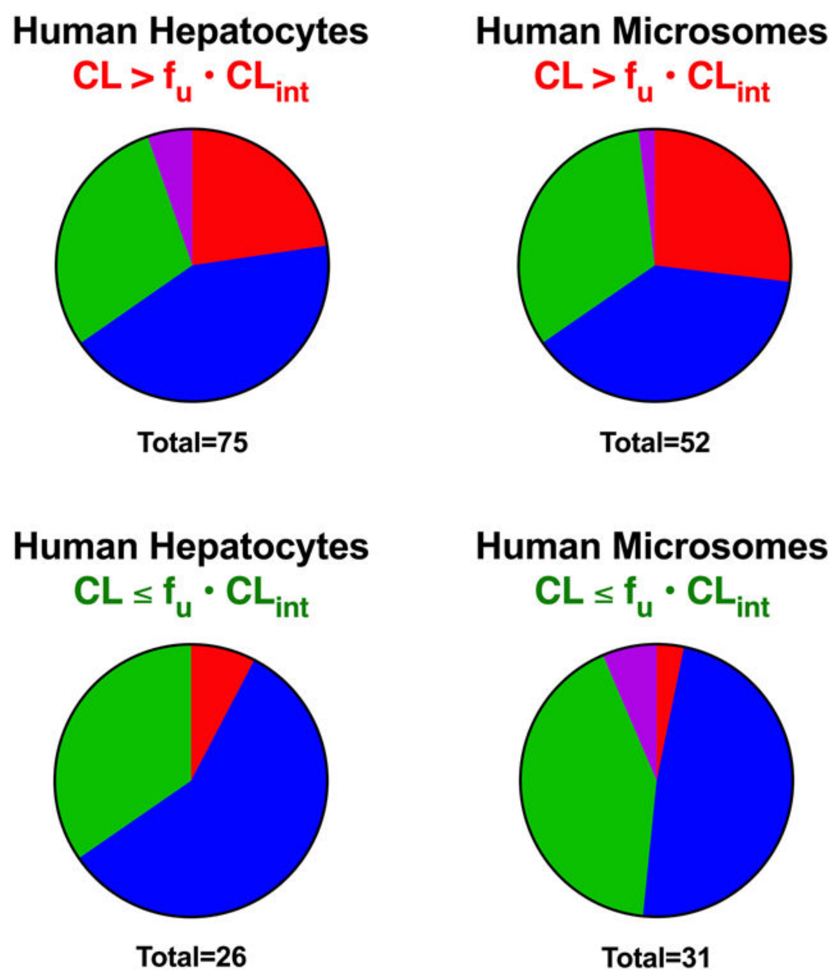

Figure 3.

Distribution by charge for compounds tabulated by Wood et al. (10) for acids (red), bases (blue), neutrals (green) and zwitterions (purple) in human hepatocytes and human microsomes.

Table I.

Analysis of reported data from Wood et al. (10) for comparison of the product of $f_{u,B}$ and $CL_{int,\ in\ vitro}$ with $CL_{H,\ in\ vivo}$

Human Hepatocytes			Human Microsomes		
	$CL_H > f_{u,B} \cdot CL_{int,invitro}$	**$CL_H \quad f_{u,B} \cdot CL_{int,invitro}$**		**$CL_H > f_{u,B} \cdot CL_{int,invitro}$**	**$CL_H \quad f_{u,B} \cdot CL_{int,invitro}$**
All Drugs (n = 101)	**74.3%** (n=75 of 101)	**25.7%** (n=26 of 101)	**All Drugs** (n = 83)	**62.7%** (n=52 of 83)	**37.3%** (n=31 of 83)
BDDCS Class 1 (n = 60)	**71.7%** (n=43 of 60)	**28.3%** (n=17 of 60)	**BDDCS Class 1** (n = 47)	**66.0%** (n=31 of 47)	**34.0%** (n=16 of 47)
Rat Hepatocytes			**Rat Microsomes**		
	$CL_H > f_{u,B} \cdot CL_{int,invitro}$	**$CL_H \quad f_{u,B} \cdot CL_{int,invitro}$**		**$CL_H > f_{u,B} \cdot CL_{int,invitro}$**	**$CL_H \quad f_{u,B} \cdot CL_{int,invitro}$**
All Drugs (n = 127)	**78.7%** (n=100 of 127)	**21.3%** (n=27 of 127)	**All Drugs** (n = 71)	**62.0%** (n=44 of 71)	**38.0%** (n=27 of 71)
Marketed Drugs (n = 38)	**47.4%** (n=18 of 38)	**52.6%** (n=20 of 38)	**Marketed Drugs** (n = 34)	**35.3%** (n=12 of 34)	**64.7%** (n=22 of 34)
BDDCS Class 1 (n = 21)	**47.6%** (n=10 of 21)	**52.4%** (n=11 of 21)	**BDDCS Class 1** (n = 17)	**29.4%** (n=5 of 17)	**70.6%** (n=12 of 17)

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Table II.

Analysis of reported data from Wood et al. (10) for comparison of the product of $f_{u,B}$ and $CL_{int,\ in\ vitro}$ with $CL_{H,\ in\ vivo}$ in terms of predictability within 2-fold

Human Hepatocytes			**Human Microsomes**		
	$CL_H > f_{u,B} \cdot CL_{int,invitro}$	$CL_H \quad f_{u,B} \cdot CL_{int,invitro}$		$CL_H > f_{u,B} \cdot CL_{int,invitro}$	$CL_H \quad f_{u,B} \cdot CL_{int,invitro}$
All Drugs 2-fold: **30.7%** (n=31 of 101)	2-fold: **16.0%** (n=12 of 75)	2-fold: **73.1%** (n=19 of 26)	**All Drugs** 2-fold: **42.2%** (n=35 of 83)	2-fold: **23.1%** (n=12 of 52)	2-fold: **74.2%** (n=23 of 31)
BDDCS Class 1 2-fold: **35.0%** (n=21 of 60)	2-fold: **16.3%** (n=7 of 43)	2-fold: **82.4%** (n=14 of 17)	**BDDCS Class 1** 2-fold: **48.9%** (n=23 of 47)	2-fold: **25.8%** (n=8 of 31)	2-fold: **93.8%** (n=15 of 16)
Rat Hepatocytes			**Rat Microsomes**		
	$CL_H > f_{u,B} \cdot CL_{int,invitro}$	$CL_H \quad f_{u,B} \cdot CL_{int,invitro}$		$CL_H > f_{u,B} \cdot CL_{int,invitro}$	$CL_H \quad f_{u,B} \cdot CL_{int,invitro}$
All Drugs 2-fold: **25.2%** (n=32 of 127)	2-fold: **11.0%** (n=11 of 100)	2-fold: **77.8%** (n=21 of 27)	**All Drugs** 2-fold: **43.7%** (n=31 of 71)	2-fold: **22.7%** (n=10 of 44)	2-fold: **77.8%** (n=21 of 27)
Marketed Drugs 2-fold: **57.9%** (n=22 of 38)	2-fold: **38.9%** (n=7 of 18)	2-fold: **75.0%** (n=15 of 20)	**Marketed Drugs** 2-fold: **61.8%** (n=21 of 34)	2-fold: **33.3%** (n=4 of 12)	2-fold: **77.3%** (n=17 of 22)
BDDCS Class 1 2-fold: **71.4%** (n=15 of 21)	2-fold: **50.0%** (n=5 of 10)	2-fold: **90.9%** (n=10 of 11)	**BDDCS Class 1** 2-fold: **82.4%** (n=14 of 17)	2-fold: **60.0%** (n=3 of 5)	2-fold: **91.7%** (n=11 of 12)

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Table III.

Distribution by charge for drugs violating and not violating boundary conditions as tabulated by Wood et al. (10) for A. hepatocyte measurements and B. microsome measurements

A.

Human Hepatocytes

	$CL_{H,in\ vivo} > f_{u,B} \cdot CL_{int,invitro}$	$CL_{H,in\ vivo} \quad f_{u,B} \cdot CL_{int,invitro}$
All Drugs (n = 101)	**n=75** (74.3% of 101)	**n=26** (25.7% of 101)
Acids (n = 19)	**n=17** (22.7% of 75) (89.5% of 19)	**n=2** (7.7% of 26) (10.5% of 19)
Bases (n = 47)	**n=32** (42.7% of 75) (68.1% of 47)	**n=15** (57.7% of 26) (31.9% of 47)
Neutrals (n = 31)	**n=22** (29.3% of 75) (71.0% of 31)	**n=9** (34.6% of 26) (29.0% of 31)
Zwitterions (n = 4)	**n=4** (5.3% of 75) (100% of 4)	**n=0** (0% of 26) (0% of 4)

B.

Human Microsomes

	$CL_{H,in\ vivo} > f_{u,B} \cdot CL_{int,invitro}$	$CL_{H,in\ vivo} \quad f_{u,B} \cdot CL_{int,invitro}$
All Drugs (n = 83)	**n=52** (62.7% of 83)	**n=31** (37.3% of 83)
Acids (n = 15)	**n=14** (26.9% of 52) (93.3% of 15)	**n=1** (3.2% of 31) (6.7% of 15)
Bases (n = 35)	**n=20** (38.5% of 52) (57.1% of 35)	**n=15** (48.4% of 31) (42.9% of 35)
Neutrals (n = 30)	**n=17** (32.7% of 52) (56.7% of 30)	**n=13** (41.9% of 31) (43.3% of 30)
Zwitterions (n = 3)	**n=1** (1.9% of 52) (33.3% of 3)	**n=2** (6.5% of 31) (66.6% of 3)

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Table IV.

Regression of $CL_{in\ vivo\ predictive}$ vs $CL_{in\ vivo,\ measured}$ with changing hepatic blood flow and percent predictions falling within 2-fold for: A. the 26 drugs not violating Eq. 5 for hepatocyte measurements and B. the 31 drugs not violating Eq. 5 for microsome measurements from the Wood et al. (10) data set

A.

Matrix	n	Q_H (ml/min/kg)	r^2	Slope	Y-Intercept	Percent Within 2-Fold
Human Hepatocytes	26	15	0.660	0.485	2.40	73.1%
Human Hepatocytes	26	20	0.649	0.600	2.49	73.1%
Human Hepatocytes	26	25	0.638	0.697	2.54	73.1%
Human Hepatocytes	26	30	0.626	0.780	2.55	73.1%
Human Hepatocytes	26	40	0.605	0.916	2.53	73.1%
Human Hepatocytes	26	50	0.588	1.02	2.49	73.1%

B.

Matrix	n	Q_H (ml/min/kg)	r^2	Slope	Y-Intercept	Percent Within 2-Fold
Human Microsomes	31	15	0.601	0.397	3.84	77.4%
Human Microsomes	31	20	0.591	0.521	4.05	74.2%
Human Microsomes	31	25	0.576	0.633	4.14	67.7%
Human Microsomes	31	30	0.558	0.736	4.16	67.7%
Human Microsomes	31	40	0.523	0.920	4.07	61.3%
Human Microsomes	31	50	0.492	1.08	3.91	58.1%