Commentary

Clearance prediction methodology needs fundamental improvement: trends common to rat and human hepatocytes/microsomes and implications for experimental methodology

Wood FL, Houston JB and Hallifax D

Centre for Applied Pharmacokinetic Research, Division of Pharmacy and Optometry, School of Health Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Science Centre, Manchester, M13 9PT, UK.
Global assessment of in vitro assay performance for prediction of clearance

Corresponding author:

D Hallifax, Centre for Applied Pharmacokinetic Research, Division of Pharmacy and Optometry, School of Health Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Science Centre, Manchester, M13 9PT, UK.

Telephone: +44 (0)161 275 8347

Fax: +44 (0)161 275 8349

Email: David.Hallifax@manchester.ac.uk

Number of text pages: 31

Number of tables: 4

Number of figures: 6

Number of references: 78

Number of words in Abstract: 250

Number of words in Introduction: 838

Number of words in Discussion: 2192

Non-standard abbreviations: AFE (average fold error); BDDCS (Biopharmaceutics Drug Disposition Classification System); CL_b (clearance in blood); CL_p (clearance in plasma); CL_int (intrinsic clearance); CYP (cytochrome P450); ESF (empirical scaling factor): fu_b (fraction unbound in blood): fu_p (fraction unbound in plasma); HLM (human liver microsomes); PBSF (physiologically based scaling factor); Q_h (blood flow); R_b (blood:plasma concentration ratio; RLM (rat liver microsomes); UGT (uridine 5'- diphospho-glucuronosyltransferase); fm_UGT (fraction metabolised by UGT)
Abstract

Although prediction of clearance using hepatocytes and liver microsomes has long played a decisive role in drug discovery, it is widely acknowledged that reliably accurate prediction is not yet achievable despite the predominance of hepatically cleared drugs. Physiologically mechanistic methodology tends to underpredict clearance by several-fold and empirical correction of this bias is confounded by imprecision across drugs. Understanding of the causes of prediction uncertainty has been slow, possibly reflecting poor resolution of variables associated with donor source and experimental methods, particularly for the human situation. Hallifax et al. (Pharm. Res. 27: 2150-2161, 2010) reported that among published human hepatocyte predictions there was a tendency for underprediction to increase with increasing \textit{in vivo} intrinsic clearance, suggesting an inherent limitation using this particular system. This implied an artefactual rate limitation \textit{in vitro}, although preparative effects on cell stability and performance were not yet resolved from assay design limitations. Here, to resolve these issues further, we present an up-to-date and comprehensive examination of predictions from published rat as well as human studies (n= 128 and 101, hepatocytes and n= 71 and 83, microsomes, respectively) to assess system performance more independently. We report a clear trend of increasing underprediction with increasing \textit{in vivo} intrinsic clearance which is similar both between species and between \textit{in vitro} systems. Hence, prior concerns arising specifically from human \textit{in vitro} systems may be unfounded and the focus of investigation in future should be to minimise the potential \textit{in vitro} assay limitations common to whole cells and subcellular fractions.
Introduction

For more than a decade numerous studies have reported prediction of clearance of drugs from human and rat hepatic in vitro systems (with increasing emphasis on hepatocytes rather than microsomes), all of which have indicated a tendency for underprediction on the basis of physiologically mechanistic scaling (Shibata et al., 2002; Hallifax et al., 2005; Ito and Houston, 2005; Riley et al., 2005; Brown et al., 2007; Stringer et al., 2008; Chiba et al., 2009; Hallifax et al., 2010). These studies used both intra- and inter-laboratory datasets for drugs which were mostly commercially available small molecules (MW 200-600) with a predominant metabolic route of clearance. With an increasing emphasis on human (hepatic) in vitro systems, in vivo intrinsic clearance (CL\text{int}) of these drugs ranged over about five orders of magnitude (1-100,000 ml/min/kg).

To limit the negative impact of prediction inaccuracy on drug discovery and human dosing decisions, empirical correction of prediction bias, as has been suggested, offers a practical and justifiable improvement (Poulin et al., 2012; Sohlenius-Sternbeck et al., 2012; Yamagata et al., 2016). But considerable uncertainty remains, reflecting the poor precision which accompanies the bias seen in published datasets. Reducing both the imprecision and bias requires understanding of their causes and for prediction methodology to progress, a mechanistic approach must be maintained and improved. There have been a number of suggested potential causes of underprediction, but many have not been met with incisive investigation.

The impact of liver model choice is still questioned despite demonstration of marginal effect between the well-stirred and parallel tube models, for a large dataset (Hallifax et al., 2010). Only very high clearance compounds are significantly impacted and those are inevitably a minor proportion of a typical dataset. While the modelling of hepatic clearance has been extended to include transmembrane processes (Chiba et al., 2009), this is more applicable to more recently developed, less permeable, drugs and in any case, cannot be as widely assessed due to the general lack of distinction of uptake processes in available in vitro data. Related to liver models, the extent of protein binding is often cited as a factor in prediction accuracy, due possibly to perceived inherent binding measurement inaccuracy, a lack of equilibrium in vivo or facilitated uptake of drug (Soars et al., 2007b; Ring et al., 2011; Poulin et al., 2012; Bowman and Benet, 2016). For highly lipophilic drugs, Poulin et al. proposed a methodology relating plasma and hepatic albumin binding based on
postulated involvement of bound drug with the uptake process (Poulin et al., 2012; Poulin and Haddad, 2013). However, this semi-mechanistic methodology has been shown to offer no improvement on the conventional physiological approach (Yamagata et al., 2016).

Human hepatocytes are inherently variable in drug clearance capability in vitro, reflecting a number of unavoidable factors: donor phenotypic variability, potentially detrimental processing (preparation and storage) and lability of metabolising enzymes and uptake transporters – all sources of variation and possibly bias (Hallifax and Houston, 2009). Prediction assay methodology itself, lacking in standardisation, is probably another source of variation (and bias); given the extremely wide range of in vivo CL\textsubscript{int} involved, system artefacts might be expected. Addressing such issues, Hallifax et al. (2010) highlighted a tendency for prediction accuracy from human hepatocytes (but not microsomes) to decrease with increasing in vivo CL\textsubscript{int}, among an extensive dataset from published studies, suggesting in vitro artefacts (eg. cofactor exhaustion) or in vitro permeability rate limitation to explain underprediction. A lack of relationship between prediction accuracy and permeability was later demonstrated (Hallifax et al., 2012) focussing attention on assay methodology. Bowman and Benet (2016), in a recent comparison of published studies using human hepatocytes and microsomes, saw only slight evidence of difference in prediction accuracy between drugs classified (BDDCS) as transporter substrates or not, or between drugs segregated as highly bound in blood or not. Having confirmed the lack of resolution between sources of uncertainty, they highlighted a need for improved experimental methodology.

Use of rat in vitro systems for measurement of CL\textsubscript{int} has been superseded by the now widely available human equivalent. Although some studies have indicated a tendency towards underprediction of clearance for rat as well as that more widely acknowledged for human (Huang et al., 2010; Sohlenius-Sternbeck et al., 2012), minimal inter-individual and processing variability in rat implies much less prediction uncertainty (Iwatsubo et al., 1996; Iwatsubo et al., 1997; Wilson et al., 2003; Ito and Houston, 2005; Riley et al., 2005; Hallifax and Houston, 2009). It is therefore of considerable potential value to appraise the limitations of clearance prediction in rat, in parallel to human, to enable more incisive understanding of experimental uncertainty. Going beyond previous assessments of prediction of clearance and to attempt to resolve source- and experimentally-based variation and bias, we have compiled the most extensive datasets to date for inter-species (rat and human) comparison of in vitro
(hepatocyte and microsome) predictions of $\text{CL}_{\text{int}}$ from the literature. This commentary critically examines the accuracy, precision and trends in prediction of clearance between the foundational \textit{in vitro} species assessing the implications for future progress in prediction methodology.

\section*{Data collation}

Datasets for human ($n=101$, hepatocytes; $n=83$, microsomes) and rat ($n=128$ hepatocytes; $n=71$, microsomes) \textit{in vitro} $\text{CL}_{\text{int}}$ and \textit{in vivo} CL were compiled based on examination of existing published datasets and a further search of the literature. Both approved pharmaceuticals and investigatory proprietary compounds were considered provided that complementary \textit{in vitro} and \textit{in vivo} data were available. Criteria for inclusion of \textit{in vivo} data were that reported blood clearance ($\text{CL}_b$) or plasma clearance ($\text{CL}_p$) was determined from intravenous dosing and that $\text{CL}_b$ did not exceed hepatic blood flow ($Q_H$). \textit{In vitro} $\text{CL}_{\text{int}}$ determined from either metabolite formation or substrate depletion over a range of substrate concentrations, or from single concentration substrate depletion time profiles was considered. \textit{In vitro} data included both suspended hepatocytes and liver microsomes, reflecting the prevalent use of these systems in the pharmaceutical industry. Microsomal data represents that from incubations with exogenous NADPH (Phase I metabolism) only. Hepatocyte data represents incubations conducted in the absence of serum; in each instance the use of fresh or cryopreserved cells was recorded.

For human hepatocyte data, two key review articles, Hallifax et al. (2010) and Paixao et al. (2010) were identified; from these the original sources were examined and data not previously included were added (for e.g. Hallifax et al. (2010) included only predictions from cryopreserved hepatocytes). Most additional data came from subsequently published studies (Sohlenius-Sternbeck et al., 2010; Akabane et al., 2012a; Akabane et al., 2012b; Sohlenius-Sternbeck et al., 2012); some data came from earlier studies not previously included (Hallifax et al., 2005; Hallifax et al., 2008). For human liver microsomes (HLM), original sources from Ito and Houston (2004) and Hallifax et al. (2010) were examined, with supplementation from Obach (1999), Cubitt et al. (2009), Gertz et al. (2010) and Sohlenius-Sternbeck et al. (2010). Additional data for both human hepatocyte and HLM were provided by R Stringer (Stringer 2006, thesis, University of Manchester) as data supplemental to Stringer et al. (2008). The rat hepatocyte datasets encompassed a previous compilation (Ito and Houston 2005) and several subsequent original research articles; the majority of data were from Huang et al. (2010) and
Sohlenius-Sternbeck et al. (2010). For rat liver microsomes (RLM), original source data cited by Houston (1994), Houston and Carlile (1997) and Ito and Houston (2004) were collated together with data from Jones and Houston (2004), De Buck et al. (2007) and Huang et al. (2010). The complete datasets (with sources) are given in Supplements 1 (human) and 2 (rat). No data was duplicated by source; where more than one original source provided data for a particular drug, mean CL$\text{int}$ was calculated.

The datasets were considered to predominantly comprise highly permeable drugs of which the vast majority would be expected to be cleared by metabolism without rate limitation by transport, although dependency on hepatic uptake transport for clearance would not necessarily preclude inclusion of predictions for the purposes of this analysis. In 2015, Varma et al. proposed a drug classification system for predicting the major clearance route, based on charge, permeability and molecular weight – the Extended Clearance Classification System (ECCS). Using the assignments they presented, the human datasets in the present study (as far as could be assigned – 73 and 72 % hepatocytes and microsomes, respectively) comprised about 86 % deemed to have metabolism as the primary route of clearance – which supports our initial assumption. Of the remainder, half (7 %) could be assigned as rate limited by hepatic uptake transport and half (7 %) dependent on renal clearance. For rat, about 79 and 100 % (hepatocytes and microsomes, respectively) of the commercial drugs which were ECCS pre-categorised (60 and 65 % of commercial drugs, hepatocytes and microsomes, respectively) were predominantly cleared by metabolism. A large proportion of the rat datasets were proprietary compounds but on the basis of their physico-chemical properties at least 85 % for hepatocytes and 100 % for microsomes (assuming all ‘AZ’ compounds were highly permeable as denoted by Varma et al. [>5x10$^6$ cm/sec]) were inferred as predominantly cleared by metabolism. As the above categorisation is inherently imprecise, no particular individual drug prediction was excluded following the initial collation criteria.

**In vitro fraction unbound**

In studies where the fraction unbound in either microsomes (fu$_\text{mic}$) or hepatocytes (fu$_\text{heps}$) was experimentally determined and reported alongside CL$_\text{int}$, this value was applied in prediction of in vivo unbound CL$_\text{int}$ (CL$_\text{int,u}$). Where fu$_\text{mic}$ or fu$_\text{heps}$ were unreported, these values were estimated using
lipophilicity relationship algorithms (Equations 1 (Hallifax and Houston (2006) and 2 (Kilford et al. (2008) respectively).

\[
fu_{\text{mic}} = \frac{1}{1 + P \cdot 10^{0.072 \cdot \log P/D^2 + 0.067 \cdot \log P/D - 1.126}}
\]

\[
fu_{\text{heps}} = \frac{1}{1 + 125 \cdot V_R \cdot 10^{0.072 \cdot \log P/D^2 + 0.067 \cdot \log P/D - 1.126}}
\]

where \( P \) is the microsomal protein concentration, \( \log P/D \) is either the \( \log P \) value for basic and neutral drugs or the \( \log D \) value for acidic drugs and \( V_R \) is the volume ratio of hepatocytes to medium (0.005 for 1 x \( 10^6 \) cells/ml (Brown et al., 2007)).

**Scaling in vitro intrinsic clearance to whole liver**

*In vitro* \( CL_{\text{int}} \) values were scaled to the *in vivo* whole liver equivalent using Equation 3 (Hallifax et al., 2010), where the physiologically based scaling factor (PBSF) is the microsomal average recovery factor for microsomal predictions and hepatocellularity for hepatocyte predictions, and \( LW \) is the liver weight/kg bodyweight.

\[
\text{Predicted in vivo } CL_{\text{int,u}} = \frac{\text{in vitro } CL_{\text{int}} \cdot \text{PBSF} \cdot LW}{fu_{\text{mic}} \text{ or } fu_{\text{heps}}}
\]

Microsomal recovery factors of 40 mg microsomal protein/g liver (Hakooz et al., 2006) and 60 mg microsomal protein/g liver (Houston and Carlile, 1997) were used for human and rat respectively; hepatocellularity of 120 x \( 10^6 \) hepatocytes/g liver was used for both human (Hakooz et al., 2006) and rat (Bayliss et al., 1999) and \( LW \) was 21.4 g/kg bodyweight for human and 40 g/kg bodyweight for rat (Davies and Morris, 1993).

**Determination of in vivo intrinsic clearance**

*In vivo* \( CL_{\text{int,u}} \) was derived from hepatic clearance (\( CL_h \)) (blood), fraction unbound in blood (\( fu_b \)) and \( Q_H \) with a given value of 20.7 ml/min/kg for human (Davies and Morris, 1993) and 100 ml/min/kg for rat
Where applicable and data available, CL\textsubscript{h} was determined by subtracting renal clearance from total CL\textsubscript{b}. Where sources provided CL\textsubscript{p} and fraction unbound in plasma (fu\textsubscript{p}), CL\textsubscript{b} and fu\textsubscript{b} were calculated using reported blood/plasma concentration ratio (R\textsubscript{b}) (CL\textsubscript{p}/R\textsubscript{b} and fu\textsubscript{p}/R\textsubscript{b} respectively). Where R\textsubscript{b} was unavailable, it was assumed to be equal to 1 for a basic or neutral compound and 0.55 (1 - haematocrit) for an acidic compound. If data from multiple studies were available for the same compound, the arithmetic means of CL\textsubscript{b} and fu\textsubscript{b} were used in the calculation of CL\textsubscript{int,u}.

\textit{In vivo} CL\textsubscript{int,u} was derived using both the well-stirred and parallel tube models of hepatic clearance to assess the impact of liver model on the predictive accuracy of \textit{in vitro} data. As the difference in bias between these two liver models (representing both extremes of drug hepatic dispersion) was found to be marginal, consistent with previous studies (Jones and Houston, 2004; Riley et al., 2005; Brown et al., 2007), data from the well-stirred model (Equation 4) is presented.

\[ \text{In vivo \ } CL_{\text{int,u}} = \frac{CL_h}{fu_b \left(1 - \frac{CL_h}{QH}\right)} \]  

\textbf{Assessment of accuracy and precision of predictions}

The overall bias in predictions was assessed by calculation of the average fold error (AFE) (Equation 5). Root mean squared error (RMSE) (Equation 6) was used as a measure of precision.

\[ AFE = 10 \left(\frac{\sum \log_{\text{predicted}}}{\sum \log_{\text{observed}}}\right) \]  

\[ \text{RMSE} = \sqrt{\frac{1}{n} \sum (\text{predicted-observed})^2} \]  

where \( n \) = number of predictions.

As underprediction yields an AFE below 1, underprediction was also expressed as fold-underprediction (inverse of AFE). The percentage of CL\textsubscript{int,u} predictions within (and beyond) two-fold of \textit{in vivo} was used as an additional indicator of predictive accuracy, consistent with previous
publications (Obach, 1999; Naritomi et al., 2001; McGinnity et al., 2004; Stringer et al., 2008; Sohlenius-Sternbeck et al., 2012; Chan et al., 2013).

Calculation of empirical scaling factors

The empirical scaling factors (ESFs) required to equate predicted \( \text{CL}_{\text{int,u}} \) with observed \( \text{CL}_{\text{int,u}} \) for individual compounds within each dataset were calculated using Equation 7.

\[
\text{ESF} = \frac{\text{observed } \text{CL}_{\text{int,u}}}{\text{predicted } \text{CL}_{\text{int,u}}} \quad (7)
\]

Average ESF (for \( \text{CL}_{\text{int,u}} \) and \( \text{fu}_p \) subsets) was calculated using Equation 8 (log average)

\[
\text{Average ESF} = 10^{\frac{\sum \log \text{observed}}{n}} \quad (8)
\]

Segregation of predictions into subsets

For the human dataset, hepatocyte predictions were segregated into those derived from freshly isolated (\( n=52 \)) or cryopreserved cells (\( n=93 \)), to enable comparison of predictive accuracy between such preparations. Although several original research studies have performed such a comparison on small numbers of drugs, finding few significant differences (Diener et al., 1995; Li et al., 1999; Hewitt et al., 2000; Lau et al., 2002; Naritomi et al., 2003; McGinnity et al., 2004; Blanchard et al., 2005; Floby et al., 2009), this compilation provided an opportunity to address this question on a larger scale. In order to eliminate any bias associated with the inclusion of different substrates, a dataset comprising only of compounds common to both fresh and cryopreserved hepatocyte predictions (\( n=43 \)) was also evaluated.

A recent review by Jones et al. (2015) indicated that the pharmaceutical industry has less confidence in predictions of non-cytochrome P450 (CYP) mediated clearance predictions than their CYP-mediated counterparts. To test this assumption, human hepatocyte predictions of substrates for uridine 5’-diphospho-glucuronosyltransferases (UGTs), the major non-CYP metabolic enzyme family, were segregated from the remainder of the dataset (predominantly CYP substrates). Glucuronidated compounds were identified and categorised by fraction metabolised by UGT (\( \text{fm}_{\text{UGT}} \)) based on published in vitro and in vivo data (Miners and Mackenzie, 1991; Kaiser et al., 1992; Laethem et al., 1995; Soars et al., 2002).
Prompted by the recent publication by Bowman and Benet (2016), the relationship between protein binding and accuracy of in vivo clearance predictions (from both human hepatocytes and human liver microsomes) was also reassessed. In this study, the $f_{up}$, as opposed to the $f_{ub}$ was used as the measure of protein binding to circumvent the influence of drug binding to red blood cells. In addition, consistent with Bowman and Benet (2016), drugs were segregated by BDDCS class to examine potential relationships between drugs identified as substrates of uptake or efflux transporters, protein binding, and underprediction (human). For human hepatocyte and human liver microsome (HLM) predictions respectively, 99/101 and 83/83 drugs were able to be classified by BDDCS according to Benet et al. (2011) and Hosey et al. (2016). In addition, the BDDCS assignments were used to distinguish drug type for the analysis of ESF (both species). For rat, 125/128 (hepatocytes) and 65/71 (RLM) assignments were made including the proprietary compounds based on our assessment of the actual/likely physico-chemical properties. To support the examination of drug properties, drugs (human and rat hepatocyte dataset) were identified as acidic, basic or neutral.

**Comparison of human and rat in vitro prediction of intrinsic clearance**

Using the criteria detailed above, predictions of in vivo $CL_{int,u}$ were recorded for 101 drugs from human hepatocyte data (Figure 1A) and for 83 drugs from HLM data (Figure 1B); 66 drugs were common to both systems in human. In vivo $CL_{int,u}$ predictions for 128 compounds were made from rat hepatocyte data (Figure 1C) and for 71 compounds from rat liver microsome (RLM) data (Figure 1D); 52 compounds were common to both systems in rat. In vivo and in vitro clearance data and references for individual compounds are given in the Supplement (tables 1 and 2 for human and rat respectively).

In vivo $CL_{int,u}$ was predominantly underpredicted in hepatocytes and microsomes for both human and rat. In both species underprediction was greatest in hepatocytes with an average fold-underprediction of 4.2 in human and 4.7 in rat; microsomes showed less overall bias with average fold-underprediction of 2.8 in human and 2.3 in rat (Table 1). However, despite evidence of less average bias in microsomes, the percentage of predictions within two-fold of observed was similar across systems and species, ranging between 20 and 30 %. In both species, microsomes showed a greater incidence of overprediction (predicted values more than two-fold above observed) than hepatocytes.
Precision as represented by RMSE was similar between human hepatocytes and HLM; precision for rat was much less than for human, least of all for RLM (Table 1).

In both species, microsomes appear to display a wider range of CL\textsubscript{int,u} predictions than hepatocytes. In human, predicted CL\textsubscript{int,u} in hepatocytes ranged from approximately 1-1,000 ml/min/kg compared to approximately 0.1-10,000 ml/min/kg in microsomes (Figure 1, A and B respectively). In rat, predicted CL\textsubscript{int,u} appears to be limited to approximately 10,000 ml/min/kg in hepatocytes, but reaches 100,000 ml/min/kg in microsomes (Figure 1, C and D respectively). The greater imprecision implied by RMSE for rat may have at least in part reflected the greater upper range (hence greater error) in this species. An apparent intercept of prediction trend at the line of unity occurred at about 10 ml/min/kg for human and about 100 ml/min/kg for rat, possibly reflecting the general difference in metabolic rate expected between these two species.

**Comparison of predictions from fresh and cryopreserved human hepatocytes**

Division of the complete human hepatocyte dataset into \textit{in vivo} CL\textsubscript{int,u} predictions from fresh and cryopreserved preparations revealed no meaningful differences in both prediction bias and precision (Table 2). This finding was consolidated by the similar outcome from analysis of only those drugs (n=43) common to both types of preparation (Table 2, Figure 2). There was, therefore, clear evidence that the cryopreservation process has no influence on prediction accuracy.

**Relationship between empirical scaling factor and \textit{in vivo} clearance**

To further characterise the underprediction of \textit{in vivo} CL\textsubscript{int,u} in human and rat hepatocytes and liver microsomes, ESFs were calculated for individual compounds in each system and species and plotted as a function of observed CL\textsubscript{int,u} (Figure 3).

A similar trend of increasing ESF with increasing \textit{in vivo} CL\textsubscript{int,u} was observed for human and rat hepatocytes (Figure 3, A and C), indicating clearance-dependent underprediction. For microsomes, clearance-dependency was also apparent, with comparable magnitude between the two \textit{in vitro} systems (Figure 3, B and D).

Comparison of the average ESF between segregated levels of \textit{in vivo} CL\textsubscript{int,u} showed an exponential progression of underprediction of a similar magnitude between human hepatocytes and microsomes up to 10,000 ml/min/kg, despite the high variance (Table 3). Above 10,000 ml/min/kg, detailed
comparison was not justified due to few individual data, although a lower limitation in prediction by hepatocytes, compared to microsomes, was apparent. For rat, there was also quantitative agreement between the systems, although – as for human – there was marginally less underprediction evident for microsomes compared to hepatocytes. In addition, there was a progression of underprediction similar to human, albeit out of step by an order of magnitude (in terms of observed $\text{CL}_{\text{int,u}}$ subgroup) – possibly reflecting species differences in metabolic rates (as noted above). Together, these quantitative prediction profiles provide clear evidence of strong species-independent and, to a large extent, system-independent trends in clearance-dependent underprediction.

Examining the ESF trends in terms of drug type, as denoted by the BDDCS, there was extensive overlap between permeable drugs cleared by metabolism (Class 1) and similar drugs which were potential hepatic uptake substrates and which might have their clearance limited by this (Class 2) (Figure 3). For human, the trend of increasing ESF with $\text{in vivo}$ $\text{CL}_{\text{int}}$ appeared to be independent of drug type, although this was clearer for hepatocytes compared with microsomes; in the latter system, a number of Class 2 or 3 drugs were predicted accurately or overpredicted, possibly reflecting enhanced access to metabolic enzymes in this system (Figure 3, A and B). A similar lack of drug type dependency was evident also for rat, although a number of the most highly cleared drugs were potentially dependent on transport for clearance (Class 2 to 4). The drug type assignment according to BDDCS is necessarily imprecise and some of the drugs designated as BDDCS Class 2 could be otherwise designated as dependent on metabolism, rather than transport, according to the alternative ECCS system (Varma et al., 2015) by virtue of being neutral or basic compounds; this would include a considerable number of the proprietary compounds in the rat datasets. As such, there would be a predominance of compounds dependent on metabolic clearance. Comparing human with rat, the trend in increasing ESF with $\text{in vivo}$ $\text{CL}_{\text{int}}$ was generally independent of species and to a large extent, $\text{in vitro}$ system.

Use of empirical scaling factors between $\text{in vitro}$ and $\text{in vivo}$ from pre-clinical species has been suggested as a pragmatic refinement for methodology for human prediction (Naritomi et al., 2003). In the current analysis, similarity in ESF between rat and human might appear to support this, at least in general terms. However, for those drugs common to both rat and human datasets ($n=24$, hepatocytes; $n=17$, microsomes) there was no apparent correlation between the species for either
system (data not shown), indicating a lack of drug dependency in the scaling factor and hence persistence of considerable uncertainty on an individual drug basis.

**Prediction of in vivo clearance of UGT substrates**

Within the human hepatocyte dataset, drugs subject to glucuronidation were categorised as high (≥ 0.75), medium high (0.50-0.75), medium low (0.25-0.50) or low (<0.25) fmUGT. As a single group, glucuronidated drugs span a large range of in vivo CL_{int,u} (<10 - >1,000 ml/min/kg) and show comparable underprediction to drugs metabolised predominantly by other enzymes (CYP). There appears to be no relationship between fmUGT and underprediction of in vivo CL_{int,u} (Figure 4).

**Prediction of in vivo clearance of acidic, basic and neutral drugs**

There was extensive overlap of prediction accuracy for drugs identified in groups according to ionic character for prediction made for both human and rat hepatocytes (Figure 5) and hence this criterion was considered too simplistic for distinguishing prediction trends.

**Assessment of extent of underprediction in relation to fu_p and BDDCS**

To assess the extent of any relationship between binding in blood (assumed to be mostly due to binding to plasma protein), ESF was plotted against fu_p for both human hepatocyte and microsome datasets (Figure 6, A and B). Visually, there was no trend in prediction accuracy with fu_p for either dataset, although there was a clear tendency for BDDCS Class 2 drugs to be more highly bound than those of Class 1, as highlighted by Bowman and Benet (2016). The equivalent relationship for total blood binding (fu_b) was also examined but, as there were only very minor differences (data not shown) this was not analysed further.

Segregating the drugs by level of fu_p showed that the majority (90%) of drugs had fu_p values either within the range 0.01-0.1 (40%) or within the range 0.1-1 (50%). Average ESF was between 3 and 4 for hepatocytes and between 2 and 3 for microsomes, across these two levels of fu_p (Table 4). There were relatively few drugs with fu_p in the lower range of 0.001-0.01 and, excluding one/two highly aberrant values for hepatocytes and microsomes, ESF was about 6 (Table 4). For BDDCS Class 1 drugs specifically, ESF was between 3 and 6 for fu_p across the ranges 0.01-0.1 and 0.1-1 for hepatocytes, and between 2 and 4 across the same ranges, for microsomes (Table 4). For the same fu_p ranges, ESF for Class 2 drugs was between 4 and 13 for hepatocytes and between 1 and 3 for
microsomes (Table 4). The greater ESF values obtained for the lowest fuP level for all drugs (6.0 and 5.6 for hepatocytes and microsomes, respectively) were largely reflected in Class 2 drugs (8.2 and 6.3). A single compound in the microsome dataset was identified as BDDCS Class 4 but was excluded from the tabular analysis. Taken together, these observations indicate a lack of relationship between fuP and prediction accuracy and have no bearing on the trend of clearance-dependent prediction described earlier.

It is recognised that some historical fuP values may be biased by lack of assay pH control (Kochansky et al., 2008). An adjustment of fuP values for bases (average 2.6-fold increase, Kochansky et al., 2008) in this dataset would cause some skew towards overall greater fuP for the values for the range 0.1-1, due to the relatively weak binding of these compounds; however, exclusion of bases would not impact the lack of relationship between fuP and ESF for acidic and neutral drugs. Because the data cannot be retrospectively corrected with respect to exact assay conditions, such an adjustment was not made.

**Current status of in vitro prediction of clearance**

Prediction of clearance is a key component of drug discovery but as this report and many others verify, in vitro measured CLint (using human/rat hepatocytes/hepatic microsomes) extrapolated to in vivo is not reliably quantitative on a physiologically mechanistic basis; a general problem of some 2-5-fold underprediction has been increasingly recognised for more than a decade (Shibata et al., 2002; Hallifax et al., 2005; Ito and Houston, 2005; Riley et al., 2005; Brown et al., 2007; Stringer et al., 2008). Although this problem can be circumvented by empirical corrections based on available datasets across a range of drugs, as has been reported (Sohlenius-Sternbeck et al., 2012; Yamagata et al., 2016), a high level of uncertainty remains for any individual case. This situation cannot be resolved without thorough understanding of the underlying causes of bias and imprecision which characterise prediction of clearance, as highlighted in a report by Bowman and Benet (2016).

The use of in vitro systems has progressed from microsomes to hepatocytes and from the rat preclinical species to human over several decades, but recognition of the performance and limitations of component processes, let alone the causes of underprediction, has been slow. In 2010, Hallifax et al. showed that predictions from human hepatocytes tended to be increasingly poorer with increasing in vivo CLint, a less distinct trend was observed for human liver microsomes, indicating that the intact cell
system was inherently unsuitable for handling highly cleared drugs. Whether this implication was specific to human hepatocytes was not resolvable due to the paucity of reported data for rat at that time. However, the position has changed regarding studies in rat and a viable comparison with human has become realistic and hence a key component of this commentary.

This current assessment shows an average underprediction of \( \text{in vivo} \, \text{CL}_{\text{int}} \) by human hepatocytes \((n=101)\) of 4.2-fold, which confirms the bias reported by Hallifax et al. (2010) \((n=89)\). But, in addition, this study shows a similar (4.7-fold) underprediction from rat hepatocytes \((n=128)\) and in both cases, despite the anticipated imprecision, there is a strong trend of clearance-dependency in prediction accuracy. Quantitatively, prediction accuracy ranges from unbiased (approximately 1-fold) for drug clearance below 10 ml/min/kg \( \text{in vivo} \, \text{CL}_{\text{int}} \) to about either 20- (rat) or 30-fold (human) underprediction for drug clearance between 1,000 and 10,000 ml/min/kg. Since their initial use in clearance experiments, it has been accepted that human hepatocytes have been inherently more variable and subjected to more preparative and experimental variation than their rat counterparts; an additional caveat has been the possible mismatch between enzyme activity in donors and healthy volunteers and clinical patients, with consequential uncertainties (Iwatsubo et al., 1996; Iwatsubo et al., 1997; Wilson et al., 2003; Ito and Houston, 2005; Riley et al., 2005; Hallifax and Houston, 2009). By contrast, early rat hepatocyte studies indicated a lack of bias (Houston and Carlile, 1997; Ito and Houston, 2004) and consequently, underprediction of CL has been considered specific to the human situation. Now, using accumulated data from over a decade, we have shown that this is not the case and that clearance-dependent prediction bias is effectively species independent, implying that the methodology for determining \( \text{CL}_{\text{int}} \) in hepatocytes is inadequate specifically for moderate to highly cleared drugs.

In microsomes, the average underprediction of \( \text{in vivo} \, \text{CL}_{\text{int}} \) was less than that in hepatocytes for both species, at 2.3-fold for rat \((n=71)\) and 2.8-fold for human \((n=83)\), although the proportion of predictions within 2-fold was similar to hepatocytes. However, as for hepatocytes, there was a clear trend of clearance-dependency in prediction accuracy with a similar exponential slope of increasing underprediction with \( \text{in vivo} \, \text{CL}_{\text{int}} \). Both of these findings — extent of average underprediction and clearance-dependency in microsomes — were unexpected. Previously, underprediction from microsomes has been reported as greater than from hepatocytes and seemingly explained by
absence of additional potentially critical clearance pathways (Engtrakul et al., 2005; Riley et al., 2005; Hallifax et al., 2010). Based on the present, most extensive study to date, it must be considered that the negative consequences of selectivity of enzyme pathways in microsomes is offset by other factors such as removal of rate-limiting access of drug to metabolic enzymes. Furthermore, the BDDCS analysis indicates that the trends in bias were apparently independent of the importance of clearance mechanism. Clearance-dependent prediction bias of a similar magnitude by both cellular and subcellular systems suggests influential factors beyond the above.

Aside from experimental limitations, potential problems within the physiological scaling methodology remain pertinent to this analysis. Here, as in most studies, predictions used the well-stirred liver model because the parallel tube liver model (at the other extreme of drug hepatic dispersion) offered only marginal reduction in bias and precision (not shown), consolidating previous conclusions (Hallifax et al., 2010). The discussion is necessarily limited to conventional non-permeability-limited models of hepatic clearance for two main reasons: the historical predominance of drugs not limited by permeability in their hepatic uptake and the lack of distinction of non-metabolic rate-limiting processes within the available in vitro data. Extended liver clearance models may be appropriate for an increasing number of drugs due to reliance on uptake transport and, indeed, lack of in vitro distinction of such processes has been suggested as a reason for underprediction of clearance (Chiba et al., 2009; Patilea-Vrana and Unadkat, 2016; Korzekwa and Nagar, 2017). This report, however, attempts to focus on more general prediction trends. Beyond liver model selection, binding of drug to plasma protein is another important physiological factor in prediction methodology that has been re-appraised recently. The $f_{ub}$ is potentially highly influential in determining in vivo $\text{CL}_{int}$ from clearance, especially where $f_{ub}$ is low (<0.05); the extent of binding within blood, or at least to plasma proteins, has been reported to correlate with underprediction (Soars et al., 2007b; Ring et al., 2011). There has been a perception that the $f_{ub}$ is difficult to measure at or below about 0.05 and this may be seen as a source of imprecision if not also bias. Although Riccardi et al. (2015) demonstrated that such low levels can be measured accurately with sufficiently controlled experiments, historical data might still be questioned. In addition, it has been shown that historical data might be erroneous where assay pH was not adequately controlled (Kochansky et al., 2008). This would appear to affect the $f_{up}$ of basic compounds more than others, although basic drugs show no bias in prediction relative to acids and neutrals. There is some lack of agreement in the literature on whether extensive binding is related to
poor prediction of CL; Hallifax and Houston (2012) demonstrated a lack of relation between binding and prediction, based on analysis within the database reported in 2010, in contrast to earlier reports (Chiba et al., 2009). More recently though, Bowman and Benet (2016) found a tendency for drugs categorised as highly bound to have greater prediction bias than lesser bound drugs, particularly for BDDCS Class 2, although their analysis used sub-groups of relatively small numbers. The present study, examining trends among the entire datasets for these most recent databases (human hepatocytes and microsomes), appears not to support substantial involvement of the degree of binding to plasma proteins. Alternatives to fraction unbound ie. mechanistic involvement of bound drug in uptake (Poulin et al., 2012), has been discussed elsewhere (Hallifax and Houston, 2012) and is beyond the scope of this report.

Preparative experimental steps, such as cryopreservation of human hepatocytes, have been seen as potentially detrimental, but within the human hepatocyte dataset in this study, predictions using cryopreserved hepatocytes were not biased compared to predictions from freshly isolated hepatocytes - focussing attention on downstream experimental procedures. This consolidates previous reports by Diener et al. (1995), Li et al. (1999), Hewitt et al. (2000), Lau et al. (2002), Naritomi et al. (2003), McGinnity et al. (2004), Blanchard et al. (2005) and Floby et al. (2009). For hepatocyte assays, false clearance prediction of drugs which rely on uptake transport might be expected either due to unrepresentative transporter activity \emph{in vitro} (Soars et al., 2007a) or absolute loss of activity due to instability \emph{in vitro}. In the datasets presented here, the impact of such substrates on the trend in prediction bias appears to be limited; the majority of drugs included are considered to be cleared predominantly by metabolic (particularly CYP) enzymes without rate limitation by uptake transport. Those compounds considered liable to transport dependence largely followed the same trend which was similar in magnitude between hepatocytes and microsomes (for both human and rat). Predictions for drugs identified as UGT substrates within the hepatocyte dataset were not clearly resolved from others again indicating no particular hepatocyte bias over microsomes for these pathways.

\textbf{Future prospects to refine \emph{in vitro} prediction of clearance}

A number of factors can be speculated to cause the clearance-dependent underprediction now established in both human and rat hepatocytes and microsomes. The increase in prediction bias seen
with increasing \textit{in vivo} CL_{int} has been previously speculated as due to an increasing incidence of permeation rate limitation among high-turnover drugs (Hallifax et al., 2010). This would be supported by the similar trend in the present study which involves many drugs not limited by active uptake. However, Hallifax et al. (2012) showed no discernible relationship between prediction accuracy and either of several independent measures of passive permeability for a wide range of drugs. Huang et al. (2010) showed that among a number of highly permeable proprietary compounds, those which were also efflux substrates gave greater underprediction. Umehara and Camenisch (2012) suggested using a combination of hepatocytes and microsome assays to distinguish where clearance was rate limited by permeation. While the phenomenon of permeation rate limitation to hepatic clearance \textit{in vitro} may nevertheless occur, the present analysis provides no definite evidence of a permeation rate limitation and, indeed, the similar trend in underprediction with increasing clearance between hepatocytes and microsomes suggests involvement of an alternative, common explanation.

Cofactor depletion has been suggested as a possible cause of clearance-dependent underprediction when using hepatocytes with high-turnover drugs (Swales et al., 1996; Swales and Utesch, 1998; Steinberg et al., 1999; Hengstler et al., 2000; Hewitt et al., 2000; Hewitt and Utesch, 2004; Hallifax et al., 2010; Foster et al., 2011). But this would not explain the similar trends in underprediction between hepatocytes and microsomes without considerable co-incidence given the different media used (cell culture medium vs. NADPH or regenerating system). Cofactor depletion has been a suggested consequence of cryopreservation and human hepatocytes (often cryopreserved) tend to be used with lower viability (typically not exceeding 80-90\% in the studies included here, where reported) than rat hepatocytes, implying greater potential loss of cofactors in these preparations (potentially reflected in cells deemed viable or not). But overall, the lack of any prediction bias among numerous studies for cells prepared this way does not support a significant role for cofactor loss associated with cryopreservation.

For typical drug depletion assays, sub-optimal substrate concentration would be a source of error (imprecision) among predictions and, if there was a tendency to use concentrations approaching or greater than \(K_M\) values for key pathways, this might manifest as a source of bias. In a review by Klopf and Worboys (2010), the widely practiced experimental expediency of a fixed substrate concentration of the order of 1 \(\mu\)M for all drugs screened for metabolic stability was highlighted. For some drugs, this
practice might exclude otherwise influential high affinity, low capacity enzyme sites with resultant poor predictions. Highly metabolically cleared drugs are often substrates of the CYP-2D and 3A families for which $K_M$ values at around or below 1 µM are not uncommon (Lewis and Ito, 2010); such cases, if ignored, could lead to clearance dependent prediction.

The phenomenon of the unstirred water layer (UWL) has been extensively discussed with respect to intestinal permeability in vitro systems (Karlsson and Artursson, 1991; Naruhashi et al., 2003; Avdeef et al., 2004; Korjamo et al., 2009) but has been little discussed with regard to metabolic clearance methodology, possibly due to segregation of drug discovery functions within companies. However, the UWL should be considered for hepatocyte assays at least, considering the present problem with high turnover drugs. Diffusion through the UWL might be rate limiting for such compounds, although, again, a degree of coincidence between the external environment of the whole cell and that of its microsomal fraction would be implied.

Ultimately, the in vitro causes of underprediction are likely to be multi-factorial. Further investigation into possible experimental causes of underprediction of clearance – and its clearance-dependence – is highly desirable in the light of the findings of this report. As the trend towards larger molecules with multiple charges and increasing involvement of dispositional transporters continues, more variable factors are added to the situation. While a broader view of the role of transporters and their interrelationship with enzymes will be important, detailed and systematic investigation needs to be focussed on certain other fundamental factors such as passive permeation mechanism, integrity of membrane and unstirred water barriers and their relative impact on rates. Other basic assay parameters such as substrate concentration dependence in $CL_{int}$ also need to be considered. Some of these potentially instrumental phenomena have recently been investigated in our laboratory and the findings will reported in due course.
Acknowledgement

The authors wish to thank James Harrison for useful contributions to the database compilation.

Author contributions

Participated in research design: Wood, Hallifax and Houston.

Performed data analysis: Wood and Hallifax.

Wrote or contributed to the writing of the manuscript: Wood, Hallifax and Houston.
References


DMD # 77040


Footnote

This work was funded by the Centre for Applied Pharmacokinetic Research consortium membership which included GSK, Janssen, Lilly and Pfizer.

F Wood current address: The Institute of Cancer Research, Cotswold Road, Belmont, Sutton, Surrey, SM2 5NG, UK.
Figure legends

Figure 1. Prediction of in vivo CL\textsubscript{int,u} in hepatocytes (A and C) and microsomes (B and D) in human (A and B) and rat (C and D). Dashed lines represent unity and dotted lines a two-fold margin of error. A single data point above predicted CL\textsubscript{int,u} of 100,000 ml/min/kg in RLM has been omitted for the purpose of comparable graphical representation.

Figure 2. Comparison of predicted in vivo CL\textsubscript{int,u} in fresh and cryopreserved human hepatocytes (drugs common to both preparations only). Dashed lines represent unity and dotted line represents unity.

Figure 3. Relationship between empirical scaling factor and observed CL\textsubscript{int,u} according to BDDCS classification for hepatocytes (A and C) and microsomes (B and D) in human (A and B) and rat (C and D). Compounds are represented as BDDCS Class 1 (♦), Class 2 (□), Class 3 (▲), Class 4 (∗) or unclassified (●). BDDCS assignments for propriety compounds (n= 88, rat hepatocytes; n= 37 rat microsomes) were judged on available physico-chemical information provided with source publications.

Figure 4. Comparison of predicted CL\textsubscript{int,u} in human hepatocytes with observed CL\textsubscript{int,u}. Drugs subject to glucuronidation are identified as high (≥ 0.75) (●), medium high (0.50-0.75) (∗), medium low (0.25-0.50) (▲) and low (<0.25) (■) fm\textsubscript{UGT}; all other compounds are represented as (◇). Dashed line represents unity and dotted lines a two-fold margin of error.

Figure 5. Relationship between empirical scaling factor (ESF) and observed CL\textsubscript{int,u} according to drug ionic character for human (A) and rat (B) hepatocytes. Compounds are represented as acidic (●), basic (▲), neutral (□) or unclassified (x).

Figure 6. Relationship between fu\textsubscript{p}, empirical scaling factor (ESF) and BDDCS class in (A) human hepatocytes and (B) HLM. Drugs are represented as BDDCS Class 1 (♦), Class 2 (□) and Class 3 (▲).
Table 1. Accuracy and precision of *in vivo* CL\(_{int,u}\) predictions in human and rat hepatocytes and liver microsomes as represented by AFE, average fold-underprediction, RMSE and percentage of predictions that fall within-, above- and below two-fold of observed *in vivo* CL\(_{int,u}\); n = number of drugs.

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hepatocytes</td>
<td>Microsomes</td>
</tr>
<tr>
<td>n</td>
<td>101</td>
<td>83</td>
</tr>
<tr>
<td>AFE</td>
<td>0.24</td>
<td>0.36</td>
</tr>
<tr>
<td>Average fold-underprediction</td>
<td>4.2</td>
<td>2.8</td>
</tr>
<tr>
<td>RMSE</td>
<td>3548</td>
<td>3524</td>
</tr>
<tr>
<td>% predictions within two-fold of observed</td>
<td>24</td>
<td>25</td>
</tr>
<tr>
<td>% predictions more than two-fold above observed</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>% predictions more than two-fold below observed</td>
<td>69</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Hepatocytes</td>
<td>Microsomes</td>
</tr>
<tr>
<td>n</td>
<td>128</td>
<td>71</td>
</tr>
<tr>
<td>AFE</td>
<td>0.21</td>
<td>0.43</td>
</tr>
<tr>
<td>Average fold-underprediction</td>
<td>4.7</td>
<td>2.3</td>
</tr>
<tr>
<td>RMSE</td>
<td>36203</td>
<td>63280</td>
</tr>
<tr>
<td>% predictions within two-fold of observed</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>% predictions more than two-fold above observed</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>% predictions more than two-fold below observed</td>
<td>71</td>
<td>56</td>
</tr>
</tbody>
</table>
Table 2. Accuracy and precision of *in vivo* CL\textsubscript{\textit{int,u}} predictions in fresh and cryopreserved human hepatocytes as represented by AFE, average fold-underprediction, RMSE and percentage of predictions that fall within-, above- and below two-fold of observed *in vivo* CL\textsubscript{\textit{int,u}}. Analyses of the complete dataset and of a reduced dataset of common drugs only are presented; n = number of compounds.

<table>
<thead>
<tr>
<th></th>
<th>Complete Dataset</th>
<th>Common Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh</td>
<td>Cryopreserved</td>
</tr>
<tr>
<td>n</td>
<td>52</td>
<td>93</td>
</tr>
<tr>
<td>AFE</td>
<td>0.25</td>
<td>0.23</td>
</tr>
<tr>
<td>Average fold-underprediction</td>
<td>3.9</td>
<td>4.3</td>
</tr>
<tr>
<td>RMSE</td>
<td>2891</td>
<td>3013</td>
</tr>
<tr>
<td>% predictions within two-fold of observed</td>
<td>29</td>
<td>22</td>
</tr>
<tr>
<td>% predictions more than two-fold above observed</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>% predictions more than two-fold below observed</td>
<td>63</td>
<td>72</td>
</tr>
</tbody>
</table>
Table 3. Average individual empirical scaling factor for predicted CL_{\text{int,u}} according to level of observed CL_{\text{int,u}} for human and rat hepatocytes and liver microsomes.

<table>
<thead>
<tr>
<th>Observed CL_{\text{int,u}} (ml/min/kg)</th>
<th>Empirical Scaling Factor (log average [n])</th>
<th>Human Hepatocytes</th>
<th>Human Microsomes</th>
<th>Rat Hepatocytes</th>
<th>Rat Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 10</td>
<td></td>
<td>0.61 [21]</td>
<td>0.70 [17]</td>
<td>0.13 [3]</td>
<td>0.086 [3]</td>
</tr>
<tr>
<td>10-100</td>
<td></td>
<td>3.9 [32]</td>
<td>1.8 [20]</td>
<td>1.6 [12]</td>
<td>0.83 [8]</td>
</tr>
<tr>
<td>100-1000</td>
<td></td>
<td>7.1 [40]</td>
<td>4.6 [34]</td>
<td>3.2 [67]</td>
<td>1.7 [34]</td>
</tr>
</tbody>
</table>
Table 4. Average individual empirical scaling factor for predicted CL_{int,u} according to f_u and BDDCS classification for human hepatocytes and liver microsomes. Numbers in italic indicate the exclusion of a single compound in this class, numbers in bold indicate exclusion of two compounds from this group.

<table>
<thead>
<tr>
<th>Category</th>
<th>Empirical Scaling Factor (log average [n])</th>
<th>f_u</th>
<th>0.001-0.01</th>
<th>0.01-0.1</th>
<th>0.1-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>6.0 [9]</td>
<td>4.0 [37]</td>
<td></td>
<td></td>
<td>3.3 [53]</td>
</tr>
<tr>
<td>BDDCS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class 3</td>
<td>[0]</td>
<td>0.032 [1]</td>
<td></td>
<td></td>
<td>0.61 [5]</td>
</tr>
<tr>
<td>Microsomes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>5.6 [7]</td>
<td>2.1 [35]</td>
<td></td>
<td></td>
<td>2.6 [39]</td>
</tr>
<tr>
<td>BDDCS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class 1</td>
<td>4.3 [2]</td>
<td>2.7 [13]</td>
<td></td>
<td></td>
<td>3.8 [31]</td>
</tr>
<tr>
<td>Class 3</td>
<td>[0]</td>
<td>0.013 [1]</td>
<td></td>
<td></td>
<td>0.22 [3]</td>
</tr>
</tbody>
</table>
Figure 1

A

B

C

D

Predicted CL_{int,u} (ml/min/kg)

Observed CL_{int,u} (ml/min/kg)

Predicted CL_{int,u} (ml/min/kg)

Observed CL_{int,u} (ml/min/kg)

Predicted CL_{int,u} (ml/min/kg)

Observed CL_{int,u} (ml/min/kg)

Predicted CL_{int,u} (ml/min/kg)

Observed CL_{int,u} (ml/min/kg)
Figure 2
Figure 4
Figure 5