1521-009X/43/12/1917–1928$25.00
Drug Metabolism and Disposition

http://dx.doi.org/10.1124/dmd.115.066431
Drug Metab Dispos 43:1917–1928, December 2015

Minireview

Low-Turnover Drug Molecules: A Current Challenge for Drug Metabolism Scientists

J. Matthew Hutzler, Barbara J. Ring, and Shelby R. Anderson

Q^2 Solutions, a Quintiles Quest Joint Venture, Bioanalytical and ADME Laboratories, Indianapolis, Indiana

Received July 22, 2015; accepted September 10, 2015

ABSTRACT

In vitro assays using liver subcellular fractions or suspended hepatocytes for characterizing the metabolism of drug candidates play an integral role in the optimization strategy employed by medicinal chemists. However, conventional in vitro assays have limitations in their ability to predict clearance and generate metabolites for low-turnover (slowly metabolized) drug molecules. Due to a rapid loss in the activity of the drug-metabolizing enzymes, in vitro incubations are typically performed for a maximum of 1 hour with liver microsomes to 4 hours with suspended hepatocytes. Such incubations are insufficient to generate a robust metabolic response for compounds that are slowly metabolized. Thus, the challenge of accurately estimating low human clearance with confidence has emerged to be among the top challenges that drug metabolism scientists are confronted with today. In response, investigators have evaluated novel methodologies to extend incubation times and more sufficiently measure metabolism of low-turnover drugs. These methods include plated human hepatocytes in monoculture, and a novel in vitro methodology using a relay of sequential incubations with suspended cryopreserved hepatocytes. In addition, more complex in vitro cellular models, such as HepatoPac (Hepregen, Medford, MA), a micropatterned hepatocyte-fibroblast coculture system, and the HμREL (Beverley Hills, CA) hepatic coculture system, have been developed and characterized that demonstrate prolonged enzyme activity. In this review, the advantages and disadvantages of each of these in vitro methodologies as it relates to the prediction of clearance and metabolite identification will be described in an effort to provide drug metabolism scientists with the most up-to-date experimental options for dealing with the complex issue of low-turnover drug candidates.

Introduction

The science of drug metabolism and pharmacokinetics (DMPK) has developed into a critical discipline under the broad spectrum of drug discovery and development. A primary objective of drug metabolism scientists is to effectively integrate human in vitro metabolism data with preclinical in vivo disposition data in order to predict the pharmacokinetic outcomes of drug molecules in patients, which is a challenging endeavor. Toward this effort, implementation of in vitro DMPK screening assays (e.g., metabolic stability) early in discovery phase programs became the paradigm, which has had significant positive impact on pharmacokinetic properties, resulting in marked improvement in later-stage drug candidate attrition (Kola and Landis, 2004). Due to these successes, modern drug discovery programs have generally adopted a parallel optimization strategy for absorption, distribution, metabolism, and excretion (ADME) properties along with pharmacological target potency in an effort to expedite the process of discovering suitable drug candidates for clinical investigations (Di et al., 2008; Hop et al., 2008). Because of these advances, selection of a drug candidate with a reasonably low-dose and dose-frequency projection is the expectation to enable decision making around investment in expensive clinical programs.

dx.doi.org/10.1124/dmd.115.066431.

Clearance is the volume of blood that is completely cleared of drug per unit time (typically, ml/min or ml/min/kg) when it passes through a clearing organ such as the liver. Clearance is the most important pharmacokinetic parameter since it is a critical component used in estimating the half-life and oral bioavailability of a drug (Obach, 2001, 2011). For example, absorption, along with first-pass metabolic clearance, will determine bioavailability of an orally administered drug. Clearance is also coupled with the volume of distribution to estimate the effective half-life of a drug, which is a pharmacokinetic parameter used in estimating the necessary frequency of dosing. Thus, development of in vitro and allometric scaling methodologies for predicting clearance and assessment of their accuracy (e.g., within 2- or 3-fold of actual) has been an intense focus of drug companies in an effort to continuously modify and improve these methods (Hosea et al., 2009; Ring et al., 2011). Twenty years after first proposing the use of in vitro methodologies to predict in vivo clearance (Houston, 1994) there are multiple literature examples where scaling from liver microsomal intrinsic clearance (Cl_{int}) provided a good prediction of the pharmacokinetic profile of a drug (Kuperman et al., 2001; Obach et al., 2007; Hutzler et al., 2010). However, the increased availability of fresh and cryopreserved hepatocytes, which contain a complete set of hepatic clearance (Cl_h) pathways, has resulted in this in vitro system being increasingly used for metabolic clearance predictions (Brown et al., 2007; Hallifax et al., 2010). However, it has been noted that data from

ABBREVIATIONS: ADME, absorption, distribution, metabolism, and excretion; Cl_h, hepatic clearance; Cl_{int}, intrinsic clearance; Cl_{total}, total clearance in vivo; DMPK, drug metabolism and pharmacokinetics; HLM, human liver microsome; Q_h, liver blood flow.

hepatocyte incubations, similar to data using microsomes, generally underpredicts in vivo clearance. Thus, there are additional, and as yet unknown, factors unaccounted for in these predictions that have not been improved despite the presence of a complete metabolic milieu in hepatocyte systems. While this observation is not the focus of this review, it is discussed at length in Hallifax et al. (2010).

A main shortcoming of current in vitro systems, which use human liver microsomes (HLMs) and hepatocytes to assess metabolism, is that incubation times are limited due to loss of enzymatic activity of the drug-metabolizing enzymes over time, which precludes the ability to obtain an estimate of intrinsic clearance for low-turnover (slowly metabolized) compounds. For example, in a standard metabolic stability study using HLMs with incubation times limited to roughly 1 hour (Foti and Fisher, 2004), sufficient metabolism (e.g., >10% substrate depletion to ensure detectable loss beyond biologic and bioanalytical noise) may not be observed to conclude metabolic turnover. Thus, in vitro clearance must be reported as a less-than value, which is of no practical use other than to say the compound of interest likely will have low clearance in vivo. As stated in a recent review by Di and Obach (2015), for a standard 4-hour human hepatocyte suspension assay, the lower limit of scaled Cl_h is roughly 6.3 ml/min/kg or ~30% of human liver blood flow (Q_h) (assumptions and scaling rules unknown). This is inadequate from a pharmacokinetic prediction standpoint, particularly for low volume of distribution drugs, where small changes in clearance can have a profound impact on estimated half-life. Furthermore, undetectable in vitro clearance precludes the ability to differentiate clearance properties of a subset of late-stage analogs within a chemical series poised for further development. This failure may lead to costly microdosing approaches in the clinic, a time-consuming and complicated venture. Adding to this limitation, it appears that the prevalence of low-turnover drugs is on the rise since medicinal chemistry strategies for synthesizing drug molecules that are less susceptible to metabolism are fairly straightforward. This trend is readily apparent in the observation by scientists at Pfizer who have recently reported that as much as 30% of their drug candidates have Cl_{int} values that are less than 10 ml/min/kg (Di et al., 2012). When clearance cannot be confidently estimated using in vitro substrate depletion assays, metabolism scientists are limited to approaches such as allometric scaling or monitoring metabolite formation. Despite reported successes (Ring et al., 2011), allometric scaling is time-consuming, costly, and increases animal usage. The allometric scaling approach is also less than ideal when considering potential species differences in metabolic clearance mechanisms, which has led to poor predictions of human drug disposition (Martignoni et al., 2006; Diamond et al., 2010; Zhang et al., 2011). Furthermore, current physiologically based pharmacokinetic models like SimCYP (Certara, St. Louis, MO) or GastroPlus (Simulations Plus, Lancaster, CA) require in vitro–derived enzyme kinetic input data in their models to predict human clearance, and with slowly metabolized drugs it is difficult to obtain reliable data unless key metabolites can be identified and quantitated for enzyme kinetic (V_{max}, K_m) assessment. While this approach is well-established, it requires access to often intractable authentic metabolite standards for calibration curve generation, which is typically not supported until early human clinical studies when predominant circulating metabolites are identified. Nonetheless, methodologies such as quantitative NMR have been reported, which may help to address this issue (Walker et al., 2014). In addition to predicting metabolic clearance, the U.S. Food and Drug Administration (USFDA, 2012) and European Medicines Agency (EMA, 2013) guidances emphasize that an understanding of predominant metabolites and metabolic pathways (i.e., clearance mechanisms) needs to be obtained for human, such that coverage of these metabolites can be confirmed in the preclinical species used for required in vivo toxicology studies prior to exposing humans. Observation of relevant human metabolites for low-turnover compounds in conventional in vitro systems is an equally important issue that deserves attention. Thus, in vitro methodologies that can reliably predict human-relevant metabolites for compounds that exhibit low turnover will be discussed.

In this review, we offer a critical comparison of several published methodologies employed to obtain estimations of clearance for low-turnover drug molecules using human hepatocytes, as well as their use in predicting relevant human metabolites. The advantages and disadvantages of utilizing suspended hepatocytes, a relay method in which the compound is sequentially incubated with suspended hepatocytes for 4 hours a day for up to 5 days, plated cryopreserved human hepatocytes, and coculture hepatocyte systems such as HepatoPac (Hepregen Corporation, Medford, MA) and HμREL (Beverley Hills, CA) and flow systems are discussed. It is anticipated that with the further development of novel in vitro systems, the need to conduct animal studies to enable human pharmacokinetic parameter predictions, and expensive exploratory clinical studies at risk to obtain human pharmacokinetics and metabolic pathways for low-turnover drugs, may be reduced.

Use of Hepatocytes to Estimate Clearance

Within the DMPK discipline, both HLMs and hepatocytes have been used to predict in vivo clearance, with hepatocytes having apparently become the preferred in vitro system for predicting total metabolic clearance and relevant metabolism because they contain the full complement of oxidative/reductive, hydrolytic, and conjugative drug-metabolizing enzymes present in the liver (Dalvie et al., 2009). In justifying this approach, hepatocyte use has been shown to be predictive of human metabolic clearance for a variety of cytochrome P450 and uridine 5′-diphospho-glucuronosyltransferase substrates (McGinnity et al., 2004). However, other groups have reported that accuracy of clearance predictions have not improved with the use of hepatocytes over that of HLMs, despite this being a more physiologically relevant system, where an underprediction bias in Cl_{int} values of 4- to 5-fold was noted (Hallifax et al., 2010). One source of potential inaccuracy may be impaired cellular uptake of the drug into the hepatocytes, particularly in those cases where active hepatic transport is involved in drug disposition (Di et al., 2013). It may also be that the differences in preparation of the hepatocytes may contribute to the observed poor correlations with in vivo clearance data. Underpredictions may also be partly due to cofactor exhaustion (Hallifax et al., 2010). Notwithstanding these observations, hepatocytes have become increasingly preferred as a matrix to study total metabolism, particularly since current drug discovery efforts have been focused on reducing reliance on the cytochrome P450 enzymes for clearance. An additional caveat in the use of hepatic matrices to predict total clearance predictions is the assumption that liver metabolism is the predominant route of drug clearance. Thus, should extra-hepatic metabolism and/or clearance (renal) be occurring, the use of in vitro systems derived from liver tissue (both HLMs and hepatocytes) would obviously underpredict total clearance (Di et al., 2013). For the purposes of this review, in vivo clearance is assumed to be 100% hepatic metabolism for model comparison.

A common practice for obtaining hepatocytes historically has been to order them fresh from vendors. However, this process is hindered by availability of suitable donor livers, which is not predictable and thus may delay timely delivery of metabolism data. Furthermore, fresh hepatocytes are from a single donor and have not been evaluated extensively for metabolic competency; therefore, there is a risk of

obtaining cells in which metabolism, as it relates to the population average, is low due to enzyme polymorphism, donor medical history, or factors such as the process of tissue procurement. The advancement of cryopreservation techniques has resulted in a more convenient and well-characterized source of hepatocytes with appropriate enzymatic activity. For example, McGinnity et al. (2004) at AstraZeneca observed that cryopreserved human and dog hepatocytes retained roughly 94% and 81% of the intrinsic clearance capability observed in freshly prepared hepatocytes. In addition, Smith et al. (2012) observed that cryopreserved hepatocytes generally had activity equal to (if not higher than) freshly isolated hepatocytes when comparing donor-matched hepatocytes. Hutzler et al. (2014) also reported that for aldehyde oxidase substrates cryopreserved hepatocytes generally provided higher activity than fresh hepatocytes from the same donor when used for incubation 24–48 hours later. Furthermore, cryopreserved hepatocytes are often pooled from multiple donors, offering a system to examine the metabolism of an average subject, with the goal of muting the effect of enzymatic differences between individuals (which at their most extreme may exhibit polymorphically expressed enzymes). Thus, because of the convenience and the preponderance of evidence demonstrating acceptable metabolic competency, cryopreserved hepatocytes that are pooled from multiple donors (typically, $n \geq 10$ donors) are the current gold standard in vitro metabolism system for determining hepatic intrinsic clearance.

While the implementation of hepatocytes in metabolism testing strategies has become routine, there are limitations for use of cryopreserved hepatocytes in suspension because viability drops rapidly following isolation (Skett, 1994). Smith et al. (2012) tested the metabolic activity of numerous enzymes in human hepatocyte suspensions that have been cryopreserved. Specific activities of CYP1A2, CYP2C9, CYP2D6, CYP3A, and 5′-diphospho-glucuronosyltransferase enzymes were determined following incubation periods from 0.5 to 10 hours and a pharmacodynamic parameter (IT_{50}, or time at which 50% activity has been lost) was derived for each enzyme tested using an inhibitory effect sigmoid E_{max} model to estimate the rate of enzyme activity loss. It should be noted that enzyme theory suggests an enzyme that is static (i.e., not engaged in substrate metabolism) may cause enzymatic damage due to futile cycling and the production of reactive oxygen species. Thus, the IT_{50} values generated may have overestimated enzymatic instability in this system. Regardless, the IT_{50} values can be used as a harbinger to indicate enzyme stability with time. In the Smith et al. (2012) study, the mean IT_{50} values were 2.69 ± 0.39, 4.47 ± 0.50, and 3.03 ± 1.16 hours for CYP1A2, CYP2C9, and CYP2D6, respectively, and 1.62 ± 0.09 and 1.39 ± 0.48 hours for CYP3A and 5′-diphospho-glucuronosyltransferase, respectively. This rapid decline in activity indicates a short window in time in which the hepatocytes are enzymatically competent. Thus, incubation times for cryopreserved hepatocytes in suspension should be limited to between 2 and 4 hours. The limits of this in vitro system have come to the forefront, and thus in vitro assay methodologies that prolong incubation times are required for slowly metabolized compounds.

In Vitro Models to Assess Low Clearance

Calculating Intrinsic Clearance. To perform intrinsic clearance studies with hepatocytes, either formation of metabolites or depletion of the parent test drug from the incubation is followed. Since these studies are often performed earlier in drug research when metabolic routes are not well-characterized and metabolite standards are not available, substrate depletion is the most commonly used methodology (Obach and Reed-Hagen, 2002). Following in vitro incubations with hepatocytes, a rate of depletion ($k_{dep} = \ln 2/t_{1/2}$) of the test article from the incubation is generated, which is then used to predict the in vitro Cl_{int} value of the compound (ml/min/kg) using in vitro scaling factors, and ultimately to predict hepatic clearance (the in vitro Cl_h value).

For the purpose of comparing published in vitro models and their performance in predicting clearance, the reported data from six studies were evaluated (Blanchard et al., 2005; Hallifax et al., 2010; Di et al., 2012, 2013; Smith et al., 2012; Chan et al., 2013). It is important to note when comparing different reports of hepatic clearance predictions using in vitro results that the data analyses were performed with a variety of scaling factors and approaches. Thus, to enable a more effective side-by-side comparison of the previously reported data, recalculations of the in vitro Cl_{int} and Cl_h values were conducted to ensure that the same scaling factors and assumptions were made across data sets. There are differing opinions on the modeling techniques to use for clearance predictions; however, the pros and cons of different approaches are outside the purpose of this paper (Fagerholm, 2007; Hallifax and Houston, 2009). It is also acknowledged that there is inherent variability in these in vitro estimates, from both laboratory and biologic sources. However, this analysis uses point estimates for both the in vitro and in vivo data in order to make these comparisons. Furthermore, a static modeling approach was taken that does not take into account the dynamic in vivo and in vitro environment in which metabolism is actually occurring. However, this approach is routinely used by DMPK scientists, and thus was used to focus on the techniques and outcomes in order to compare the various in vitro models for slow metabolic clearance.

To evaluate the prediction accuracy of the reported in vitro hepatocyte models, two comparisons were made: 1) scaled in vitro Cl_{int} versus back-calculated in vivo Cl_{int} from the literature total clearance in vivo (Cl_{total}) data (using the well-stirred model); and 2) scaled Cl_h generated from in vitro data (in vitro Cl_h) versus literature values for Cl_{total} (in vivo Cl_{total}). To predict Cl_{int} from in vitro data, scaling factors of 120 million hepatocytes/g liver (Hosea et al., 2009) and 25.7 g liver/kg (Davies and Morris, 1993) were used

$$\text{In vitro } Cl_{int} = k_{dep} \times \frac{\text{ml incubation}}{\text{hepatocytes in millions}} \times \frac{120 \quad \text{million hepatocytes}}{\text{g liver}} \times \frac{25.7 \text{ g liver}}{\text{kg}} \quad (1)$$

To calculate in vitro Cl_h from in vitro data, eq. 2 (the well-stirred model) was used (Rowland et al., 1973), with or without the incorporation of the unbound fraction of drug in the blood ($f_{uB} = f_{up}/R_B$, where f_{up} = fraction unbound in plasma and R_B = red blood cell partitioning) and liver blood flow (Q_h = 20.7 ml/min/kg) (Davies and Morris, 1993)

$$\text{In vitro } CL_h = \frac{Q_h \times f_{uB} \times Cl_{int}}{[Q_h + (f_{uB} \times Cl_{int})]} \quad (2)$$

In addition, to calculate in vivo Cl_{int} from observed Cl, a back-calculation using the rearranged well-stirred model (eq. 3) was used, where Cl = in vivo Cl_{total} [or in the case of analysis of data from Chan et al. (2013), Cl = in vitro Cl_h to back-calculate in vitro Cl_{int}]

$$\text{In vivo } Cl_{int} = \frac{Cl}{f_{uB} \times [1 - (Cl/Q_h)]} \quad (3)$$

Thus, data reported from the various in vitro systems reviewed herein were recalculated to assure that model evaluation was performed using a common platform (Table 1). The only deviation from this was for the literature review concerning the evaluation of the suspended hepatocyte model for clearance estimations (Hallifax et al., 2010). These data also included a correction of binding in the hepatocyte matrix as an added scaler to eq. 1, which was not teased out from the data reported herein.

TABLE 1

Clearance parameters from the literature and calculated Cl_{int} and Cl_h values (ml/min/kg) for the 27 compounds analyzed

	$CL_{in\ vivo}$[a]	$CL_{int,in\ vivo}$[b]	fuB[c]	R_B[d]	Suspended CL_{int}[e]	Suspended CL_h		Relay Method CL_{int}[g]	Relay Method CL_h		Plated CL_{int}[h]	Plated CL_h		HepatoPac Cl_{int}[i]	HepatoPac CL_h	
						With Binding[f]	Without Binding[f]		With Binding[f]	Without Binding[f]		With Binding[f]	Without Binding[f]		With Binding[f]	Without Binding[f]
	ml/min/kg	*ml/min/kg*														
Alprazolam	0.59[1]	1.6	0.38[1]	0.76[1]	2.1	0.77	1.9	—	—	—	—	—	—	4.0	1.4	3.3
Antipyrine	0.57[2]	0.60	0.97[2]	—	0.67	0.63	0.65	1.3	1.2	1.2	—	—	—	—	—	—
Atazanavir	8.7[1]	108	0.14[1]	1.0[1]	—	—	—	—	—	—	—	—	—	142.3	10.2	18.1
Atomoxetine	3.9[1]	205	0.024[1]	0.55[1]	—	—	—	—	—	—	—	—	—	70.9	1.5	16.0
Diazepam	0.38[1]	18.3	0.021[1]	0.71[1]	6.6	0.14	5.0	15.0	0.31	8.7	2.8[1]	0.06	2.5	12.2	0.26	7.7
Diclofenac	4.2[1]	583	0.009[1]	0.55[1]	86.8	0.76	16.7				—	—	—	294.0	2.4	19.3
Disopyramide	0.9[3]	6.3	0.15[3]	1.2[2]	—	—	—	4.8	0.70	3.9	—	—	—	—	—	—
Flecanide	9.1[1]	37.1	0.44[1]	0.89[1]	—	—	—	—	—	—	—	—	—	2.5	1.1	2.3
Glimepiride	0.62[1]	70.3	0.009[1]	0.55[1]	9.4	0.09	6.5	—	—	—	—	—	—	72.2	0.64	16.1
Ketoprofen	1.2[2]	74.9	0.017[2]	—	11.0	0.19	7.2	17.0	0.29	9.3	—	—	—	—	—	—
Lidocaine	9.2[1]	46.4	0.36[1]	0.84[1]	15.3	4.3	8.8	—	—	—	—	—	—	29.1	6.9	12.1
Meloxicam	0.15[1]	30.7	0.005[1]	1.22 [1]	—	—	—	—	—	—	—	—	—	4.6	0.02	3.8
Metroprolol	13.3[2]	46.5	0.80[2]	—	5.3	3.5	4.2	—	—	—	—	—	—	—	—	—
Midazolam	4.6[1]	204	0.029[2]	0.69[1]	138	3.4	18.0	—	—	—	58.2[2]	1.6	15.3	238.4	5.2	19.0
Naproxen	0.11[1]	6.1	0.018[2]	—	1.4	0.03	1.3	18.0	0.32	9.6	—	—	—	—	—	—
Prednisolone	2.4[2]	9.7	0.28[1]	1.0[1]	30	6.0	12.2				—	—	—	6.7	1.7	5.1
Ranitidine	2.9[2]	4.4	0.77[2]	—	3.0	2.1	2.6	3.1	2.1	2.7	—	—	—	—	—	—
Riluzole	3.5[1]	361	0.012[1]	1.7[1]	—	—	—	—	—	—	—	—	—	96.0	1.1	17.0
Risperidone	3.6[1]	26.2	0.16[1]	0.67[1]	—	—	—	—	—	—	—	—	—	34.1	4.4	12.9
Theophylline	1.1[1]	2.3	0.48[1]	0.85[1]	2.6	1.2	2.3	2.8	1.3	2.5	—	—	—	3.2	1.4	2.8
Timolol	11[2]	48.9	0.48[2]	—	4.4	1.9	3.6	14.0	5.1	8.4	—	—	—	—	—	—
Tolbutamide	0.17[1]	1.6	0.11[1]	0.55[1]	0.38	0.04	0.37	7.4	0.75	5.5	1.1[1]	0.12	1.1	4.6	0.47	3.8
Verapamil	13.3[2]	310	0.12[2]	—	33.4	3.4	12.8	—	—	—	—	—	—	—	—	—
Voriconazole	3.8[1]	11.1	0.42[1]	1.0[3]	—	—	—	—	—	—	—	—	—	25.3	7.0	11.4
Warfarin	0.045[1]	2.5	0.018[1]	0.55[1]	—	—	—	4.2	0.08	3.5	—	—	—	3.6	0.06	3.0
Zolmitriptan	6.7[1]	13.0[1]	0.75[4]	1.0[1]	—	—	—	3.5	2.3	3.0	—	—	—	—	—	—
Zolpidem	4.3[2]	31.9	0.17[2]	—	8.0	1.3	5.8	—	—	—	—	—	—	—	—	—

[a]The superscript numbers represent the following references: 1, Chan et al. (2013); 2, Hallifax et al. (2010); 3, Di et al. (2012).
[b]Cl_{int} in vivo back-calculated from Cl_{total} using eq. 3, except for zolmitriptan; the superscript number 1 represents Di et al. (2012).
[c]Data from the literature or calculated from reported f_u and R_b ($f_{ub} = f_u/R_b$). The superscript numbers represent the following: 1, f_u from Chan et al. (2013); 2, f_{ub} referenced in Hallifax et al. (2010); 3, Aitio (1981); 4, Dixon and Warrander (1997).
[d]The superscript numbers represent the following references: 1, Chan et al. (2013); 2, Hinderling et al. (1974); or 3, not available (assumed to be 1).
[e]Represents results from Hallifax et al. (2010).
[f]Cl_h calculated using the well-stirred model (eq. 2) either with or without f_{ub}.
[g]Represents results from Di et al. (2012, 2013).
[h]The superscript numbers represent the following references: 1, Smith et al. (2012); 2, Blanchard et al. (2005) (scaled in vitro data using eq. 1).
[i]In vitro Cl_{int} value obtained from back-calculated in vitro scaled CL_h (Chan et al., 2013) using the well-stirred model (modified eq. 3).
—, no data available.

Suspended Hepatocytes. While the use of hepatocytes has improved the underprediction bias of clearance noted with microsomes, there still generally remains a bias toward underprediction (Brown et al., 2007), which worsens as clearance increases (Hallifax et al., 2010). There has been speculation that this may relate to possible loss of the drug uptake transporters in cryopreserved hepatocytes (Lundquist et al., 2014). For slowly metabolized compounds, the rapid loss of enzymatic function alone limits the use of this system since it is difficult to reliably detect small losses of test article from the incubations; however, other factors such as transporter function should also be kept in mind when using suspended hepatocytes to predict total clearance.

Hallifax et al. (2010) published a review evaluating the prediction accuracy of hepatocyte incubations to assess clearance for 89 compounds from a variety of literature sources. For this evaluation, data from many researchers were included, and thus it is acknowledged that the incubation conditions were variable, which may impact some conclusions from these prediction comparisons. Furthermore, the free fraction in the hepatocyte matrix was included in the calculations, which was not included in analyses of other hepatocyte modeling techniques reported herein. In a re-evaluation of a subset of these compounds ($n = 17$), where in vitro Cl_{int} is compared with in vivo Cl_{int} based on the calculations described here (eqs. 1 and 3), the conclusion of Hallifax et al. (2010) was confirmed, which indicates that there is a clearance-dependent underprediction bias exacerbated as in vivo Cl increases (Fig. 1A; Table 1). In a re-examination of compounds that exhibit low intrinsic clearance ($Cl_{int} \leq 15$ ml/min/kg), this bias was less pronounced (Fig. 1B). Thus, using Cl_{int} to compare in vitro data to in vivo data, suspended hepatocyte incubations performed well for low-clearance compounds when metabolic loss was reliably detected (i.e., the major assumption using this in vitro system), but generally trends toward underprediction for higher-clearance compounds.

Using the well-stirred model (eq. 2) with this data set to predict Cl_h from in vitro Cl_{int}, the conclusion of Hallifax et al. (2010) was again confirmed, with the incorporation of the free fraction yielding on average an underprediction of in vivo Cl (Fig. 2A; Table 1). Focusing on those compounds with reported low in vivo Cl ($n = 13$; $\leq$5 ml/min/kg; ~25% of Q_h), underprediction of clearance was again observed when the free fraction was included in the well-stirred model with prediction accuracies of 38% and 54% within 2- and 3-fold, respectively (Fig. 2, B and C). Strikingly, when the free fraction was not included in the well-stirred model, a strong overprediction bias was observed (Fig. 2, A and B, open symbols). It should be noted that the reliability of the in vitro data (e.g., half-life extrapolation rules) used for these predictions of low-clearance compounds was not reported. Data from an in vitro substrate depletion assay becomes less reliable as extrapolation of the $t_{1/2}$ for substrate loss extends beyond the length of the incubation; therefore, overextrapolation from these studies is a possibility. For the aforementioned examinations, in vitro assay conditions were used, and subsequent data evaluation and analysis rules were unable to be assessed. It is clear that in vitro clearance must be sufficiently robust to accurately measure suspended hepatocytes following maximal 4-hour incubation. If this is not the case, then incubation conditions such as the number of cells or the length of incubation must be optimized to accurately estimate in vitro metabolic clearance.

Relay Method. Since short-term incubations with hepatocytes have shown good, albeit mixed, success in the prediction of the clearance of test articles when there is sufficient metabolism, one approach reported by Di et al. (2012, 2013) to resolve the incubation time limitation issue for suspended hepatocytes is to perform a series of up to five 4-hour incubations, or a relay. In this system, incubations of pooled hepatocytes at 0.5×10^6 cells/ml are performed in 0.5 ml incubation volumes in 24-well plates. The incubation mixture at the end of a 4-hour incubation period is centrifuged to pellet the hepatocytes with the supernatant subsequently drawn off and frozen until the next incubation period. This supernatant is thawed the next day and 300 μl introduced into a freshly prepared cryopreserved hepatocyte suspension (0.5×10^6 cells/ml) to continue the incubation period (Di et al., 2012, 2013). This technique allows the evaluation of low-clearance compounds over a period up to 20 hours (5 days of 4-hour incubation/day) (Di et al., 2013), and since every 4 hours a newly thawed hepatocyte suspension is used, the cells are metabolically competent over the total time period.

Evaluating the Di et al. (2012, 2013) data (Fig. 1A; Table 1), and using in vitro Cl_{int} values to compare with in vivo Cl_{int} (from total Cl using eq. 3), the predictions appear somewhat closer to the line of unity than when using a single incubation of suspended hepatocytes, albeit

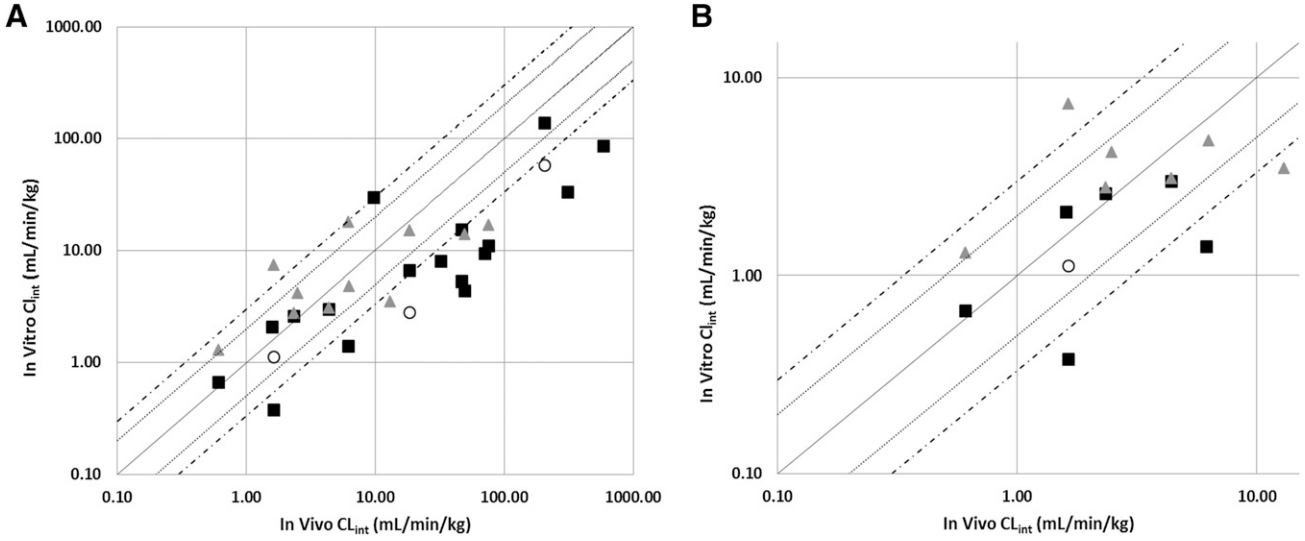

Fig. 1. In vivo Cl_{int} versus in vitro Cl_{int} generated in hepatocytes in suspension (standard and relay) and plated monoculture. The solid lines represent the line of unity; the dashed lines represent 2- and 3-fold differences from the line of unity. (A) Full data set. (B) Data set focused on low in vivo $Cl_{int} \leq 15$ ml/min/kg. ■, suspended hepatocytes (Hallifax et al., 2010); $n = 17/n = 6$ for total and low Cl_{int}, respectively. ▲, relay method with suspended hepatocytes (Di et al., 2012, 2013); $n = 11/n = 7$ for total and low Cl_{int}, respectively. ○, plated hepatocytes (Blanchard et al., 2005; Smith et al., 2012); $n = 3/n = 1$ for total and low Cl_{int}, respectively.

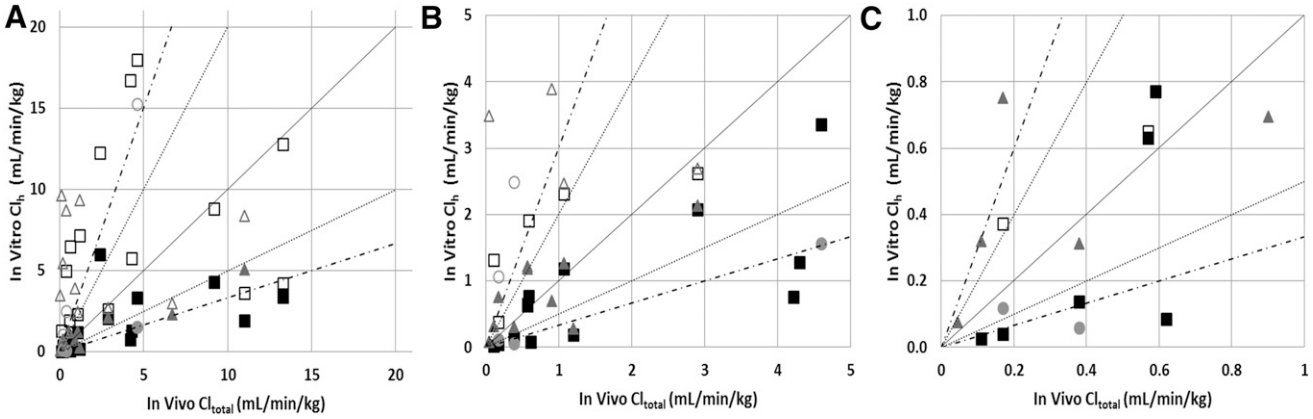

Fig. 2. In vivo Cl_{total} versus in vitro Cl_h generated in hepatocytes in suspension (standard and relay) and plated monoculture. The solid lines represent the line of unity; the dashed lines represent 2- and 3-fold differences from the line of unity. (A) Full data set. (B) Data set focused on low Cl ≤ 5 ml/min/kg. (C) Data set focused on very low Cl ≤ 1 ml/min/kg for ease of visualization. ■, suspended hepatocytes with f_{uB} correction, □, suspended hepatocytes without f_{uB} correction in the well-stirred model (eq. 2) (Hallifax et al., 2010); $n = 17/n = 13$ for total and low clearance, respectively. ▲, suspended hepatocyte relay model with f_{uB} correction, Δ, relay model without f_{uB} correction using the well-stirred model (eq. 2) (Di et al., 2012, 2013); $n = 11/n = 7$ for total and low clearance, respectively. ●, plated hepatocytes with f_{uB} correction, ○, plated hepatocytes without f_{uB} correction in the well-stirred model (eq. 2) (Blanchard et al., 2005, Smith et al., 2012); $n = 3/n = 2$ for total and low clearance, respectively.

with fewer compounds evaluated. Moreover, there is a similar trend in underprediction bias with high-clearance compounds. This underprediction bias was not as pronounced for compounds with Cl_{int} values of ≤15 ml/min/kg. Using the well-stirred model to predict Cl_h, a pattern similar to that observed with suspended hepatocytes was observed (Fig. 2A). For the low-clearance compounds, the predictions that included f_{uB} in the well-stirred model (eq. 2) exhibited 56% and 78% prediction accuracies within 2- and 3-fold, respectively. While underprediction bias is observed with the relay method for high-clearance compounds, similar to that seen with suspended hepatocytes, this bias is reduced or eliminated for low-clearance compounds. A similarly performed relay system using cryopreserved rat or dog hepatocytes with compounds, which exhibited an array of clearance values from low to high, gave similar performance outcomes. This is an important finding, since the successful use of in vitro preclinical models to predict in vivo outcomes helps to assure the relevance of the human clearance predictions using human in vitro models (Di et al., 2013).

Contrary to using standard hepatocyte suspension incubations to predict clearance, this suspended hepatocyte relay model allows the extension of incubation times up to 20 hours for low-clearance compounds. Evaluation of this limited data set suggests that use of the relay system for low-clearance compounds exhibits more accurate predictions of in vivo Cl_{int} compared with traditional hepatocyte suspensions (78% and 54% predicted within 3-fold for relay versus traditional suspended, respectively) (Table 1). However, a limitation in using the relay method in a discovery setting is the use of many vials of expensive hepatocytes. In addition, it is relatively labor intensive (4 to 5 consecutive days in laboratory). To offset the cost incurred though the use of up to five vials of human hepatocytes, a large number of compounds would need to be evaluated simultaneously.

Plated Hepatocytes. An alternative approach for extending the incubation time when using hepatocytes is to plate the hepatocytes into a monolayer or sandwich culture on a collagen-coated plate (12-, 24-, 48-, or 96-well design), typically at a cell density in the range of $0.3–1.0 \times 10^6$ cells/ml (Lau et al., 2002; Griffin and Houston, 2005; Smith et al., 2012). Plating hepatocytes enables them to recover from the isolation procedure and develop more liverlike function over a longer period of time. In addition, there have been several reports of utilization of hepatocyte monolayer culture systems in sandwich configuration in an effort to develop and then maintain liver-specific function for extended periods (e.g., days to weeks), such as albumin secretion and enzyme activity (Dunn et al., 1989; Koebe et al., 1994). However, data in this model for the purposes of estimating metabolic drug clearance over an extended incubation period for low-clearance drugs are currently lacking. One of the first reports proposing the use of a hepatocyte monolayer culture system to enable more accurate assessment of clearance for low-turnover drugs was that by Griffin and Houston (2005). In this series of studies, the Cl_{int} values of seven compounds were compared in freshly isolated rat hepatocyte suspensions and monolayer cultures (Griffin and Houston, 2005). While the in vitro Cl_{int} of only two (tolbutamide and 7-ethoxycoumarin) of the seven compounds tested correlated well (within 2-fold) with the in vivo clearance data, the overall rank order of Cl_{int} between compounds was the same in both models. Interestingly, the prediction of in vitro Cl_{int} for high-turnover compounds was lower for monolayer cultures compared with that observed in hepatocyte suspension, presumably due to rate-limited uptake of compound into the static (i.e., no shaking) monolayer hepatocytes and the reduced surface area for drug diffusion, thereby impacting the apparent enzyme kinetics (increased K_m and/or decreased V_{max}). Comparatively, when drug was incubated with hepatocytes in suspension with shaking (900 rpm), more rapid dispersion may take place, resulting in higher observed intrinsic clearance. The possibility of differential transporter expression in suspended compared with plated hepatocytes contributing to differences in cellular uptake of drug is also a consideration, where lack of efflux transport out of suspended hepatocytes may lead to higher rates of metabolism. This is speculative, and certainly does not apply to drugs that are not substrates for transport. Intriguingly, monolayer cultures gave a higher estimation of in vitro Cl_{int} for the low-turnover drug *S*-warfarin compared with suspensions (following equal incubation time of 60 minutes). Thus, the conclusion was made that hepatocyte monolayer cultures may be more suitable for predicting the Cl_{int} of low-clearance compounds (below 0.1 μl/min/10^6 cells), although this was based on a single observation using rat hepatocytes (Griffin and Houston, 2005), thus there is a need for further testing of this approach. Blanchard et al. (2005) also directly compared the metabolic clearance (fitting drug depletion to Michaelis-Menten constants) in suspensions and plated cultures of human hepatocytes prepared from three donors using naloxone, midazolam, and bosentan. As reported by Griffin and Houston (2005) using rat hepatocytes, the Cl_{int} of the high-clearance compound, naloxone, was higher in human hepatocyte suspensions compared with primary cultures for two of the three donors. The suggested explanation for

this observation was the continuous mixing of the suspensions, resulting in an increased rate of drug uptake into the suspended cells relative to the primary cultures. This explanation is comparable to the aforementioned work of Griffin and Houston (2005), with assay conditions for suspension incubations (shaking at speeds of up to 900 rpm) being quite different from monolayer cultures (no shaking). It is also the authors' experience that shaking versus nonshaking has a large impact on turnover rates (unpublished observations). In contrast, to the results with the high-clearance compound, the clearance of the two other (medium and low-clearance) compounds was comparable in both systems, which supports the notion that plated systems may be suitable for generating sufficient turnover for low-clearance drugs. Confounding these results was the fact that within the three donors tested there was variable activity, and only three test compounds were evaluated.

In a comprehensive assessment of metabolic profiles and temporal stability of phase I and II activities in human hepatocytes, Smith et al. (2012) evaluated the time-course of activity under various conditions in donor-matched livers that were incubated in both suspension formats, as well as following plating as adherent monolayers. In these studies, cryopreserved human hepatocytes from four donors were seeded in 24-well collagen-coated plates (0.4×10^6 cells/well). Following a 4-hour attachment period, specific activities of CYP1A2 (phenacetin) and CYP3A (testosterone) were assessed following 4, 6, 8, 12, 24, 48, and 72 hours of preincubation. Activities for these two enzymes were sustained for roughly 12 hours after plating and, on average, activities dropped to 38% ± 6% and 50% ± 31% of the 4-hour postattachment activity by 24 hours for CYP1A2 and CYP3A4, respectively (Smith et al., 2012). Similar to their assessment of intrinsic activity for enzymes in suspended hepatocytes previously discussed (inhibitory E_{max} model), the mean IT_{50} values from plated cells from the four donors were 21.3 ± 2.1 and 28.8 ± 20.4 hours for CYP1A2 and CYP3A, respectively (Smith et al., 2012). These results indicate increased enzyme stability relative to suspensions, where the IT_{50} values for these two enzymes were 2.69 and 1.62 hours, respectively. While this assessment of the time-course of enzyme activity in cultured human hepatocytes was limited in scope (i.e., only CYP1A2 and CYP3A activity was studied), the authors speculate that monolayer cultures sustain liverlike physiology, which prolongs metabolic enzyme activity relative to suspended hepatocytes. Diazepam and tolbutamide were incubated in both suspended and plated hepatocytes from the same donor to support this conclusion. While the 2-hour incubation in suspended hepatocytes resulted in insufficient turnover for both substrates, in vitro Cl_{int} values of 0.917 ± 0.377 and 0.364 ± 0.253 μl/min/10^6 cells were able to be obtained for diazepam and tolbutamide, respectively, following incubation in plated hepatocytes for up to 24 hours (Smith et al., 2012). However, the basal activities in hepatocytes in monolayer culture relative to suspension are still in question, and some believe that activities generally decrease substantially once cells are plated in monoculture, despite prolonged activity. Thus, the ability to capture clearance for diazepam and tolbutamide is likely a function of incubation time and not enhanced basal activity in a monolayer culture.

Similar to the analysis of data with hepatocytes in suspension, eqs. 1 and 2 were used to analyze the Blanchard et al. (2005) and Smith et al. (2012) data with respect to Cl_{int} of these three low-clearance compounds, which were common to the data set in Table 1 [midazolam; mean of four observations (Blanchard et al., 2005); diazepam and tolbutamide; mean of three observations (Smith et al., 2012)]. While this data set is very limited, with respect to Cl_{int} prediction there is clear underprediction bias for two of the three compounds, the exception being tolbutamide (Fig. 1A). When examining calculated in vitro Cl_h without f_{uB} correction, an overprediction of 3- to 6-fold is observed (Figs. 2, A and B; Table 1). However, when calculated Cl_h is corrected for f_{uB}, predictions are within 3-fold for midazolam (2.9-fold) and tolbutamide (1.4-fold), while a 6-fold underprediction for diazepam is observed (Fig. 2, A–C; Table 1), suggesting that correction for f_{uB} may be appropriate for low-turnover compounds.

Overall, this limited data set suggests that plated hepatocytes may be an in vitro system option for drug molecules requiring longer incubation times to achieve sufficient turnover. However, it is worth keeping in mind the possible loss in enzyme activity over the first 24 hours following plating (Smith et al., 2012). In addition, Richert et al. (2006) reported that gene expression for a number of phase I and II metabolic enzymes and transporters is profoundly decreased in cultured hepatocytes following plating, presumably due to oxidative stress responses. Current recommended practices are thus to begin incubations following visual verification of successful cell attachment, which tends to be in the 2–4 hour window following seeding, in an effort to capture enzyme activity near its peak, and minimize underpredictions of metabolic clearance. Also, due to the reported loss in enzyme activity over time, incubations using plated monoculture hepatocytes for clearance estimations should be limited to 24 hours or less, which may still be an insufficient incubation time for drug molecules that are metabolized extremely slowly.

Hepatocyte Coculture: HepatoPac (Hepregen). To address the relatively short timeframe (e.g., 4–24 hours) of metabolic enzyme activity in standard hepatocyte suspension or monolayer cultures, technologies such as the hepatocyte-fibroblast coculture system have been developed (Khetani and Bhatia, 2008). In this innovative in vitro approach, a micropatterned coculture of human hepatocytes is created by seeding hepatocytes on collagen-patterned matrices that enable selective cell adhesion, a concept that began development in the early 1990s (Bhatia et al., 1994). Following a 2 to 3 hour period, unattached cells are washed off, leaving ~10,000 adhered hepatocytes on 37 individual 500 μm collagen-coated islands (24-well configuration). This plate configuration is flexible, and can be scaled to 96-well plates. The next day, supportive mouse 3T3-J2 fibroblasts are seeded to create the cocultures. With this design, hepatocytes have been shown to maintain good morphology, viability, and liver-specific cytochrome P450 and phase II enzyme gene expression for up to 6 weeks (Khetani and Bhatia, 2008). In addition, liver-specific functions such as albumin secretion and urea synthesis were maintained for several weeks, compared with pure hepatocytes where these activities were only maintained for a few days. The longevity of drug-metabolizing enzyme activity and general cellular health has enabled numerous ADME applications, including the ability to conduct long-term incubations for low-turnover drug molecules (Chan et al., 2013) and to study the interplay between drug metabolism and transport (Ramsden et al., 2014).

The first report to evaluate the hepatocyte-fibroblast (HepatoPac) coculture system for measuring metabolism of low-turnover drugs was published by Chan et al. (2013). In this evaluation, 17 compounds with in vivo nonrenal clearance values ranging from low (≤5 ml/min per kg) to intermediate (>5 and <15 ml/min/kg) were incubated in the HepatoPac coculture system in a 96-well format, with 5000 hepatocytes per well. Incubations were carried out continuously for 7 days without media change, and three separate individual hepatocyte donors were evaluated to account for individual variability in enzyme activity. An evaluation of the Chan et al. (2013) data for HepatoPac was performed using the common modeling approaches outlined previously (Figs. 3 and 4; Table 1). This method yielded an excellent correlation between the in vitro Cl_{int} compared with the in vivo Cl_{int} (Fig. 3A). All five compounds (alprazolam, warfarin, tolbutamide, theophylline, and prednisolone), in which the modeled in vivo Cl_{int} was low (<15 ml/min/kg), predicted within 3-fold of the actual value (Fig. 3B). In scaling to in vitro Cl_h using

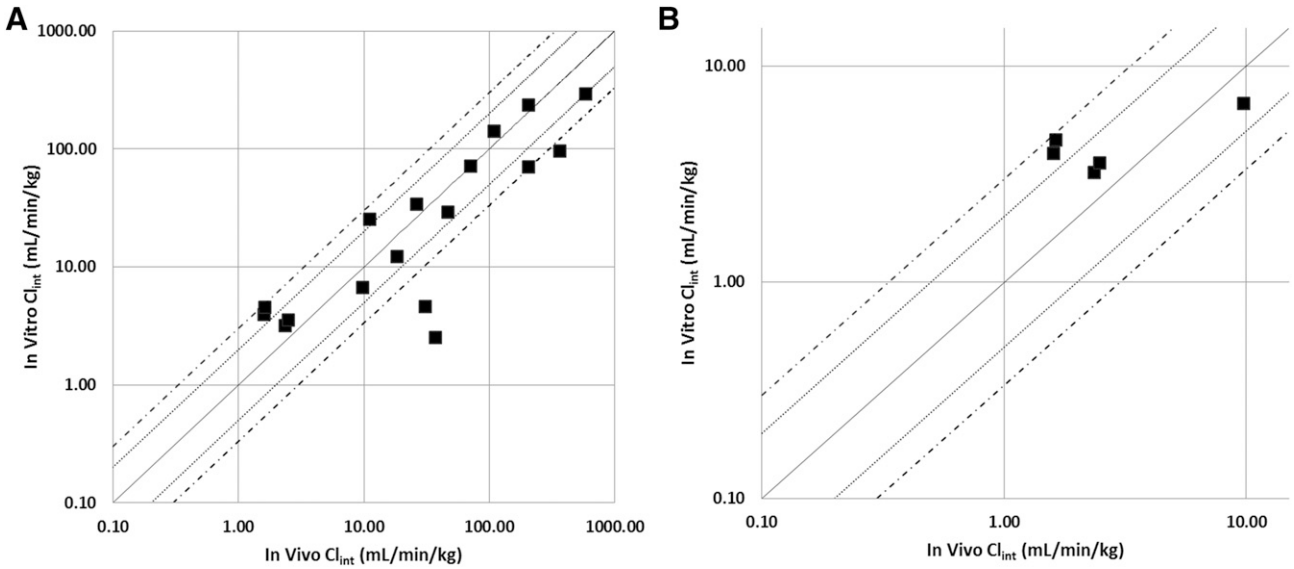

Fig. 3. In vivo Cl_{int} versus in vitro Cl_{int} generated in the coculture HepatoPac system (Chan et al., 2013). The solid lines represent the line of unity; the dashed lines represent 2- and 3-fold differences from the line of unity. (A) Full data set. (B) Data set focused on low $Cl_{int} \leq 15$ ml/min/kg.

f_{uB} binding correction in the well-stirred model (eq. 2), over the full range of compounds, within 2- and 3-fold prediction accuracies were 71% and 82%, respectively (Fig. 4A). Focusing on the 14 compounds that exhibit low in vivo Cl (≤5 ml/min/kg), the accuracies were 71% and 86%, respectively (Fig. 4B). It is noted that this favorable outcome was observed despite the fact that only 5000 hepatocytes were present in each well (96-well format) compared with the relay system in which 250,000 hepatocytes were used per well (24-well format). Even accounting for well size differences, there are significantly fewer hepatocytes present in the HepatoPac system. It should also be noted that the recalculated outcome compares even more favorably than that reported by Chan et al. (2013), in which comparisons to in vivo nonrenal Cl were made and only f_{up} was incorporated into the well-stirred model, whereas herein comparisons were made to total in vivo Cl using f_{uB} in the well-stirred model. The lack of impact of using in vivo hepatic Cl for comparison instead of total Cl likely reflects the Chan et al. (2013) observation that few of these compounds had a significant renal component in their clearance.

Again, as in the suspended hepatocyte evaluation where no incorporation of protein binding into the well-stirred model resulted in significant overprediction, this observation also held true for the HepatoPac system. When f_{uB} was not factored into the calculations, predictions were considerably less accurate [only one (theophylline) of six compounds predicted within 3-fold for the low-clearance compounds], with a definite trend toward overprediction (Fig. 4B). Chan et al. (2013) noted that underprediction was observed when incorporating protein binding for the intermediate to high-clearance compounds; however, this is not the finding when using total Cl and f_{uB}. In any case, there were only six compounds in this category, and thus this observation merits further investigation.

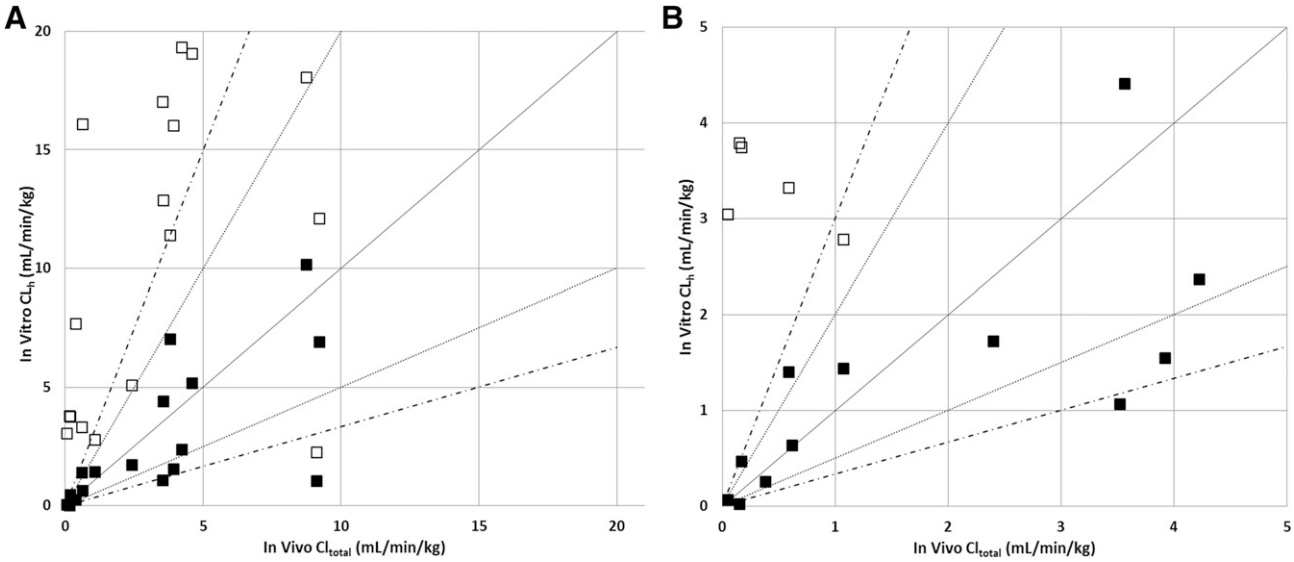

Fig. 4. In vivo Cl_{total} versus in vitro Cl_h generated in the coculture HepatoPac system. The solid lines represent the line of unity; dashed lines represent 2- and 3-fold differences from the line of unity. (A) Full data set. (B) Data set focused on low Cl ≤ 5 ml/min/kg. ■, in vitro data generated with f_{uB} correction in the well-stirred model, □, in vitro data generated without f_{uB} correction in the well-stirred model (eq. 2).

Evaluation of this data set suggests that use of the HepatoPac system for the low-clearance compounds is an excellent choice for accurate predictions of in vivo Cl_{total} (100%, 71%, and 86% predicted within 3-fold for HepatoPac, suspension, and relay methods, respectively) (Table 1). It appears that incorporation of f_{uB} into the well-stirred model (eq. 2), significantly improved prediction accuracy across metabolic clearance categories. Limitations of using this method in a discovery setting include the higher expense and increased labor intensiveness relative to cryopreserved suspended hepatocytes. However, for later-staged chemical series where metabolism is consistently hard to measure, and for differentiation of late-staged candidates within the same series, a coculture model may currently be the best option.

Donor Selection Considerations. As previously mentioned, variability in drug-metabolizing enzyme expression and activity between hepatocyte preparations is a concern, especially when considering that metabolic clearance rate data from hepatocytes are commonly used for prediction of human clearance as an important component of pharmacokinetic predictions. Thus, it is important to use donor hepatocytes that are thoroughly characterized from an activity perspective, in particular for polymorphically expressed cytochrome P450 (e.g., CYP2C9, CYP2C19, and CYP2D6), and for non-P450 enzymes such as glucuronosyltransferases and aldehyde oxidase (Hutzler et al., 2014). One approach to addressing this is to use pooled hepatocytes, typically composed of 10–20 donors. This approach is also commonly employed for hepatocyte suspensions and various subcellular fractions (e.g., liver microsomes or cytosol) to acquire data from what is perceived to be the average human. In plated hepatocyte systems, using pooled hepatocytes that are also plateable has been a challenge because not all donors provide plateable hepatocytes, and plating efficiencies may be variable between donors. Thus, a recommendation when using plated hepatocytes systems (standard plating or coculture systems) is to test multiple donors, as was conducted in the aforementioned HepatoPac clearance work where three separate donors were plated and clearance data from each were averaged for predictive purposes (Chan et al., 2013). A more novel approach is the use of pooled cryoplateable hepatocytes, which are now available from multiple vendors. The availability of pooled plateable hepatocytes theoretically means that average enzyme activities can be acquired similar to hepatocytes suspensions. The plating efficiency of each donor in the pool, as well as how the cells in this format communicate and how active they are, is a question that needs further validation and research to ensure the system truly represents an equal representation of each donor. Nonetheless, this plateable pooled approach is beginning to be used more often.

In Vitro Systems for Metabolite Generation. Following the release of the Food and Drug Administration guidance for safety testing of drug metabolites (USFDA, 2008), and later the European Medicines Agency guidance on nonclinical safety studies (EMA, 2009), a number of publications evaluating the predictive power of human in vitro systems to predict human circulatory and excretory metabolites emerged (Anderson et al., 2009; Dalvie et al., 2009; Leclercq et al., 2009), exemplifying the importance of a strategy for this assessment. The evaluation of human circulating metabolites enables one to address the metabolite in safety testing thresholds by assessing whether major human metabolites (defined as those representing $\geq$10% total circulating drug material) have been appropriately exposed and tested for safety in preclinical toxicology studies (Nedderman et al., 2011; Gao et al., 2013). In addition, the Food and Drug Administration (USFDA, 2012) and European Medicines Agency (EMA, 2013) drug interaction guidances reiterate that an understanding of human metabolism, including both metabolic clearance pathways and circulating human metabolites, should be understood prior to large-scale clinical trials. For drugs with moderate to higher clearance, the assessment of human metabolic pathways is typically conducted early in drug development using in vitro systems such as microsomes and/or suspended hepatocytes. Optimally, an understanding of the primary human metabolic pathways should occur early in the drug development process, with in vitro cross-species metabolite data customarily being generated prior to preclinical toxicity studies and clinical testing, and in vivo metabolite scouting data (e.g., human plasma and urine) being generated as part of the single and multiple ascending dose (early phase 1 studies) human clinical trials. To validate this approach for a given drug candidate, in vivo metabolism is typically assessed in the relevant preclinical species to ensure that the selected in vitro system (whether microsomes or hepatocytes) appropriately represents the observed in vivo pathways. Additionally, phenotyping of the enzymes responsible for the primary metabolic clearance routes is evaluated using in vitro systems (such as recombinant enzymes with relative activity scaling factors, selective inhibitors of drug metabolism enzymes, and/or liver bank cross-liver correlation analyses) to determine the potential for victim-based drug-drug interactions (DDI) and the potential for pharmacokinetic variability in drug clearance due to polymorphic expression of drug-metabolizing enzymes.

As previously noted, the successful use of early ADME screens to reduce metabolic liability and enable once-a-day dosing has resulted in an increased percentage of lower turnover rates for compounds in pharmaceutical drug portfolios. This change has resulted not only in added challenges in predicting drug clearance, but also in understanding the human-relevant metabolites for these lower-clearance drugs. For low-clearance drugs, the traditional in vitro systems (e.g., HLMs or hepatocytes) are inadequate for generating a predictive metabolite profile for a drug that is slowly, but extensively, metabolized. The inability to measure drug metabolites in vitro can thereby force companies to proceed into clinical testing prior to being able to effectively evaluate the likely human metabolic pathways, including whether the species selected (typically, one rodent and one non-rodent) for in vivo toxicity testing were in fact representative of the human metabolism. This represents a potential safety risk for human subjects, highlighting the requirement for novel metabolically competent in vitro models where drugs can be incubated for sufficient periods of time to enable turnover more representative of the in vivo systems.

A thorough evaluation of the ability of traditional in vitro systems including HLMs, S9 fractions, and suspended hepatocytes to predict major human metabolites (defined as metabolites representing $\geq$10% of total circulating drug-related material or $\geq$10% of excreted dose) for 48 drugs possessing quantitative assessments of in vivo human metabolism was conducted by Dalvie et al. (2009). It should be noted that the reported compounds represent a broad range of clearances, and without access to reported clearance values it is not possible to comment specifically on the success rates of the various methods for low-clearance compounds. Based on this assessment, HLM, S9 fraction, and hepatocytes successfully produced major in vivo human metabolites (both circulating and excretory) for 33%, 44%, and 54% of the compounds assessed, respectively. The predictive value increased when considering only primary metabolites (metabolites only one biotransformation step removed from parent drug) but decreased when evaluating only secondary metabolites (metabolites greater than one biotransformation step removed from parent drug) with HLM, S9, and hepatocytes successfully yielding the primary metabolites 66%–69% of the time, but secondary metabolites only 12%–56% of the time. The lower predictive value for metabolites requiring multiple, sequential metabolic processes very likely reflects the limited incubation times, and thereby metabolite residence times, for these systems. It is also possible that the high concentrations of substrate often used in incubations (e.g., 10–20 μM or higher) for metabolite identification

purposes may compete out low-level primary metabolite for enzyme, assuming the same enzyme is also involved in the secondary metabolism. Thus, while these traditional in vitro systems were fairly successful at generating relevant human metabolites, there is certainly much room for improvement.

Wang et al. (2010) expanded the Dalvie et al. (2009) analysis, evaluating 27 of the original 48 drugs (again representing a range of clearances) using a micropatterned coculture system from Hepregen. Incubations were conducted in 24-well plates using approximately 30,000 cells per well, and drug was incubated for up to 7 days with no change of media. Based on this analysis, HLM, S9, suspended hepatocytes, and cocultured hepatocytes successfully yielded the major excretory metabolites ($n = 39$ metabolites) 49%, 56%, 64%, and 82% of the time, and circulatory metabolites ($n = 40$ metabolites) 43%, 48%, 53%, and 75% of the time, respectively. These data suggest that the in vitro systems showed only slightly better predictive power for excretory metabolites than for circulatory metabolites, and that the predictive value increases when moving to the cocultured hepatocyte system with prolonged incubation times. The cocultured system again yielded primary metabolites (88–94% success rate) more effectively than secondary metabolites (67%–74% success rate), and showed notable improvement over the traditional HLM, S9, and suspended hepatocyte systems with shorter incubation times (69%–75% and 25%–57% success rates for primary and secondary metabolites, respectively). These data strongly suggest that the micropatterned, cocultured hepatocytes yield more in vivo relevant human metabolites than the standard in vitro systems. Again, it is not possible to comment specifically on the success rates for low-clearance compounds, but that said, the prolonged longevity of the cocultured hepatocytes would be expected to provide an advantage when assessing metabolic pathways for such low-clearance compounds. A nice example of the value of long-term incubations (up to 7 days) for two lower-clearance drugs using micropatterned cocultures was reported in Wang et al. (2010) (Fig. 5), where human-relevant metabolites for linezolid (in vivo Cl = 1 ml/min/kg) and ziprasidone (in vivo Cl = 5 ml/min/kg) were generated in more abundance when compared with 4-hour suspension incubations. Additionally, Ballard et al. (2014) evaluated a select subset of the previously reported compounds (a total of five compounds, three of which had not yielded all of the major in vivo human metabolites in the cocultured hepatocyte system) using the suspended hepatocyte relay method. For this evaluation, 4-hour incubations were conducted in 24-well plates using approximately 250,000 cells per well, with metabolite generation assessed after a total of 2, 3, or 5 relays (corresponding to 8, 12, and 20 hours of incubation). For these five compounds, the cocultured hepatocyte system and relay method generated 10 and 13 of the 16 relevant human metabolites, for success rates of 63% and 81%, respectively. Although this reflects a limited data set, it was noted that no new metabolites were generated after 2 relays (8 hours), suggesting that fewer relays may be required for metabolite generation than for clearance predictions.

As a targeted example of a low-clearance compound, Ramsden et al. (2014) presented the metabolism of faldaprevir, an inhibitor of the hepatitis C virus, using the HepatoPac cocultured hepatocyte system. Faldaprevir was initially reported to be metabolically stable in vitro (Duan et al., 2012), whereas the ^{14}C-human ADME study revealed two abundant monohydroxylated fecal metabolites together accounting for 41% of the dose (Chen et al., 2014). To better characterize the observed in vivo metabolism, ^{14}C-faldaprevir was evaluated in the HepatoPac system using a 24-well plate format with approximately 32,000 cells per well and sampling times up to 96 hours. Metabolites generated using HepatoPac were quantitatively compared with those reported in feces for the ^{14}C-human ADME study. HepatoPac successfully yielded five of the seven reported faldaprevir fecal metabolites, with two monohydroxylated products (M2a and M2b) representing the largest

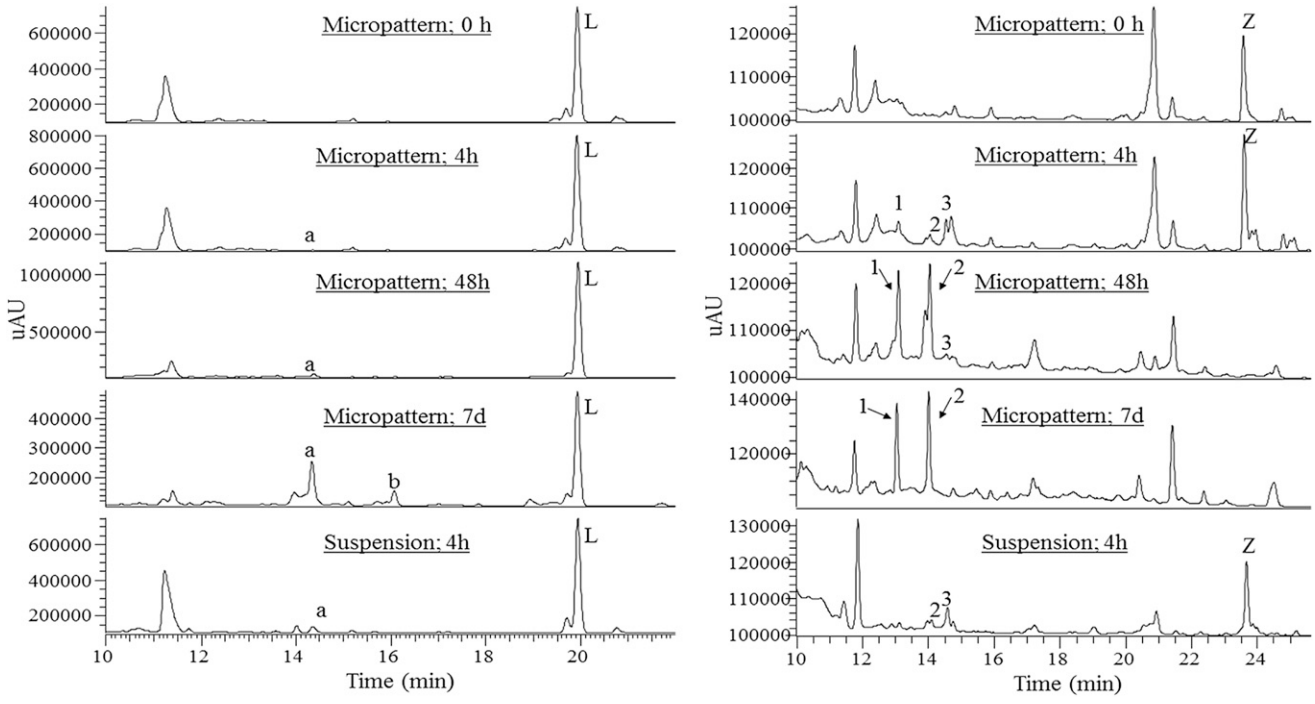

Fig. 5. High-performance liquid chromatography UV chromatogram of linezolid (left) and ziprasidone (right) in micropatterned human hepatocyte cocultures (0, 4, and 48 hours, and 7 days) and suspended human hepatocytes (4 hours). L, linezolid; a and b, morpholine ring-opened acid metabolites; Z, ziprasidone; 1, *N*-dealkylziprasidone *S*-oxide; 2, ziprasidone *S*-oxide; 3, *S*-methyldihydroziprasidone. Used by permission from R.S. Obach, and previously published in Drug Metabolism and Disposition (Wang et al., 2010).

metabolites in the HepatoPac system (accounting for 5% and 10% of the dose, respectively) and in the feces (accounting for 22% and 19% of the dose, respectively). The remaining five fecal metabolites were minor, individually accounting for <2% of the dose. Interestingly, the HepatoPac system also yielded faldaprevir glucuronide (accounting for ~2% of dose). Direct glucuronidation was a major clearance pathway in rat, but was not observed in the ^{14}C-human ADME study, either reflecting the lack of direct glucuronidation in humans or excretion of faldaprevir glucuronide into feces with subsequent hydrolysis. The authors suggest that based on in vitro to in vivo correlation using rat HepatoPac incubations, the human HepatoPac results suggest that direct glucuronidation may play a role in faldaprevir clearance in humans, thereby providing additional mechanistic information not directly obtained from the ^{14}C-human ADME study.

An overall evaluation of all of the reported data sets clearly suggests that use of the longer duration hepatocyte systems, such as the cocultured hepatocytes or the suspended hepatocyte relay method, provides improved predictive power for human-relevant metabolites, whether excretory or circulatory, over traditional in vitro methods. As more data are generated, it seems very likely that these systems will prove particularly valuable when assessing metabolic pathways, as well as enzyme phenotyping, for low-clearance compounds such as demonstrated by the faldaprevir case study. Additionally, coculture vendors such as Hepregen and HμREL now offer 24-, 48-, and 96-well plates containing both preclinical species and human hepatocytes to enable cross-species comparisons using a single plate. Given the trend toward lower-clearance drug candidates, such alternate in vitro systems are likely to become increasingly mainstream approaches.

Future Perspective. Despite considerable progress in recent years in developing new in vitro approaches and technologies to enable longer incubation times and measurement of intrinsic clearance with higher confidence, more research is certainly needed to further improve extrapolation to the in vivo situation. For example, most of the studies performed to evaluate different models for estimating intrinsic clearance have done so using predominantly cytochrome P450 substrates. While this family of drug-metabolizing enzymes remains the most important in drug metabolism, other metabolic enzymes such as UDP-glucuronosyltransferases and aldehyde oxidase are becoming increasingly relevant (Hutzler et al., 2013; Oda et al., 2015; Zientek and Youdim, 2015). Thus, the long-term stability and activity of these and other non-P450 enzymes in the discussed monoculture and coculture models still deserve better characterization. A direct comparison of various in vitro models using the same lot of hepatocytes also has yet to be reported. Given known distinct donor composition and thus enzyme activities between lots of hepatocytes, the most direct comparison for the performance of the reviewed hepatocyte models is one in which the different models are compared using the same lot of hepatocytes. In addition, phenotyping for enzyme involvement in drug metabolism is especially challenging for low-turnover drugs, in particular for early discovery in the absence of knowledge about predominant metabolites or access to metabolite standards. An in vitro model that enables longer duration incubations with the appropriate concentration of cytochrome P450 inhibitor that maintains concentration (e.g., minimal inhibitor depletion) and selectivity throughout long-term incubations still needs to be developed. Finally, microfluidic flow systems coupled with cocultured hepatocytes (e.g., HμREL) seem to be an area of emerging science, and may be used more routinely in drug metabolism laboratories in the near future. For example, Novik et al. (2010) demonstrated that integration of fluid flow with a hepatocyte coculture system provides the ability to differentiate compounds for clearance rates, including buspirone (high clearance), sildenafil (medium clearance), and indomethacin (low clearance). In addition, this coculture dynamic flow system showed an increased metabolite formation rate for several cytochrome P450 enzymes compared with both static systems and the monoculture flow system. Most recently, a model integrating drug metabolism with disease (e.g., inflammatory liver models) using a three-dimensional liver bioreactor culture system has been published (Sarkar et al., 2015), where hydrocortisone intrinsic clearance was predicted that correlated with human data and key metabolites were observed. A similar three-dimensional dynamic flow model has been shown to maintain human hepatocyte function for up to 7 weeks while generating more reproducible estimates of clearance for 7-ethoxycoumarin compared with hepatocytes in suspension (Choi et al., 2014). Finally, human hepatocytes seeded in a microfluidic LiverChip system were investigated for their phenotype, expression levels of 22 drug metabolism and transporter genes, and metabolic capacity in comparison with two-dimensional static monocultures (Vivares et al., 2015). The LiverChip device consists of a liver tissue–engineered array of perfused open-well bioreactors, each containing an integrated micropump for controlling flow of culture medium. The results demonstrated stable cellular function, and mRNA levels of major genes involved in drug metabolism were maintained over a 7-day culture period. Also, cytochrome P450 activity was comparable or higher (depending on the cytochrome P450 enzyme) after 4 days of culture compared with 1 day of culture in two-dimensional static conditions. Certainly, the in vitro metabolism field seems to be moving toward dynamic flow systems, some being coupled with cocultures of hepatocytes as the model of choice for definitive characterization of drug candidates. However, more optimization and validation for in vitro-in vivo extrapolation purposes is needed for low-turnover compounds.

Overall, it appears that most simplistic models for studying drug metabolism (e.g., microsomes and cryopreserved hepatocyte suspension), while serving a very important purpose in drug discovery, have effectively peaked in terms of their predictive utility. Thus, the current wave of drug metabolism research is now focused on identifying liverlike models containing hepatocytes with prolonged drug metabolism capacity that are more predictive of the in vivo situation for metabolic clearance and metabolite identification, in particular, for low-turnover drug molecules.

Acknowledgments

The authors thank Steven Wrighton for discussions and review of the manuscript.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Hutzler, Ring, Anderson.

References

Aitio ML (1981) Plasma concentrations and protein binding of disopyramide and mono-*N*-dealkyldisopyramide during chronic oral disopyramide therapy. *Br J Clin Pharmacol* **11**: 369–375.

Anderson S, Luffer-Atlas D, and Knadler MP (2009) Predicting circulating human metabolites: how good are we? *Chem Res Toxicol* **22**:243–256.

Ballard TE, Orozco CC, and Obach RS (2014) Generation of major human excretory and circulating drug metabolites using a hepatocyte relay method. *Drug Metab Dispos* **42**: 899–902.

Bhatia SN, Toner M, Tompkins RG, and Yarmush ML (1994) Selective adhesion of hepatocytes on patterned surfaces. *Ann N Y Acad Sci* **745**:187–209.

Blanchard N, Alexandre E, Abadie C, Lavé T, Heyd B, Mantion G, Jaeck D, Richert L, and Coassolo P (2005) Comparison of clearance predictions using primary cultures and suspensions of human hepatocytes. *Xenobiotica* **35**:1–15.

Brown HS, Griffin M, and Houston JB (2007) Evaluation of cryopreserved human hepatocytes as an alternative in vitro system to microsomes for the prediction of metabolic clearance. *Drug Metab Dispos* **35**:293–301.

Chan TS, Yu H, Moore A, Khetani SR, and Tweedie D (2013) Meeting the challenge of predicting hepatic clearance of compounds slowly metabolized by cytochrome P450 using a novel hepatocyte model, HepatoPac. *Drug Metab Dispos* **41**:2024–2032.

Chen LZ, Rose P, Mao Y, Yong CL, St George R, Huang F, Latli B, Mandarino D, and Li Y (2014) Mass balance and metabolite profiling of steady-state faldaprevir, a hepatitis C virus NS3/4 protease inhibitor, in healthy male subjects. *Antimicrob Agents Chemother* **58**:2369–2376.

Choi K, Pfund WP, Andersen ME, Thomas RS, Clewell HJ, and LeCluyse EL (2014) Development of 3D dynamic flow model of human liver and its application to prediction of metabolic clearance of 7-ethoxycoumarin. *Tissue Eng Part C Methods* **20**:641–651.

Dalvie D, Obach RS, Kang P, Prakash C, Loi CM, Hurst S, Nedderman A, Goulet L, Smith E, and Bu HZ, et al. (2009) Assessment of three human in vitro systems in the generation of major human excretory and circulating metabolites. *Chem Res Toxicol* **22**:357–368.

Davies B and Morris T (1993) Physiological parameters in laboratory animals and humans. *Pharm Res* **10**:1093–1095.

Di L, Atkinson K, Orozco CC, Funk C, Zhang H, McDonald TS, Tan B, Lin J, Chang C, and Obach RS (2013) In vitro–in vivo correlation for low-clearance compounds using hepatocyte relay method. *Drug Metab Dispos* **41**:2018–2023.

Di L, Kerns EH, Ma XJ, Huang Y, and Carter GT (2008) Applications of high throughput microsomal stability assay in drug discovery. *Comb Chem High Throughput Screen* **11**:469–476.

Di L and Obach RS (2015) Addressing the challenges of low clearance in drug research. *AAPS J* **17**:352–357.

Di L, Trapa P, Obach RS, Atkinson K, Bi YA, Wolford AC, Tan B, McDonald TS, Lai Y, and Tremaine LM (2012) A novel relay method for determining low-clearance values. *Drug Metab Dispos* **40**:1860–1865.

Diamond S, Boer J, Maduskuie TP, Jr, Falahatpisheh N, Li Y, and Yeleswaram S (2010) Species-specific metabolism of SGX523 by aldehyde oxidase and the toxicological implications. *Drug Metab Dispos* **38**:1277–1285.

Dixon R and Warrander A (1997) The clinical pharmacokinetics of zolmitriptan. *Cephalalgia* **17** (**Suppl 18**):15–20.

Duan J, Yong CL, Garneau M, Amad M, Bolger G, De Marte J, Montpetit H, Otis F, Jutras M, and Rhéaume M, et al. (2012) Cross-species absorption, metabolism, distribution and pharmacokinetics of BI 201335, a potent HCV genotype 1 NS3/4A protease inhibitor. *Xenobiotica* **42**: 164–172.

Dunn JC, Yarmush ML, Koebe HG, and Tompkins RG (1989) Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration. *FASEB J* **3**:174–177.

European Medicines Agency (EMA) (2009) ICH guideline M3 (R2) on non-clinical safety studies for the conduct of human clinical trials and marketing authorisation for pharmaceuticals. EMA, London.

European Medicines Agency (EMA) (2013) Guideline on the investigation of drug interactions. EMA, London.

Fagerholm U (2007) Prediction of human pharmacokinetics—evaluation of methods for prediction of hepatic metabolic clearance. *J Pharm Pharmacol* **59**:803–828.

Foti RS and Fisher MB (2004) Impact of incubation conditions on bufuralol human clearance predictions: enzyme lability and nonspecific binding. *Drug Metab Dispos* **32**:295–304.

Gao H, Jacobs A, White RE, Booth BP, and Obach RS (2013) Meeting report: metabolites in safety testing (MIST) symposium-safety assessment of human metabolites: what's REALLY necessary to ascertain exposure coverage in safety tests? *AAPS J* **15**:970–973.

Griffin SJ and Houston JB (2005) Prediction of in vitro intrinsic clearance from hepatocytes: comparison of suspensions and monolayer cultures. *Drug Metab Dispos* **33**:115–120.

Hallifax D, Foster JA, and Houston JB (2010) Prediction of human metabolic clearance from in vitro systems: retrospective analysis and prospective view. *Pharm Res* **27**:2150–2161.

Hallifax D and Houston JB (2009) Methodological uncertainty in quantitative prediction of human hepatic clearance from in vitro experimental systems. *Curr Drug Metab* **10**:307–321.

Hinderling PH, Bres J, and Garrett ER (1974) Protein binding and erythrocyte partitioning of disopyramide and its monodealkylated metabolite. *J Pharm Sci* **63**:1684–1690.

Hop CE, Cole MJ, Davidson RE, Duignan DB, Federico J, Janiszewski JS, Jenkins K, Krueger S, Lebowitz R, and Liston TE, et al. (2008) High throughput ADME screening: practical considerations, impact on the portfolio and enabler of in silico ADME models. *Curr Drug Metab* **9**:847–853.

Hosea NA, Collard WT, Cole S, Maurer TS, Fang RX, Jones H, Kakar SM, Nakai Y, Smith BJ, and Webster R, et al. (2009) Prediction of human pharmacokinetics from preclinical information: comparative accuracy of quantitative prediction approaches. *J Clin Pharmacol* **49**: 513–533.

Houston JB (1994) Utility of in vitro drug metabolism data in predicting in vivo metabolic clearance. *Biochem Pharmacol* **47**:1469–1479.

Hutzler JM, Linder CD, Melton RJ, Vincent J, and Daniels JS (2010) In vitro-in vivo correlation and translation to the clinical outcome for CJ-13,610, a novel inhibitor of 5-lipoxygenase. *Drug Metab Dispos* **38**:1113–1121.

Hutzler JM, Obach RS, Dalvie D, and Zientek MA (2013) Strategies for a comprehensive understanding of metabolism by aldehyde oxidase. *Expert Opin Drug Metab Toxicol* **9**:153–168.

Hutzler JM, Yang YS, Brown C, Heyward S, and Moeller T (2014) Aldehyde oxidase activity in donor-matched fresh and cryopreserved human hepatocytes and assessment of variability in 75 donors. *Drug Metab Dispos* **42**:1090–1097.

Khetani SR and Bhatia SN (2008) Microscale culture of human liver cells for drug development. *Nat Biotechnol* **26**:120–126.

Koebe HG, Pahernik S, Eyer P, and Schildberg FW (1994) Collagen gel immobilization: a useful cell culture technique for long-term metabolic studies on human hepatocytes. *Xenobiotica* **24**: 95–107.

Kola I and Landis J (2004) Can the pharmaceutical industry reduce attrition rates? *Nat Rev Drug Discov* **3**:711–715.

Kuperman AV, Kalgutkar AS, Marfat A, Chambers RJ, and Liston TE (2001) Pharmacokinetics and metabolism of a cysteinyl leukotriene-1 receptor antagonist from the heterocyclic chromanol series in rats: in vitro-in vivo correlation, gender-related differences, isoform identification, and comparison with metabolism in human hepatic tissue. *Drug Metab Dispos* **29**:1403–1409.

Lau YY, Sapidou E, Cui X, White RE, and Cheng KC (2002) Development of a novel in vitro model to predict hepatic clearance using fresh, cryopreserved, and sandwich-cultured hepatocytes. *Drug Metab Dispos* **30**:1446–1454.

Leclercq L, Cuyckens F, Mannens GS, de Vries R, Timmerman P, and Evans DC (2009) Which human metabolites have we MIST? Retrospective analysis, practical aspects, and perspectives for metabolite identification and quantification in pharmaceutical development. *Chem Res Toxicol* **22**:280–293.

Lundquist P, Lööf J, Sohlenius-Sternbeck AK, Floby E, Johansson J, Bylund J, Hoogstraate J, Afzelius L, and Andersson TB (2014) The impact of solute carrier (SLC) drug uptake transporter loss in human and rat cryopreserved hepatocytes on clearance predictions. *Drug Metab Dispos* **42**:469–480.

Martignoni M, Groothuis GM, and de Kanter R (2006) Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opin Drug Metab Toxicol* **2**:875–894.

McGinnity DF, Soars MG, Urbanowicz RA, and Riley RJ (2004) Evaluation of fresh and cryopreserved hepatocytes as in vitro drug metabolism tools for the prediction of metabolic clearance. *Drug Metab Dispos* **32**:1247–1253.

Nedderman AN, Dear GJ, North S, Obach RS, and Higton D (2011) From definition to implementation: a cross-industry perspective of past, current and future MIST strategies. *Xenobiotica* **41**:605–622.

Novik E, Maguire TJ, Chao P, Cheng KC, and Yarmush ML (2010) A microfluidic hepatic coculture platform for cell-based drug metabolism studies. *Biochem Pharmacol* **79**:1036–1044.

Obach RS (2001) The prediction of human clearance from hepatic microsomal metabolism data. *Curr Opin Drug Discov Devel* **4**:36–44.

Obach RS (2011) Predicting clearance in humans from in vitro data. *Curr Top Med Chem* **11**: 334–339.

Obach RS, Margolis JM, and Logman MJ (2007) In vitro metabolism of CP-122,721 ((2S,3S)-2-phenyl-3-[(5-trifluoromethoxy-2-methoxy)benzylamino]piperidine), a non-peptide antagonist of the substance P receptor. *Drug Metab Pharmacokinet* **22**:336–349.

Obach RS and Reed-Hagen AE (2002) Measurement of Michaelis constants for cytochrome P450-mediated biotransformation reactions using a substrate depletion approach. *Drug Metab Dispos* **30**:831–837.

Oda S, Fukami T, Yokoi T, and Nakajima M (2015) A comprehensive review of UDP-glucuronosyltransferase and esterases for drug development. *Drug Metab Pharmacokinet* **30**:30–51.

Ramsden D, Tweedie DJ, Chan TS, Taub ME, and Li Y (2014) Bridging in vitro and in vivo metabolism and transport of faldaprevir in human using a novel cocultured human hepatocyte system, HepatoPac. *Drug Metab Dispos* **42**:394–406.

Richert L, Liguori MJ, Abadie C, Heyd B, Mantion G, Halkic N, and Waring JF (2006) Gene expression in human hepatocytes in suspension after isolation is similar to the liver of origin, is not affected by hepatocyte cold storage and cryopreservation, but is strongly changed after hepatocyte plating. *Drug Metab Dispos* **34**:870–879.

Ring BJ, Chien JY, Adkison KK, Jones HM, Rowland M, Jones RD, Yates JW, Ku MS, Gibson CR, and He H, et al. (2011) PhRMA CPCDC initiative on predictive models of human pharmacokinetics, part 3: comparative assessment of prediction methods of human clearance. *J Pharm Sci* **100**:4090–4110.

Rowland M, Benet LZ, and Graham GG (1973) Clearance concepts in pharmacokinetics. *J Pharmacokinet Biopharm* **1**:123–136.

Sarkar U, Rivera-Burgos D, Large EM, Hughes DJ, Ravindra KC, Dyer RL, Ebrahimkhani MR, Wishnok JS, Griffith LG, and Tannenbaum SR (2015) Metabolite profiling and pharmacokinetic evaluation of hydrocortisone in a perfused three-dimensional human liver bioreactor. *Drug Metab Dispos* **43**:1091–1099.

Skett P (1994) Problems in using isolated and cultured hepatocytes for xenobiotic metabolism/metabolism-based toxicity testing—Solutions? *Toxicol In Vitro* **8**:491–504.

Smith CM, Nolan CK, Edwards MA, Hatfield JB, Stewart TW, Ferguson SS, Lecluyse EL, and Sahi J (2012) A comprehensive evaluation of metabolic activity and intrinsic clearance in suspensions and monolayer cultures of cryopreserved primary human hepatocytes. *J Pharm Sci* **101**:3989–4002.

U.S. Food and Drug Administration (USFDA) (2008) Guidance for industry: Safety testing of drug metabolites. USFDA, Silver Spring, MD.

U.S. Food and Drug Administration (USFDA) (2012) Guidance for industry: Drug interaction studies—Study design, data analysis, implications for dosing, and label recommendations. USFDA, Silver Spring, MD.

Vivares A, Salle-Lefort S, Arabeyre-Fabre C, Ngo R, Penarier G, Bremond M, Moliner P, Gallas JF, Fabre G, and Klieber S (2015) Morphological behaviour and metabolic capacity of cryopreserved human primary hepatocytes cultivated in a perfused multiwell device. *Xenobiotica* **45**:29–44.

Walker GS, Bauman JN, Ryder TF, Smith EB, Spracklin DK, and Obach RS (2014) Biosynthesis of drug metabolites and quantitation using NMR spectroscopy for use in pharmacologic and drug metabolism studies. *Drug Metab Dispos* **42**:1627–1639.

Wang WW, Khetani SR, Krzyzewski S, Duignan DB, and Obach RS (2010) Assessment of a micropatterned hepatocyte coculture system to generate major human excretory and circulating drug metabolites. *Drug Metab Dispos* **38**:1900–1905.

Zhang X, Liu HH, Weller P, Zheng M, Tao W, Wang J, Liao G, Monshouwer M, and Peltz G (2011) In silico and in vitro pharmacogenetics: aldehyde oxidase rapidly metabolizes a p38 kinase inhibitor. *Pharmacogenomics J* **11**:15–24.

Zientek MA and Youdim K (2015) Reaction phenotyping: advances in the experimental strategies used to characterize the contribution of drug-metabolizing enzymes. *Drug Metab Dispos* **43**: 163–181.

Address correspondence to: Dr. J. Matthew Hutzler, Q^2 Solutions, a Quintiles Quest Joint Venture, Bioanalytical and ADME Laboratories, 5225 Exploration Drive, Indianapolis, IN 46241. E-mail: matt.hutzler@Q2LabSolutions.com