ABSTRACT: In vitro–in vivo extrapolation (IVIVE) is an important method for estimating the hepatic metabolic clearance (CL) of drugs. This study highlights a problematic area observed when using microsomal data to predict in vivo CL of drugs that are highly bound to plasma proteins, and further explores mechanisms for human CL predictions by associating additional processes to IVIVE disconnect. Therefore, this study attempts to develop a novel IVIVE calculation method, which consists of adjusting the binding terms in a well-stirred liver model. A comparative assessment between the IVIVE method proposed here and previously published methods of Obach (1999. Drug Metab Dispos 27:1350–1359) and Berezhkovskiy (2010. J Pharm Sci 100:1167–1178) was also performed. The assessment was confined by the availability of measured in vitro and in vivo data in humans for 25 drugs highly bound to plasma proteins, for which it can be assumed that metabolism is the major route of elimination. Here, we argue that a difference in drug ionization and binding proteins such as albumin (AL) and alpha-1-acid glycoprotein (AAG) in plasma and liver also needs to be considered in IVIVE based on mechanistic studies. Therefore, converting unbound fraction in plasma to liver essentially increased the predicted CL values, which resulted in much more accurate estimates of in vivo CL as compared with the other IVIVE methods tested. The impact on CL estimate was more apparent for drugs binding to AL than to AAG. This is a mechanistic rational for explaining a considerable proportion of the divergence between previously estimated and observed CL values. Human CL was predicted within 1.5-fold, twofold, and threefold of the observed CL for 84%, 96%, and 100% of the compounds, respectively. Overall, this study demonstrates a significant improvement in the mechanism-based prediction of metabolic CL for these 25 highly bound drugs from in vitro data determined with microsomes, which should facilitate the application of physiologically based pharmacokinetic (PBPK) models in drug discovery and development. © 2011 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 101:838–851, 2012

Keywords: disposition; microsomes; hepatic clearance; metabolic clearance; unbound fraction; computational ADME; in vitro–in vivo extrapolation; IVIVE; pharmacokinetics; PBPK modeling
INTRODUCTION

Prediction of hepatic metabolic clearance (CL) is important because it provides insight into the rate of elimination of drugs from the body and allows for a physiological interpretation of the potential route(s) of elimination and the magnitude of oral first-pass elimination for a candidate drug. Therefore, CL is an important parameter in selecting the size of the dose, and along with volume of distribution, it determines the half-life and therefore the frequency of dosing. Although it is convenient to use in vitro data in drug discovery, in vitro–in vivo extrapolation (IVIVE) methods are often used to scale-up the in vitro intrinsic CL (CL_{int}) data from human liver preparations for predicting in vivo CL of drugs that are mainly eliminated by metabolism. Commonly used in vitro systems for the determination of CL_{int} include microsomal incubations, hepatocyte suspensions, and plated hepatocytes.

The central issue is whether the traditional assumption for drug access to the hepatocytes holds true for IVIVE. This assumption is that equilibrium between the free and protein-bound drug is instantaneous, such that the metabolism process is driven by a constant supply of unbound drug concentration in plasma. The equilibrium model generally refers to a well-stirred or parallel tube. There is evidence that drug extraction from the liver is sensitive to changes in plasma protein binding, in particular at very low free fraction values. However, the role and definition of unbound fraction in plasma (f_{up}) has long been a subject without a clear consensus because the kinetics of plasma protein binding under in vivo conditions is not fully understood, especially for highly bound drugs. As a result, it has been questioned whether CL can be predicted from in vitro parameters in the absence of in vivo measurements. Indeed, commonly reported discrepancies in IVIVE calculations include systematic underestimation and overestimation of in vivo CL when binding corrections measured in vitro (e.g., f_{up}) and no binding corrections (i.e., direct scaling), respectively, are used, particularly for highly bound drugs. Among the most probable reasons are the omissions of extrahepatic metabolism, significant errors in experimental assessment of f_{up}, as well as CL_{int} and/or inappropriate estimations of unbound drug available for metabolism in liver. The latter is the most commonly referenced reason because many studies have reported greater drug uptake into liver than that predicted based upon the existing models using free fraction of drug in arterial serum (i.e., f_{up}).

Of the many studies that investigated this aspect, the ones investigating the impact of experimental settings on CL estimates seem to be of most importance. It has been observed that less accurate CL predictions were obtained with plasma-free and microsomal incubations as compared with those from incubations using plasma. Blanchard et al. and Chao et al. assumed that adding plasma to the in vitro incubations would better mimic the protein binding/uptake/metabolism interplay that happens under dynamic in vivo conditions. Indeed, Blanchard et al. determined CL_{int} using hepatocyte incubations containing undiluted plasma and consequently incorporated the resulting apparent CL_{int} into the well-stirred model. These authors observed that the in vivo CL values predicted with these experimental settings may increase up to 18-fold as compared with plasma-free incubations. Furthermore, such settings significantly reduced the systematic bias in the estimation of CL and provided a better correlation between the predicted and observed CL values for several highly bound drugs. Berezhkovskiy et al. suggested that the improvements could be due to the direct account of plasma protein binding in the incubation medium. These authors presented a mathematical exercise that assumed that the values of f_{up} and hence the predicted CL values would increase only when the plasma in the incubations is diluted (i.e., f_{up} would increase due to the impact of dilution). However, the calculations of Berezhkovskiy et al. (diluted plasma) and observations of Blanchard et al. (undiluted plasma) are not in total agreement.

The available evidence on the role of extracellular binding proteins on liver metabolism supports the notion that ionic interactions between the protein–drug complex and hepatocyte cell surface would supply more unbound drug to the cell membrane, and hence this should result in a greater uptake (or intracellular concentration) than that predicted based on the free concentration in plasma. In other words, we should observe an enhanced uptake of the drug by passive diffusion (or transport processes), and hence a greater than expected CL_{int} and metabolism in vivo. In this context, Burczynski et al. observed that the CL of palmitate, a compound highly bound to albumin (AL), was significantly larger in the presence of AL in incubations as compared with when no AL was added, which are in accordance with Blanchard et al. Related to this, other studies seem to indicate that adding AL in the incubation medium may result in greater nonspecific binding of the AL–drug complex as compared with controls because smaller Michaelis–Menten affinity constant (K_{m}) values were observed. Thus, these observations might result in whole-liver fu (f_{uliver}) significantly larger than f_{up} under in vivo conditions, and hence explain why the consideration of protein binding inside the whole-liver compartment could be important for more accurate IVIVE calculations of drugs eliminated principally by metabolism.

It has also been suggested that the free drug concentration in the water of plasma and intracellular liver are not equal for ionizable compounds because there is a pH difference in the extracellular and intracellular water. That is, fu between plasma and liver should also vary according to the class and pKa of a drug, as suggested recently by Berezhkovskiy.\textsuperscript{11} Therefore, an unbound fraction in plasma apparent (fu\textsubscript{p-app}) also considers the impact of drug ionization on CL estimates. This is an improvement, but preliminary calculations for highly bound drugs demonstrated that this aspect did not totally cancel the systematic underestimation of in vivo CL when fu\textsubscript{p-app} was used in the well-stirred model.

Overall, it is understood that the accurate IVIVE calculation of CL should follow the free drug concentration in liver rather than plasma. Such an approach should consider the impact of drug ionization in the extracellular and intracellular water.\textsuperscript{11} Additionally, we hypothesize that the role of ionic interactions between the extracellular protein–drug complex and cell surface on the availability of drugs in hepatocytes should also be factored for highly bound drugs.\textsuperscript{13} Together, these factors may result in differences in the unbound drug concentration between plasma and liver and hence impact the predicted CL values. Therefore, it is generally assumed that converting fu\textsubscript{p} or fu\textsubscript{p-app} to fu\textsubscript{liver} in the well-stirred model might explain a considerable proportion of the divergence between previously estimated and observed CL values. As IVIVE is an important method used for CL estimates of drugs, this study attempts to develop a novel IVIVE calculation method that can be applied to data obtained in plasma-free microsomal incubations. Another objective was to make a comparative assessment between the proposed IVIVE method and other methods published in the literature, namely, the methods of Obach\textsuperscript{2} and Berezhkovskiy\textsuperscript{11}.

**METHODS**

The overall strategy is divided into four steps. The first step consists of presenting traditional IVIVE calculation methods based on the well-stirred model as tested in the literature by Obach.\textsuperscript{2} The second step consists of presenting those methods that were recently adjusted for drug ionization with fu\textsubscript{p-app} by Berezhkovskiy.\textsuperscript{11} The third step presents the efforts of this study wherein the well-stirred model was adjusted with fu\textsubscript{liver}. Finally, the fourth step evaluates the performance of all the IVIVE methodologies investigated. The present study did not prefer one IVIVE calculation method as compared with another because the main objective was to assess the prediction of human intravenous CL using several IVIVE methods with and without binding corrections to identify the most promising calculation method. The human CL referring to plasma kinetics was predicted for a common dataset of 25 drugs, which are highly bound to plasma proteins. Various statistical criteria were applied.

**MODELING ASSUMPTIONS**

The drugs used in this study were thought to be eliminated by hepatic oxidative metabolic CL under in vivo conditions.\textsuperscript{2,10} Among others, facilitated transport processes that could possibly be responsible for drug uptake or drug extrusion from hepatocytes were neglected. Because we used microsomal data determined in plasma-free incubations, it was implicit that drug distribution from plasma to hepatocytes was not impeded by limited diffusion processes under in vivo conditions. It was also assumed that the in vivo CL of drugs follows the free-drug hypothesis according to the well-stirred model. In this case, the binding to plasma proteins was assumed to be reversible and unsaturated at the conditions studied. Here, an important objective was to verify the impact on CL estimates of drugs highly bound to plasma proteins. Therefore, the binding to the incubation medium had to be relatively small as compared with the binding to plasma proteins. Consequently, only those drugs highly bound to plasma proteins (fu\textsubscript{p} < 0.15) and not highly bound in the incubation medium (fu\textsubscript{inc} > 0.1) were investigated in this study. Because it can be argued that a highly bound drug has a value of fu\textsubscript{p} ≤ 0.05 rather than fu\textsubscript{p} ≤ 0.15 as currently assumed, this study presents the statistical analyses of the prediction methods of human CL investigated also for the subset of drugs with fu\textsubscript{p} ≤ 0.05.

**Datasets**

Concise and complete datasets for human were obtained from two sources that had the required preclinical and clinical data, and experimentally determined in vitro data on CL\textsubscript{int} and fu\textsubscript{inc} under similar experimental conditions were reported. Obach\textsuperscript{2} published a complete human dataset containing 19 drugs meeting our selection criteria (eight bases, six acids, and five neutrals) and recently a PhRMA initiative\textsuperscript{10} published a unique and novel dataset with six drugs also meeting our selection criteria (five bases and one neutral), bringing the total number of drugs studied here to 25. These two datasets reported in vitro data principally for microsomal incubations. Compounds were divided into acidic, basic, and neutral classes. For the current drugs, the observed value of fu\textsubscript{p} ranged from 0.0007 to 0.15, whereas human CL ranged from 0.04 to 15 mL/(min kg), covering a large range of properties for highly bound compounds.\textsuperscript{2,10,16–35} The dataset is compiled in Table 1.
## Table 1. Human Dataset of Drugs Used for the Prediction of CL\textsuperscript{2, 10, 16–35}

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Log (P_{ow})\textsuperscript{a}</th>
<th>pKa\textsuperscript{a}</th>
<th>(F_1)\textsuperscript{b}</th>
<th>(fu_p)\textsuperscript{a}</th>
<th>(fu_{in})\textsuperscript{a}</th>
<th>(fu_{app})\textsuperscript{c}</th>
<th>(fu_{liver})\textsuperscript{c}</th>
<th>(R_{BP})\textsuperscript{a}</th>
<th>Main Binding Proteins\textsuperscript{d}</th>
<th>Scaled Microsomal (CL_{int}) (mL/min kg)\textsuperscript{c}</th>
<th>Human Plasma CL (In Vito) (mL/min kg)\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>5.42</td>
<td>9.7</td>
<td>2.50</td>
<td>0.05</td>
<td>0.11</td>
<td>0.125</td>
<td>–</td>
<td>0.78</td>
<td>AAG</td>
<td>25</td>
<td>8.6</td>
</tr>
<tr>
<td>Propafenone</td>
<td>4.24</td>
<td>9.74</td>
<td>2.51</td>
<td>0.04</td>
<td>0.26</td>
<td>0.10</td>
<td>–</td>
<td>0.7</td>
<td>AAG</td>
<td>166</td>
<td>13</td>
</tr>
<tr>
<td>Verapamil</td>
<td>3.79</td>
<td>8.5</td>
<td>2.40</td>
<td>0.1</td>
<td>0.43</td>
<td>0.24</td>
<td>–</td>
<td>0.77</td>
<td>AAG</td>
<td>122</td>
<td>14.5</td>
</tr>
<tr>
<td>Lorcanide</td>
<td>4.55</td>
<td>9.5</td>
<td>2.50</td>
<td>0.15</td>
<td>0.52</td>
<td>0.375</td>
<td>–</td>
<td>0.77</td>
<td>AAG</td>
<td>50</td>
<td>14</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>4.9</td>
<td>9.4</td>
<td>2.50</td>
<td>0.05</td>
<td>0.15</td>
<td>0.125</td>
<td>–</td>
<td>0.86</td>
<td>AAG</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>Imipramine</td>
<td>4.8</td>
<td>9.5</td>
<td>2.50</td>
<td>0.1</td>
<td>0.18</td>
<td>0.25</td>
<td>–</td>
<td>1.1</td>
<td>AAG</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>Quinidine</td>
<td>3.44</td>
<td>10</td>
<td>2.51</td>
<td>0.123</td>
<td>0.32</td>
<td>0.308</td>
<td>–</td>
<td>0.92</td>
<td>AAG</td>
<td>3.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Clozapine</td>
<td>3.42</td>
<td>7.7</td>
<td>2.01</td>
<td>0.05</td>
<td>0.13</td>
<td>0.100</td>
<td>–</td>
<td>0.86</td>
<td>AAG</td>
<td>4.6</td>
<td>2.5</td>
</tr>
<tr>
<td>PhRMA #1</td>
<td>2.9</td>
<td>6.5</td>
<td>1.17</td>
<td>0.04</td>
<td>0.64</td>
<td>0.047</td>
<td>–</td>
<td>0.7</td>
<td>AAG</td>
<td>78.6</td>
<td>5</td>
</tr>
<tr>
<td>PhRMA #2</td>
<td>3.14</td>
<td>8.4</td>
<td>2.37</td>
<td>0.12</td>
<td>0.62</td>
<td>0.285</td>
<td>–</td>
<td>0.54</td>
<td>AAG</td>
<td>53.1</td>
<td>9</td>
</tr>
<tr>
<td>PhRMA #3</td>
<td>3.81</td>
<td>10.1</td>
<td>2.51</td>
<td>0.025</td>
<td>0.46</td>
<td>0.063</td>
<td>–</td>
<td>1</td>
<td>AAG</td>
<td>23.4</td>
<td>2.5</td>
</tr>
<tr>
<td>PhRMA #4</td>
<td>4.26</td>
<td>10.2</td>
<td>2.51</td>
<td>0.048</td>
<td>0.12</td>
<td>0.120</td>
<td>–</td>
<td>1.2</td>
<td>AAG</td>
<td>17.1</td>
<td>8.2</td>
</tr>
<tr>
<td>PhRMA #5</td>
<td>3.94</td>
<td>pKa\textsubscript{A} = 10; pKa\textsubscript{A} = 6.5</td>
<td>2.93</td>
<td>0.0009</td>
<td>0.24</td>
<td>0.0026</td>
<td>0.035</td>
<td>0.74</td>
<td>AL</td>
<td>48.6</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mainly neutrals</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diazepam</td>
<td>2.8</td>
<td>–</td>
<td>1</td>
<td>0.013</td>
<td>0.28</td>
<td>0.013</td>
<td>0.149</td>
<td>0.71</td>
<td>AL</td>
<td>2.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Midazolam</td>
<td>3.8</td>
<td>–</td>
<td>1</td>
<td>0.04</td>
<td>0.88</td>
<td>0.04</td>
<td>0.357</td>
<td>0.53</td>
<td>AL</td>
<td>160</td>
<td>6.4</td>
</tr>
<tr>
<td>Methohexital</td>
<td>1.97</td>
<td>–</td>
<td>1</td>
<td>0.09</td>
<td>0.94</td>
<td>0.09</td>
<td>0.569</td>
<td>0.67</td>
<td>AL</td>
<td>40</td>
<td>12</td>
</tr>
<tr>
<td>Triazolam</td>
<td>2.4</td>
<td>–</td>
<td>1</td>
<td>0.1</td>
<td>0.78</td>
<td>0.1</td>
<td>–</td>
<td>0.62</td>
<td>AAG</td>
<td>19</td>
<td>2.9</td>
</tr>
<tr>
<td>Zolpidem</td>
<td>2.43</td>
<td>–</td>
<td>1</td>
<td>0.13</td>
<td>0.58</td>
<td>0.13</td>
<td>0.666</td>
<td>0.76</td>
<td>AL</td>
<td>2.8</td>
<td>4.3</td>
</tr>
<tr>
<td>PhRMA #6</td>
<td>2.86</td>
<td>–</td>
<td>1</td>
<td>0.114</td>
<td>0.90</td>
<td>0.114</td>
<td>0.632</td>
<td>0.59</td>
<td>AL</td>
<td>124</td>
<td>8.9</td>
</tr>
<tr>
<td>Acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diclofenac</td>
<td>4.49</td>
<td>4</td>
<td>0.40</td>
<td>0.005</td>
<td>1</td>
<td>0.002</td>
<td>0.025</td>
<td>0.55</td>
<td>AL</td>
<td>189</td>
<td>4.2</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>3.98</td>
<td>4.4</td>
<td>0.40</td>
<td>0.01</td>
<td>0.84</td>
<td>0.004</td>
<td>0.051</td>
<td>0.55</td>
<td>AL</td>
<td>8.8</td>
<td>0.78</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>3.13</td>
<td>5.27</td>
<td>0.40</td>
<td>0.04</td>
<td>0.95</td>
<td>0.016</td>
<td>0.179</td>
<td>0.55</td>
<td>AL</td>
<td>0.9</td>
<td>0.2</td>
</tr>
<tr>
<td>Warfarin</td>
<td>3.15</td>
<td>5</td>
<td>0.40</td>
<td>0.01</td>
<td>0.47</td>
<td>0.004</td>
<td>0.051</td>
<td>0.55</td>
<td>AL</td>
<td>0.52</td>
<td>0.045</td>
</tr>
<tr>
<td>Tenidap NF</td>
<td>3.5</td>
<td>3.5</td>
<td>0.40</td>
<td>0.0007</td>
<td>0.32</td>
<td>0.0003</td>
<td>0.004</td>
<td>0.56</td>
<td>AL</td>
<td>8.3</td>
<td>0.058</td>
</tr>
<tr>
<td>Tenoxicam</td>
<td>1.9</td>
<td>pKa\textsubscript{A} = 1.1; pKa\textsubscript{A} = 5.0</td>
<td>0.16</td>
<td>0.009</td>
<td>0.78</td>
<td>0.0014</td>
<td>0.019</td>
<td>0.67</td>
<td>AL</td>
<td>1.7</td>
<td>0.038</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Experimentally determined \(\log P_{ow}\) are used in any calculation.

\textsuperscript{b} Calculated as demonstrated in the Methods section. The parameter \(F_1\) for drug ionization represents the ratio of fraction unionized between plasma (pH 7.4) and intracellular water (pH 7.0).

\textsuperscript{c} Calculated values as demonstrated in the Methods section. The value of \(fu_{app}\) was set equal to the product of \(fu_p\) with \(F_1\), whereas the value of \(fu_{liver}\) was calculated using Eq. 13. For the IVIVE method proposed here, \(fu_{app}\) was used for AAG-bound drugs, whereas \(fu_{liver}\) was used for AL-bound drugs, as explained in the Methods section.

\textsuperscript{d} Observed from the literature\textsuperscript{b–d} or assumed in the methods. For midazolam, both AL and AAG are important binding proteins,\textsuperscript{15} but only AL was considered in this study because the impact on CL estimates was most important for this protein.

\textsuperscript{e} Scaled to in vivo condition using a standard scaling factor of 900 mg protein/kg body weight.\textsuperscript{2}

\textsuperscript{f} Clearance refers to plasma kinetics, and it was assumed that this is mainly due to hepatic metabolic CL. \(P_{ow}\), \(n\)-octanol-buffer ratio.
Modeling CL for Highly Bound Drugs

Traditional IVIVE Calculation Methods Tested by Obach

Obach\(^2\) tested disregarding or including binding terms (i.e., \textit{in vitro} \(f_{up}\) and/or \(f_{inc}\)) in calculating CL referring to plasma kinetics. Using the well-stirred model, he reported a discrepancy in the levels of accuracy between the scaling approaches. Ring et al.\(^1\) confirmed such a discrepancy. Therefore, all approaches were reinvestigated as follows:

\[
CL = \frac{Q_{liver} R_{BP} CL_{int, in vitro}}{Q_{liver} R_{BP} + CL_{int, in vitro}} \quad (1)
\]

\[
CL = \frac{Q_{liver} R_{BP} CL_{int, in vitro} f_{up}}{Q_{liver} R_{BP} + CL_{int, in vitro} f_{up}} \quad (2)
\]

\[
CL = \frac{Q_{liver} R_{BP} CL_{int, in vitro} f_{up}/f_{inc}}{Q_{liver} R_{BP} + CL_{int, in vitro} f_{up}/f_{inc}} \quad (3)
\]

where \(R_{BP}\) is the equilibrium blood-to-plasma concentration ratio, \(Q_{liver}\) is the blood flow rate to liver in human [21 mL/(min kg)], and \(CL_{int, in vitro}\) is the intrinsic clearance under \textit{in vitro} condition. These IVIVE methods scale \textit{in vitro} \(CL_{int}\) to \textit{in vivo} \(CL_{int}\) in human using a physiologically based scaling factor based on hepatic microsomal recovery from the whole liver for the conversion of the unit of \(CL_{int, in vitro}\) from \(\mu L/(min\ mg\ proteins)\) to \(mL/(min\ kg\ body\ weight)\) for \(CL_{int, in vivo}\) (i.e., 45 mg protein/g liver and 20 g liver/kg body weight)\(^2\):

\[
CL_{int, in vivo} = CL_{int, in vitro} PBSF \quad (4)
\]

IVIVE Calculation Methods Corrected by Berezhkovskiy

At steady-state equilibrium, the concentrations of unbound drug are assumed to be same in both the plasma water and organ intracellular water.\(^2\) However, this assumption does not hold for ionizable drugs because there is a pH difference between the extracellular and intracellular water (7.4 and 7.0, respectively).\(^1\) Accordingly, in plasma and the cells, the central compartment is water where ionizable molecules are present in ionic and nonionic forms, which equilibrate with other constituents (e.g., proteins and lipids). More specifically, extracellular and intracellular water represent the site of ionizable molecules; therefore, both ionic and nonionic forms are present in this constituent. Lipophilic, unionized substances may diffuse across the cell membrane; however, lipophilic, ionized substances also are candidates for transporters, which is the primary transmembrane flux mechanism.\(^11,36\) Consequently, the total concentration of unbound drug (nonionic and ionic forms) in plasma relative to intracellular water should be larger by an ionization factor \((F_1)\) at steady-state equilibrium. Berezhkovskiy\(^11\) introduced the parameter \(F_1\), which corresponds to the ratio of \(fu\) of drugs unionized between extracellular plasma \((f_{unionized, p})\) and intracellular tissue \((f_{unionized, IW})\) water. For a neutral drug, \(F_1\) is equal to unity. However, for an ionizable molecule, \(f_{unionized, p}\) and \(f_{unionized, IW}\) are not equal and can be calculated as follows for the classes of drugs investigated:

\[
f_{unionized—neutral} = 1 \quad (5)
\]

\[
f_{unionized—monoprotic—acid} = 1/[1 + (10^{pH—pK_a}]) \quad (6)
\]

\[
f_{unionized—diprotic—acids} = 1/[1 + (10^{pH—pK_a1} + 10^{2pH—pK_a1—pK_a2}]) \quad (7)
\]

\[
f_{unionized—monoprotic—base} = 1/[1 + (10^{pK_a—pH}]) \quad (8)
\]

\[
f_{unionized—diprotic—bases} = 1/[1 + (10^{pK_a1—pH} + 10^{pK_a1—pK_a2—2pH})] \quad (9)
\]

For strong monoprotic basic compounds, these equations may yield a calculated \(F_1\) up to 2.5-fold (and even greater for diprotic bases), which means that the unbound drug concentrations in water is 2.5-fold greater in intracellular liver than plasma. Inversely, for strong monoprotic acid compounds, the unbound drug concentrations in water is 2.5-fold smaller in intracellular liver than plasma (and even smaller for diprotic acids). Thus, Berezhkovskiy\(^11\) used \(f_{up-app}\) (\(f_{up-app} = \textit{in vitro} f_{up} \times F_1\)) to estimate \(f_{up-app} in vivo\). Consequently, this may lead to a noticeable change in the predicted drug CL. The correction of \(f_{up}\) for differences in unbound drug concentration in plasma and intracellular liver is also applicable to a highly bound drug (i.e., the bound concentrations are significantly larger than the unbound concentrations, thus \(fu\) in each matrix approximates the ratio between the unbound and bound concentrations). Accounting for drug ionization leads to a novel equation for CL. This aligns with the traditional description of CL, but has the CL calculated with \(f_{up-app}\) accounting for drug ionization\(^11\):

\[
CL = \frac{Q_{liver} R_{BP} CL_{int, in vitro} f_{up-app}}{Q_{liver} R_{BP} + CL_{int, in vitro} f_{up-app}} \quad (10)
\]

Only Eq. 10 was tested by Berezhkovskiy\(^11\). However, considering \(f_{inc}\) or only \(F_1\) in the well-stirred
model is also an option to test with the following equations:

\[
\text{CL} = \frac{Q_{\text{liver}} R_{BP} \text{CL}_{\text{int.in vivo}} f_{\text{u}} \text{p-app}/f_{\text{u}} \text{inc}}{Q_{\text{liver}} R_{BP} + \text{CL}_{\text{int.in vivo}} f_{\text{p-app}}/f_{\text{u}} \text{inc}} \quad (11)
\]

\[
\text{CL} = \frac{Q_{\text{liver}} R_{BP} \text{CL}_{\text{int.in vivo}} F_1}{Q_{\text{liver}} R_{BP} + \text{CL}_{\text{int.in vivo}} F_1} \quad (12)
\]

**IVIVE Calculation Method Proposed in this Study**

The method consists of converting \( f_{\text{p}} \) _in vitro_ to \( f_{\text{u}} \) _liver_ in the well-stirred model. Additional mechanisms were integrated based on the recent studies of Berezhkovskiy\(^{11}\) and Burczynski et al.\(^{13}\). First, the parameter \( F_1 \) was again used to consider differences in the unbound drug concentrations in water between plasma and intracellular liver because of the pH difference.\(^ {11}\) Second, because we were extrapolating _in vitro_ data determined in plasma-free microsomal incubations, the role of extracellular binding proteins on the uptake of drugs in hepatocytes potentially occurring _in vivo_ was also considered based on the literature,\(^ {13}\) and consequently, this also affected the estimation of \( f_{\text{u}} \) _liver_. Therefore, we argue that a difference in drug ionization and binding proteins such as AL and alpha-1-acid glycoprotein (AAG) in plasma and liver also needs to be considered to accurately estimate CL _in vivo_.

**Estimation of \( f_{\text{u}} \) _liver_**

The well-stirred liver model assumes that the drug that enters the liver is homogenously distributed in the organ. To be in agreement with this model, this concept should, therefore, also apply to binding proteins in the liver compartment and hence should impact the fu in this compartment. In other words, both drug distribution and protein binding should be considered on the basis of the whole liver. Related to this, protein-facilitated metabolism is probably a consequence of an ionic interaction process between the extracellular protein–drug complex and hepatocyte cell surface, and that process can be very rapid.\(^ {13,37}\) Consequently, this may help the system to reach distributional equilibrium more rapidly and facilitate a homogenous dispersion of the drug, and hence explain why it could be worth converting \( f_{\text{p}} \) to \( f_{\text{u}} \) _liver_ at the whole-organ level to be in agreement with the well-stirred model.

In addition, each ionic interaction between the bound drug (plasma protein–drug complex) and the hepatocyte cell surface would result in an additional unbound drug molecule available for uptake, and hence a greater intracellular concentration _in vivo_ (and CL\(_{\text{int}}\)). In other words, the main observation from Burczynski et al.\(^{13}\) suggests that there exists circumstantial evidence favoring the view that a significant portion of uptake occurs from the protein-bound fraction at the hepatocyte cell surface only. This finding is consistent with the results from another study of removal of drugs by hepatocytes.\(^ {38}\) Thus, the bound drug concentrations in the extracellular space become a reservoir of unbound drug molecules available for uptake by passive diffusion, which may result in greater CL\(_{\text{int}} \) _in vivo_ than expected. It is necessary to take into account the extracellular protein binding in plasma relative to liver (or the plasma-to-whole-liver concentration ratio (PLR) of plasma binding proteins). Accordingly, the following equation of the binding isotherm should apply at equilibrium\(^ {12}\) for estimating \( f_{\text{u}} \) _liver_ from measured values of \( f_{\text{p-app}} \) (_in vitro_ \( f_{\text{p}} \times F_1 \)) by considering a difference in both the free and bound drug concentrations:

\[
f_{\text{u}} \text{liver} = \frac{\text{PLR} \ f_{\text{p-app}}}{1 + (\text{PLR} - 1) f_{\text{p-app}}} \quad (13)
\]

The apparent value of \( f_{\text{u}} \) _liver_ can, therefore, be greater than \( f_{\text{p}} \) depending on PLR and \( F_1 \), which should likely satisfy the criteria for enhanced uptake, and consequently, for a more accurate CL estimate for highly bound drugs. Thus, the values of \( f_{\text{u}} \) _liver_ will vary with the value of \( f_{\text{p}} \) determined _in vitro_ and PLR as well as \( F_1 \). In turn, \( F_1 \) will depend on both the class and pKa of the drug, whereas PLR will depend on the difference in concentration of the main binding proteins in plasma and whole liver.

**Estimation of PLR Value**

For each drug studied, the main extracellular binding protein considered in this study is AL or AAG\(^ {16-35}\) (Table 1). However, the works of Burczynski et al.,\(^ {13}\) Qin et al.,\(^ {38}\) and Bilello et al.\(^ {39}\) suggest that protein-facilitated metabolism, and hence hepatocyte uptake of drugs, is significantly greater with AL as compared with that of AAG at physiologically relevant concentrations (more explanations are provided in the Discussion section for differences between AL and AAG). As such, the calculated value of \( f_{\text{u}} \) _liver_ will become important particularly for drugs binding mainly to AL. Consequently, in calculating CL for each drug binding mainly to AAG, \( f_{\text{u}} \) _liver_ of Eq. 13 was not used (i.e., \( f_{\text{p-app}} \) was used, which is the product of \( f_{\text{p}} \) and \( F_1 \), as considered in Eq. 11). For drugs binding to AL, the estimation of \( f_{\text{u}} \) _liver_ with the above equation requires the value of PLR, which is estimated just below. The level of AL in the intracellular liver is negligible as compared with that of the extracellular (interstitial) space and hence of plasma.\(^ {40-42}\)
Therefore, the PLR value for this binding protein was obtained by converting the extracellular fluid-to-plasma concentration ratio into whole-liver–plasma ratio to be in agreement with the well-stirred model, and the resulting value was 13.3.36 It should be noted that the impact of intracellular binding was neglected based on the aforementioned mechanism, which suggests that additional drug uptake in hepatocytes is governed only by the differential of the extracellular protein-bound fraction in plasma relative to liver interstitial space.13,38 Accordingly, the novel IVIVE calculation method proposed in this study for drug binding mainly to AL corrects for both the specific (fu_{liver}) and nonspecific (fu_{inc}) binding that may occur under in vivo and in vitro conditions, respectively:

\[ \text{CL} = \frac{Q_{\text{liver}} R_{\text{BP}} \text{CL}_{\text{int,in vivo}} fu_{\text{liver}}/fu_{\text{inc}}}{Q_{\text{liver}} R_{\text{BP}} + \text{CL}_{\text{int,in vivo}} fu_{\text{liver}}/fu_{\text{inc}}} \] (14)

**Input Parameters**

The measured value of each input parameter used in all equations is provided in Table 1 (i.e., CL_{int}, fu_p, fu_{inc}, fu_{p,app}, fu_{liver}, F_1, pK_a, R_{BP}). Furthermore, based on the mechanistic studies published in the literature, the main binding protein (AL or AAG) was identified for each drug.2,10,16–35 When this information was not available [e.g., compounds 1–5 of the Pharmaceutical Research and Manufacturers of America (PhRMA) initiative], the binding to AAG was assumed to be preferred for basic drugs, whereas the binding to AL was assumed for acidic and neutral drugs (Table 1).

**Comparative Assessment of IVIVE Methods of Human Drug CL**

We made the first attempt to get information on the predictive performance of the proposed IVIVE calculation method (Eq. 14) by comparing its performance with the IVIVE methods published by Obach2 (Eqs. 1–3) and Berezhkovskiy11 (Eqs. 10–12). A total of seven IVIVE methods were investigated to predict human CL for the same set of drugs.

**Evaluation of Predictive Performance**

The prediction accuracy was assessed by comparing predicted versus observed values of human CL using several statistical parameters. The same statistical evaluation as already described by Poulin et al.43 was also performed in the present study. Therefore, the following statistical parameters were calculated and presented for each prediction method studied: average fold error (AFE), absolute average fold error, root mean squared error, and coefficient of correlation (r).

Furthermore, the concordance correlation coefficient (CCC) was presented, which evaluates the degree to which pairs of predicted and observed data fall on the line of unity passing through the origin. Specific fold errors of deviation between the predicted and observed values (% fold error < 1.5, 2, 3, 4, and 10) were also calculated. Finally, plots of predicted versus observed CL values were also presented.

**RESULTS**

**Comparative Assessment for Various IVIVE Methods of Human CL**

A total of seven IVIVE calculation methods of human CL were evaluated in the present study for a total of 25 drugs. All methods were compared using the same datasets, and the comparative assessment was made based on several statistical parameters. The overall statistical summary in terms of accuracy, precision, and correlation is listed in Tables 2 and 3. The plot of observed versus predicted CL values for each method and class of drugs is shown in Figures 1–7.

The proposed IVIVE calculation method was the best performing prediction method for the current dataset. This is reflected by the statistical analyses, which are in favor of this novel approach, and can be viewed graphically (Figure 1–7). No systematic underestimation or overestimation of the in vivo CL was observed with the proposed IVIVE method, in contrast to the published methods. AFE value close to unity was obtained in this study (Fig. 1). Conversely, the published IVIVE methods of both Obach2 and Berezhkovskiy11 resulted in a systematic underestimation or overestimation of CL because AFE values were lower than 0.4 or greater than 2. In this case, the published methods that included a binding correction tend to underpredict CL (AFE values lower than 0.4), in contrast to the models with no binding correction (AFE values greater than 2; Figure 2–7).

Indeed, for some drugs (up to 16 drugs), the predictions using the methods of Obach2 and Berezhkovskiy11 incorrectly estimated human CL by a factor of threefold or greater. This was not the case for the method proposed here wherein the maximum success rate in predicting the human CL was 84%, 96%, and 100% of the compounds with predictions falling within 1.5-fold, twofold, and threefold error, respectively, of the observed CL. These levels of accuracy are significantly lower for the published IVIVE methods (Tables 2 and 3). Furthermore, r and CCC, which are closer to unity for the proposed method, confirm the validity of this method. We covered a large range of drug ionization because F_1 values varied from 0.16 to 2.93 (Table 1). However, combining the parameter F_1 with fu_p, or only using F_1 in the IVIVE calculation methods, as suggested by Berezhkovskiy,11 did
PREDICTING METABOLIC CLEARANCE OF HIGHLY BOUND DRUGS

Table 2. Comparative Assessment of Several IVIVE Calculation Methods Used to Predict Human CL for the Current Dataset of 25 Drugs with $f_{up} \leq 0.15$

<table>
<thead>
<tr>
<th>IVIVE Methods</th>
<th>% ≤ 1.5-Fold</th>
<th>% ≤ Twofold</th>
<th>% ≤ Threefold</th>
<th>% ≤ Fourfold</th>
<th>% ≤ 10-Fold</th>
<th>AFE</th>
<th>AAFE</th>
<th>RMSE</th>
<th>$r$</th>
<th>CCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>All binding corrections</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>This study (Eq. 14)</td>
<td>84</td>
<td>96</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0.99</td>
<td>1.29</td>
<td>0.14</td>
<td>0.95</td>
<td>0.98</td>
</tr>
<tr>
<td>Berezhkovskiy (Eq. 11)</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>64</td>
<td>76</td>
<td>0.39</td>
<td>2.72</td>
<td>0.62</td>
<td>0.88</td>
<td>0.82</td>
</tr>
<tr>
<td>Obach (Eq. 3)</td>
<td>28</td>
<td>48</td>
<td>64</td>
<td>72</td>
<td>96</td>
<td>0.38</td>
<td>2.61</td>
<td>0.52</td>
<td>0.90</td>
<td>0.83</td>
</tr>
<tr>
<td>Binding correction for plasma only</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Berezhkovskiy (Eq. 10)</td>
<td>12</td>
<td>36</td>
<td>40</td>
<td>48</td>
<td>64</td>
<td>0.20</td>
<td>4.97</td>
<td>0.84</td>
<td>0.83</td>
<td>0.71</td>
</tr>
<tr>
<td>Obach (Eq. 2)</td>
<td>4</td>
<td>16</td>
<td>36</td>
<td>36</td>
<td>76</td>
<td>0.18</td>
<td>5.50</td>
<td>0.85</td>
<td>0.75</td>
<td>0.65</td>
</tr>
<tr>
<td>No binding correction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Berezhkovskiy (Eq. 12)</td>
<td>36</td>
<td>52</td>
<td>76</td>
<td>80</td>
<td>94</td>
<td>2.17</td>
<td>2.30</td>
<td>0.51</td>
<td>0.79</td>
<td>0.76</td>
</tr>
<tr>
<td>Obach (Eq. 1)</td>
<td>48</td>
<td>56</td>
<td>72</td>
<td>72</td>
<td>88</td>
<td>2.27</td>
<td>2.54</td>
<td>0.63</td>
<td>0.77</td>
<td>0.56</td>
</tr>
</tbody>
</table>

AFE, average fold error; AAFE, absolute average fold error; RMSE, root mean squared error; $r$, correlation coefficient; CCC, concordance correlation coefficient (global).

Table 3. Comparative Assessment of Several IVIVE Calculation Methods Used to Predict Human CL for the Current Dataset of Sixteen Drugs with $f_{up} \leq 0.05$

<table>
<thead>
<tr>
<th>IVIVE Methods</th>
<th>% ≤ 1.5-Fold</th>
<th>% ≤ Twofold</th>
<th>% ≤ Threefold</th>
<th>% ≤ Fourfold</th>
<th>% ≤ 10-Fold</th>
<th>AFE</th>
<th>AAFE</th>
<th>RMSE</th>
<th>$r$</th>
<th>CCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>All binding corrections</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>This study (Eq. 14)</td>
<td>81</td>
<td>94</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>1.07</td>
<td>1.31</td>
<td>0.15</td>
<td>0.95</td>
<td>0.98</td>
</tr>
<tr>
<td>Berezhkovskiy (Eq. 11)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>63</td>
<td>0.30</td>
<td>3.56</td>
<td>0.73</td>
<td>0.90</td>
<td>0.80</td>
</tr>
<tr>
<td>Obach (Eq. 3)</td>
<td>25</td>
<td>38</td>
<td>56</td>
<td>63</td>
<td>94</td>
<td>0.34</td>
<td>2.93</td>
<td>0.58</td>
<td>0.91</td>
<td>0.83</td>
</tr>
<tr>
<td>Binding correction for plasma only</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Berezhkovskiy (Eq. 10)</td>
<td>0</td>
<td>25</td>
<td>25</td>
<td>31</td>
<td>50</td>
<td>0.14</td>
<td>7.39</td>
<td>0.98</td>
<td>0.81</td>
<td>0.67</td>
</tr>
<tr>
<td>Obach (Eq. 2)</td>
<td>0</td>
<td>13</td>
<td>25</td>
<td>25</td>
<td>69</td>
<td>0.14</td>
<td>7.15</td>
<td>0.96</td>
<td>0.68</td>
<td>0.61</td>
</tr>
<tr>
<td>No binding correction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Berezhkovskiy (Eq. 12)</td>
<td>19</td>
<td>38</td>
<td>63</td>
<td>69</td>
<td>94</td>
<td>3.02</td>
<td>3.02</td>
<td>0.61</td>
<td>0.80</td>
<td>0.73</td>
</tr>
<tr>
<td>Obach (Eq. 1)</td>
<td>31</td>
<td>38</td>
<td>56</td>
<td>56</td>
<td>81</td>
<td>3.56</td>
<td>3.67</td>
<td>0.78</td>
<td>0.76</td>
<td>0.51</td>
</tr>
</tbody>
</table>

AFE, average fold error; AAFE, absolute average fold error; RMSE, root mean squared error; $r$, correlation coefficient; CCC, concordance correlation coefficient (global).

not demonstrate a superior prediction performance of CL as compared with this study. Alternatively, combining $F_I$ with $fu_{liver}$ was the most successful approach. This is convincing either for drugs with $f_{up} \leq 0.15$ or $f_{up} \leq 0.05$ (Tables 2 and 3). Overall, a superior degree of prediction accuracy was obtained in this study for the current dataset of drugs highly bound to plasma proteins as compared with the published IVIVE methods, which supports the current hypotheses and assumptions. It is useful to take a closer look at the performance of different classes of drugs.

Classes of Drugs

Another aspect of interest was to determine whether the IVIVE calculation methods provided a similar prediction performance across the classes of drugs. The classes of drugs investigated in the present study (acids, bases, and neutrals) presented different prediction performances particularly for the published IVIVE methods, but the proposed IVIVE method was again the most successfully tested method over the classes of drugs (Figure 1–7). In general, for the published IVIVE methods, prediction accuracy was highly dependent on the properties of the compounds investigated. In this context, more accurate predictions were obtained for basic drugs as compared with acidic and neutral compounds (at least in terms of deviation from the line of unity).

DISCUSSION

Successful IVIVE of CL of drugs is still not fully resolved despite great effort in this area.2–8,10,44–47 Recently, a PhRMA initiative that predicted human CL using traditional IVIVE calculation methods observed that several outlying drugs were highly bound to plasma proteins.10 As highly bound drugs tend to be commonplace in discovery and development, improvement in the prediction methods would be advantageous. The main focus of this study was to develop a mechanism-based IVIVE method for CL and assess the predictive performance of various methodologies reported in the literature for predicting human CL using published datasets of highly bound drugs. The
assessments were confined by the availability of measured data in humans for a set of 25 highly bound compounds. Converting $f_{ub}^2$ or $f_{ub-app}^{11}$ to $f_{liver}$ essentially increased the predicted CL values, which resulted in no systematic underestimation of in vivo CL, particularly when the binding terms were included in IVIVE. This is a mechanistic rational for explaining a considerable proportion of the divergence between previously estimated and observed CL values. Therefore, this study highlights a problematic area observed when using in vitro microsomal data to predict in vivo CL of drugs that are highly bound to plasma proteins because it further explores the mechanisms for human CL predictions by associating additional processes to the IVIVE disconnect.

Human CL of the current 25 drugs was accurately estimated in this study, giving support to the notion that the aforementioned assumptions are valid. It is noteworthy that the proposed IVIVE method should only work for compounds that have no diffusion limitation at the cellular membrane level and for which no CL mechanisms other than hepatic oxidative metabolism play a dominant role in CL (e.g., renal, biliary, nonoxidative metabolic CL, transport processes, etc.). It should be noted that the 25 drugs chosen for this study represent a set for which other CL mechanisms are known to be less important than hepatic oxidative metabolic CL, but some are known to fall outside the scope of the aforementioned

**Figure 1.** Comparison between predicted and observed human CL for the IVIVE method proposed in this study, which includes $f_{liver}/f_{int}$ (Eq. 14; $r = 0.95$ and $n = 25$). The solid line indicates the best fit (unity). Dashed lines on either side of unity include a factor of two and three. Cross (red), circles (green), and squares (blue) indicate bases, neutrals, and acids, respectively. CL is in mL/(min kg).

**Figure 2.** Comparison between predicted and observed human CL for the IVIVE method proposed by Berezhkovskiy, which includes $f_{ub-app}$ (Eq. 11; $r = 0.88$ and $n = 25$). The solid line indicates the best fit (unity). Dashed lines on either side of unity include a factor of two and three. Cross (red), circles (green), and squares (blue) indicate bases, neutrals, and acids, respectively. CL is in mL/(min kg).

**Figure 3.** Comparison between predicted and observed human CL for the IVIVE method proposed by Berezhkovskiy, which includes $F_1$ (Eq. 12; $r = 0.79$ and $n = 25$). The solid line indicates the best fit (unity). Dashed lines on either side of unity include a factor of two and three. Cross (red), circles (green), and squares (blue) indicate bases, neutrals, and acids, respectively. CL is in mL/(min kg).


assumptions, for example, nonoxidative components of metabolic CL such as the reductive metabolism of warfarin or glucuronidation of ibuprofen and active uptake of diclofenac.\textsuperscript{2,10,48} For example, it is well established that active uptake may also play a role in creating higher intracellular unbound drug concentrations, and hence higher \textit{in vivo} metabolic CL, than would be expected simply from the microsomal data. However, for diclofenac, active uptake does not significantly influence the intracellular concentrations, and hence suggests that it is not critical to consider such a process for this compound as much as the binding to extracellular proteins. Indeed, the predicted CL of this drug compares well with the observed value (3.4 vs. 4.2 mL/(min kg)). Similarly, we were able to accurately predict human CL of other drugs without considering such processes. It is acknowledged that the other processes could be a cause of discrepancy between the predicted and experimental values and could not be entirely excluded. Therefore, further investigation and more rigorous testing are needed.

According to our observations, the interaction of the current drugs with AL seems to facilitate distribution of more unbound drugs in hepatocytes and facilitates reaching a more homogenous distribution in the liver. Because no consideration of further mechanisms related to the main binding proteins was made with the other IVIVE methods tested,\textsuperscript{2,10,11} this may explain why a lower prediction performance was observed especially for the acids and neutrals. Inversely, because drugs binding to AAG seemed to require no additional correction as compared with drugs binding to AL, this may explain why the prediction performance for the basic drugs was relatively similar across the IVIVE methods tested (Figure 1–7). Obach\textsuperscript{2} also reported a discrepancy in the levels of accuracy between bases, acids, and neutrals after evaluating traditional IVIVE calculation methods with and without binding corrections in the well-stirred model. Because the bases and acids provided different conclusions, a converging approach on the use of a binding correction for predicting CL could not be reached. Obach\textsuperscript{2} therefore suggested disregarding all binding to predict CL for bases and neutrals, whereas for acids, he suggested including all binding.\textsuperscript{2} In this present study, all classes of drugs were accurately predicted by including a binding correction for the \textit{in vivo} (fu\textsubscript{liver}) and \textit{in vitro} (fu\textsubscript{inc}) conditions. This demonstrates again the advantage of the novel method proposed.

It is perhaps not a surprise that for highly bound drugs, the impact of protein binding in plasma relative to liver has such a strong influence over the
IVIVE accuracy and that for almost all the drugs evaluated here, predictions improved as compared with previous studies. The notion of changes in the unbound drug concentration with drug ionization according to the pH theory is well accepted, whereas the other notion of differences in degree of protein binding between the plasma and hepatic compartment is still not fully validated for IVIVE. The last notion could be supported from other studies on the removal of drugs by hepatocytes. For example, both AL and AAG have been reported to affect the intrinsic elimination (i.e., $\text{CL}_{\text{int}}$) of drugs. AL is known to increase the $\text{CL}_{\text{int}}$ of certain drugs, whereas AAG, in contrast to AL, limits or decreases $\text{CL}_{\text{int}}$. The resulting effect would be that AL enhances the hepatocyte uptake, whereas AAG limits the uptake for highly bound drugs. Studies on drug uptake into human T-lymphoblastoid cell lines showed similar observations. This is consistent with the present study and suggests that AL has a significantly higher effect on the drug uptake and is associated with the hepatocyte membrane at a higher degree than AAG. Related to this, specific *in vitro* studies with liver microsomes also point to protein-facilitated metabolism because the values of $K_m$ were often smaller in the presence of AL as compared with the control. Because liver microsomes and membranes of hepatocytes have a similar composition of lipids to which the AL–drug complex may aggregate, it might at least be argued that the AL–drug complex presents a greater nonspecific binding as compared with the free drug molecule.

There are probably electrostatic interactions between the extracellular AL and the membrane of hepatocytes, which are relevant in the prediction of drug uptake. In other words, positive charge on the surface of a binding protein may lead to aberrant interactions with the cell membrane due to the presence of negatively charged groups that decorate the lipid bilayer surface of most mammalian cells. This is important considering that hepatocytes have a high content of acidic phospholipids (e.g., phosphatidylserine). Therefore, it might be that $\text{CL}_{\text{int}}$ values greater than expected are observed *in vivo* for AL-bound drugs as compared with AAG-bound drugs because of differences in the surface charge interactions with the hepatocyte membrane. In fact, findings from exploratory investigations on the pharmacokinetics of proteins may be summarized as follows: (i) shifts in isoelectric point ($pI$) of approximately one unit or more can produce measurable changes in liver distribution and kinetics, and (ii) increases in net positive charge generally result in increased liver retention and increased plasma CL *in vivo*. Accordingly, Burczynski et al. reported that palmitate CL was greatest when AL ($pI = 4.9$) was used as the extracellular binding protein and lowest in the presence of AAG ($pI = 2.7$). The same trend was observed when varying the $pI$ value of AL.

Burczynski et al. and Qin et al. presented three possibilities to explain these observations. First, for highly protein-bound ligands, the protein binding and ionic interactions of the complex with hepatocytes reduce the effective diffusion distance by virtue of having a higher concentration near the cell surface for uptake, and hence intracellular concentration (and $\text{CL}_{\text{int}}$) is greater than expected. Related to this, AL may not be able to retard diffusion of drugs through the cellular stagnant diffusion layer of the hepatocyte membrane to the same degree as AAG. Second, the cell’s ionic field might enhance the dissociation of the protein–ligand complex and thus also provide more unbound ligand to the cell membrane for uptake, particularly for AL-bound drugs. This enhanced dissociation of the protein–drug complex is related to the protein–drug dissociation rate constant. Third, endocytosis of the protein–drug complex has been suspected, which may also increase the intracellular drug concentration under *in vivo* condition. Of these three possibilities, it has been suggested that the first possibility is the most probable, which means that the processes related to drug diffusion are probably the dominating processes governing the protein-facilitated metabolism. However, further investigations are necessary to clarify the relevance of these processes in CL predictions. The overall observation is that metabolism of a drug extensively bound to AL is expected to be facilitated via the AL–drug complex. This is in accordance with the estimation of $\text{fu}_{\text{liver}}$ from the PLR factor of AL. Conversely, drug penetration into the brain is not enhanced by the presence of AL in the perfusion systems. The main difference between brain and liver tissues is that nervous cells are low in acidic phospholipids as compared with hepatocytes (by a factor of 11 times), which may lead to much fewer ionic interactions between the AL–drug complex and the membranes of nervous cells as compared with hepatocytes.

Additional explanations for why an adjustment for AAG-bound compounds were not needed (in contrast to AL-bound compounds) could perhaps be that (i) the levels of AAG are much lower than AL in plasma, reducing the potential for protein–drug–cell interactions, and (ii) the PLR factor for AAG is much closer to unity as compared with AL. It becomes essential to know whether AL is the main binding protein in plasma as compared with AAG for each novel drug tested. Therefore, mechanistic plasma protein binding assays are required to identify the major binding protein in order to apply the novel IVIVE calculation method prospectively. Thus, some may conclude that there is little point in using this novel IVIVE calculation method, which requires...
additional information on the binding proteins in plasma. However, the predictive capability of this method is likely to increase as our knowledge of the main binding protein in plasma improves (Tables 2 and 3). Also, one would also expect that the binding to AAG could probably be saturable because of capacity limitations, and consequently, the binding to AL may become predominant in this context. It is possible that the drug binding to lipoproteins or globulins will also need to be adjusted.36,52

If the protein–drug–cell interaction enhances drug uptake in the hepatocytes, which may facilitate a homogenous distribution in the liver, it may help the system to reach distributional equilibrium more rapidly and hence explain why the consideration of protein binding inside the whole-liver compartment seems to work quite well for drugs binding to AL as compared with that binding to AAG. Furthermore, the observation that hepatocytes incubated with plasma may increase CLint under in vitro conditions5 may also be explained by the hypothesis of Burczynski et al.,13 and it supports our hypothesis of protein binding in liver. The addition of plasma in the incubation medium may facilitate diffusion distribution between cells and medium and bring drug distribution in the system closer to equilibrium, as postulated in the in vivo system. The impact of such a process may, in part, explain why the use of PLR factor proposed here works well for highly bound compounds.

When using the IVIVE method proposed here, there are a variety of factors that may impact the IVIVE predictions. It is imperative that CL should not be limited by \( Q_{\text{d liver}} \), and consequently, its calculation would vary particularly for strong acids and bases for which \( f_{u_p} \) values are far from unity. Indeed, the current IVIVE method seems relatively sensitive to chemical-specific input parameters used for binding (\( f_{u_p} \), \( f_{u_{inc}} \)) and ionization (pKa, and hence \( F_l \)). The latter is important because calculated pKa values are used in early drug discovery and they are not always accurate because significant differences between calculated and measured values are not uncommon. Should there be significant errors in the experimental assessment of \( f_{u_p} \), particularly for highly bound drugs, these errors would confound the predictability of IVIVE. Thus, the observation that high plasma protein binding is shown to significantly impact the reliability of IVIVE prediction performance can potentially relate to the experimental error. However, it is noted that the success of the current IVIVE method as compared with the published methods is probably due to an adjustment of the well-stirred model.

Finally, it might also be argued that that the well-stirred model does not well represent the in vivo conditions for highly bound drugs. However, here we performed a mathematical exercise comparing the predicted hepatic extraction ratio of the well-stirred model with the parallel-tube model and a physiological model of the liver (consisting of seven compartments in series, assuming there were no diffusion limitations or active transport processes involved).2,58

The conclusions of this study do not change when using the parallel-tube model because the outcomes are similar to the well-stirred model (this is in accordance with a similar comparative assessment by Obach2). However, the extraction ratio predicted by the physiological model deviated by a maximum factor of 20% to the well-stirred model for CLint values ranging from 0.1 to 1000 mL/(min kg). Therefore, we agree that the choice of the liver model can be a potential source of discrepancy, but it cannot account for all the IVIVE disconnects, as shown by the minimal differences between models in this mathematical exercise.

**CONCLUSION**

In summary, this study demonstrated the impact of both drug ionization and differential binding of drug in liver on the IVIVE predictions of human CL for highly bound drugs. In the discovery process, it is important to examine the protein binding. Obviously, CL estimates for drugs will be affected by high percentages of binding to AL as compared with AAG potentially due to differences in the surface charge interactions with the hepatocyte membrane. Consequently, for a drug binding extensively to AL, statistical bias was significantly reduced by using \( f_{u_{d liver}} \) generated in this study as compared with \( f_{u_p} \) and \( f_{u_{app}} \). The novel IVIVE calculation method was the most successful of multiple approaches. However, consideration of other datasets of drugs is required to further support the conclusions of this study, and the hypothesis of differential binding of drug in liver should be further validated with more drugs and experimental data. The current concept has important implications when using plasma-free microsomal incubation systems to estimate CLint. The concept should also work for hepatocyte suspensions and plated hepatocytes because it is applicable as long as the drug gets to the hepatocyte cell surface and the membrane uptake is not rate limiting. Thus, the novel IVIVE model might be adjusted for substances that are also transported into cells. Conversely, drugs with CL limited by \( Q_{\text{d liver}} \) or eliminated via a nonoxidative metabolic process, and for which binding to plasma proteins is nonreversible in vivo could probably not be predicted using the current concept. Finally, a recent study has developed a generic and mechanistic microsome-composition-based model used to predict \( f_{u_{inc}} \) from physicochemical data only.50 Consequently, we consider that a combination of this generic model with this present study can provide a meaningful physiological model for mechanism-based predictions of CL from IVIVE calculations.
ACKNOWLEDGMENTS

This work represents an initiative undertaken as a part of Dr. Poulin’s research program supported by Genentech, Inc. This work was also supported by a Discovery Grant from the National Sciences and Engineering Research Council of Canada for Professor Haddad.

REFERENCES


