Comparison of *In Vitro–In Vivo* Extrapolation of Biliary Clearance Using an Empirical Scaling Factor Versus Transport-Based Scaling Factors in Sandwich-Cultured Rat Hepatocytes

PENG ZOU,[1] XINGRONG LIU,[2] SUSAN WONG,[2] MEIHUA ROSE FENG,[1] BIANCA M. LIEDERER[2]

[1]Department of Pharmaceutical Sciences, University of Michigan, Ann Arbor, Michigan 48109

[2]Genentech, Inc., Drug Metabolism and Pharmacokinetics, South San Francisco, California 94080

Received 6 April 2013; revised 3 May 2013; accepted 6 May 2013

Published online 27 May 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.23620

ABSTRACT: Biliary clearance (CL_b) is often underestimated by *in vitro–in vivo* extrapolation from sandwich-cultured hepatocytes (SCHs). The objective of this study was to compare the performance of a universal correction factor with transporter-based correction factors in correcting underestimation of CL_b. The apparent *in vitro* CL_b of a training set of 21 compounds was determined using the SCH model and extrapolated to apparent *in vivo* CL_b ($CL_{b,\,app}$). A universal correction factor (10.2) was obtained by a linear regression of the predicted $CL_{b,\,app}$ and observed *in vivo* CL_b of training set compounds and applied to an independent test set ($n = 20$); the corrected CL_b predictions of 13 compounds were within twofold error of observed values. Furthermore, two transporter-based correction factors (Organic anion transporting polypeptides/multidrug-resistance-related protein 2 and diffusion/P-glycoprotein) were estimated by linear regression analysis of training set compounds. The applications of the two correction factors to the test set resulted in improved prediction precision. In conclusion, both the universal correction factor and transporter-based correction factors provided reasonable corrections of CL_b values, which are often underestimated by the SCH model. The use of transporter-based correction factors resulted in an even greater improvement of predictions for compounds with intermediate-to-high CL_b values. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:2837–2850, 2013

Keywords: biliary excretion; hepatocytes; clearance; drug transport; hepatobiliary disposition; *in vitro/in vivo* correlations (IVIVC); organic aniontransporting polypeptide transporters; P-glycoprotein

INTRODUCTION

The accurate prediction of hepatic clearance (CL_H) is an essential step in the identification of new chemical entities as drug candidates and in the estimation of human pharmacokinetics. CL_H is determined by hepatic metabolism and biliary excretion. Reasonable prediction accuracy of CL_H for compounds with high hepatic metabolism has been achieved by interspecies scaling and *in vitro–in vivo* extrapolation (IVIVE) from liver microsome or hepatocyte incubations.[1,2] By contrast, the quantitative prediction of CL_H is still very challenging for compounds with low hepatic metabolism and high biliary excretion.[2]

In vitro–in vivo extrapolation from sandwich-cultured hepatocytes (SCHs) is an approach to estimating biliary clearance (CL_b); however, low bile or blood flow and the variable expression levels of influx and/or efflux transporters can result in a 10–100-fold underestimation of CL_b in IVIVE from SCH.[3,4] Several reports reveal that the expression levels and activities of influx transporters such as Oatps, Ntcp, and Oct1 in rat hepatocytes were consistently and considerably reduced in the SCH model,[5–7] thus leading to underestimation of CL_b. Additionally, the expression levels of canalicular efflux transporters are inconsistent between different laboratories. Li et al.[8] reported a 40% decrease in the bile salt export pump (Bsep) protein level, a 50% decrease in the multidrug-resistance-related protein 2 (Mrp2) protein level, and

Additional Supporting Information may be found in the online version of this article. Supporting Information

Correspondence to: Bianca M. Liederer (Telephone +650-467-7343; Fax: 650-225-6452; E-mail: liederer.bianca@gene.com)

Journal of Pharmaceutical Sciences, Vol. 102, 2837–2850 (2013)

a fivefold increase in the breast cancer resistance protein (Bcrp) level in sandwich-cultured rat hepatocytes (SCRHs) over 5 days in culture. Tchaparian et al.[7] observed dramatically increased protein levels of P-glycoprotein (P-gp), Bcrp, and Mrp 1, 2, 3, and 4 over 4 days in culture. In Borlak's report,[5] the expression levels of canalicular transporters P-gp and Mrp2 were similar to those determined *in vivo*.

One strategy for correcting the underestimation of CL_b is to incorporate a universal empirical correction factor determined by correlating CL_b predicted from SCRHs with the observed *in vivo* CL_b.[9] A reasonable correlation between CL_b predicted by SCRHs and *in vivo* CL_b was observed among drugs that undergo similar uptake (Organic anion transporting polypeptides, Oatps) and efflux mechanisms (Mrp2 or Bcrp) such as angiotensin II receptor blockers, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)reductase inhibitors, and β-lactam antibiotics.[9–11] On the contrary, for compounds in which different combinations of influx and efflux transporters are involved in the biliary excretion, or when passive diffusion is involved, the universal correction factor might not work. To address this issue, Li et al.[4] proposed using the ratio of the protein amount of canalicular efflux transporters (Mrp2, Bsep, and Bcrp) in rat liver to that in SCRH as a correction factor. Improved prediction accuracy was achieved by this method; however, this strategy ignored the decreased expression levels of sinusoidal influx transporters in SCRHs, which might seriously affect the predictability of the SCRH model.

In the current study, a universal correction factor and a series of correction factors based on the activities of both influx and efflux transporters were examined for IVIVE of CL_b. A universal correction factor was estimated by linear regression analysis of 21 compounds in a training set. To determine whether transporter-specific correction factors can improve IVIVE, the compounds in the training set were divided into transporter-specific subgroups based on uptake and excretion mechanisms. Although both the universal correction factor and the transporter-specific correction factors provided reasonable CL_b predictions when applied to the independent test set; the use of transporter-specific correction factors resulted in a greater improvement of prediction precision.

MATERIALS AND METHODS

Chemicals

Topotecan, benazeprilat, rosuvastatin, rosuvastatin-d6, candesartan, atorvastatin, olmesartan, deferasirox, temocaprilat, irinotecan, and octreotide acetate were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Trovafloxacin and fluvastatin were obtained from Torcris Bioscience (Ellisville, Missouri) and Cayman Chemical Company (Ann Arbor, Michigan), respectively. Probenecid was supplied by Santa Cruz Biotechnology (Santa Cruz, California). [^{3}H]Taurocholate (5 Ci/mmol; purity >97%) was purchased from PerkinElmer Life and Analytical Sciences, Inc. (Waltham, Massachusetts). All other chemicals were purchased from Sigma–Aldrich (St. Louis, Missouri).

Metabolic Stability Assay

Cryopreserved rat hepatocytes (Celsis IVT, Baltimore, Maryland) were used for the hepatocyte stability assay. The cryopreserved hepatocytes were thawed in InVitroGRO^{TM} HT (Celsis IVT, Baltimore, Maryland) medium and centrifuged at 100*g* for 10 min. Cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM) (Gibco®, Grand Island, New York), and viability was assessed by the trypan blue exclusion method. Hepatocytes with viability greater than 80% were used in the study. The compounds (1 μM in DMEM) were incubated with hepatocytes (0.5×10^6 cells/mL) in a CO_2 incubator with 95% air/5% CO_2 at 37°C and 95% humidity. At 0, 10, 30, and 60 min, aliquots of the incubation mixture were taken out and the reaction was quenched by adding two volumes of acetonitrile containing 100 nM of rosuvastatin-d6. The mixtures were then centrifuged at 2095*g* for 10 min to precipitate the protein. The supernatants were diluted with two volumes of water and transferred into a 96-well assay plate to measure the disappearance of parent compounds by liquid chromatography–tandem mass spectrometry (LC–MS/MS). The predicted hepatic metabolic clearance (CL_{met}) was calculated using the following previously reported equation[2]:

$$CL_{met} = (Q_P \times f_u \times CL_{int})/(Q_P + f_u \times CL_{int}) \quad (1)$$

where Q_p represents the rat hepatic plasma flow rate (40 mL*min^{-1}*kg^{-1}), CL_{int} represents the intrinsic clearance, and f_u represents the unbound fraction in rat plasma, which was collected from the literature (Table 1).

Hepatocyte Culture

Sandwich-cultured rat hepatocytes (B-CLEAR®), which were isolated from male Wistar rats and cultured in 24-well plates, were purchased from Qualyst, Inc. (Durham, North Carolina). Hepatocytes were cultured in the medium provided by Qualyst, Inc. at 37°C in a humidified incubator with 95% air/5% CO_2. Medium was changed daily. On day 4 after hepatocyte seeding, the SCRHs were subjected to accumulation studies.

Table 1. Training Set ($n = 21$)–Observed and Predicted CL_b (Extrapolated from $CL_{b,\,app,\,in\ vitro}$)

Compounds	Major Influx Transporters	Major Efflux Transporters	$CL'_{b,app}$ ($mL^*min^{-1}*kg^{-1}$)[a]	f_u	$CL'_{b,app} \times f_u$	Predicted $CL_{b,\,app}$ (Eq. 4) ($mL^*min^{-1}*kg^{-1}$)	CL_b Corrected by ×10.2 ($mL^*min^{-1}*kg^{-1}$)	Observed *In Vivo* CL_b ($mL^*min^{-1}*kg^{-1}$)	Category Predicted/ Observed[b]
Vincristine	Diffusion (Ref. 12)	P-gp (Ref. 12)	11.5	0.4 (Ref. 13)	4.61	4.13	42.1	33.1 (Ref. 14)	H/H
Taurocholate	Ntcp (Oatps)	Bsep	19.7	0.15 (Ref. 4)	2.95	2.75	28.0	29.8 (Ref. 9)	H/H
Temocaprilat	Oatps (Ref. 15)	Mrp2 (Ref. 16)	45.7	0.0639 (Ref. 17)	2.92	2.72	27.8	34.4 (Ref. 17)	M/H
Probenecid	Oat2 (Ref. 18) (Oatps)	Mrp2 (Ref. 19)	25.2	0.116 (Ref. 20)	2.92	2.72	27.8	28.59 (Ref. 19)	M/H
Irinotecan	Oatps (Ref. 21)	P-gp(Mrp2) (Refs. 22,23)	9.44	0.2 (Ref. 24)	1.89	1.80	18.4	20.6 (Refs. 24,25)	M/M
Erythromycin	Oat2 (Ref. 26) (Oatps) (Ref. 27)	P-gp (Ref. 28)	2.34	0.78 (Ref. 29)	1.83	1.75	17.8	15.5 (Ref. 27)	M/M
Rosuvastatin	Oatps (Ref. 30)	Bcrp (Ref. 30)	44.9	0.039 (Ref. 11)	1.75	1.68	17.1	24.3 (Ref. 11)	M/M
Benazeprilat	Oatps (Refs. 15,17)	Mrp2 (Ref. 17)	7.04	0.245 (Ref. 17)	1.72	1.65	16.9	20.2 (Ref. 17)	M/M
Fexofenadine	Oatps (Ref. 31)	Mrp2(Bsep) (Ref. 31)	12.0	0.11 (Ref. 32)	1.32	1.28	13.0	11.4 (Ref. 33)	M/L
Colchicine	Diffusion (Ref. 34)	P-gp(Mrp2) (Refs. 35,36)	3.04	0.389 (Ref. 37)	1.18	1.15	11.7	9.05 (Ref. 35)	L/L
Trovafloxacin	N.A.	N.A.	7.36	0.11 (Ref. 38)	0.810	0.794	8.09	8.39 (Ref. 38)	L/L
Enalaprilat	Oatps (Ref. 39)	Mrp2 (Ref. 39)	1.51	0.42 (Ref. 17)	0.634	0.624	**6.36**	2.96 (Ref. 17)	L/L
Atorvastatin	Oatps (Ref. 40)	Mrp2 (Ref. 40)	9.76	0.0567 (Ref. 41)	0.553	0.546	5.56	6.2 (Ref. 42)	L/L
Mitoxantrone	Diffusion (Ref. 43)	Bcrp(P-gp) (Refs. 43,44)	8.24	0.05 (Ref. 45)	0.412	0.408	4.16	7.2 (Ref. 46)	L/L
AAFE ($n = 14$)								1.26	
Lomefloxacin	N.A.	N.A.	0.480	0.722 (Ref. 47)	0.347	0.344	**3.50**	0.253 (Ref. 47)	L/L
Fluvastatin	Oatps (Ref. 48)	Mrp2 (Ref. 49)	29.1	0.00986 (Ref. 41)	0.287	0.285	2.91	3.78 (Ref. 41)	L/L
Indomethacin	Oat2 (Ref. 50)	P-gp (Ref. 51)	120	0.002 (Ref. 52)	0.241	0.240	**2.44**	0.45 (Ref. 53)	L/L
Octreotide	N.A.	P-gp (Ref. 54)	0.302	0.41 (Ref. 55)	0.124	0.124	**1.27**	7.44 (Ref. 56)	L/L
Deferasirox	Diffusion	Mrp2 (Ref. 57)	10.4	0.01 (Ref. 58)	0.104	0.104	**1.06**	5.6 (Ref. 57)	L/L
Olmesartan	Oatps (Ref. 59)	Mrp2 (Ref. 59)	3.44	0.01 (Ref. 10)	0.0344	0.0344	**0.351**	2.22 (Ref. 10)	L/L
Candesartan	N.A.	N.A.	1.55	0.00651 (Ref. 17)	0.0101	0.0101	**0.103**	3.03 (Ref. 17)	L/L
AAFE of total training set ($n = 21$)								2.20	

[a] $CL'_{b,\,app}$ (mL/min/kg) was derived by scaling *in vitro* apparent CL_b ($mL^*min^{-1}*mg^{-1}$ of protein) with physiological parameters: 200 mg protein/g rat liver tissue and 40 g rat liver tissue/kg body weight.

[b] CL_b categories: low, *in vivo* CL_b ≤30% (12 $mL^*min^{-1}*kg^{-1}$) of liver plasma flow; moderate, 30%–70% (12–28 $mL^*min^{-1}*kg^{-1}$) of liver plasma flow; and high, ≥70% (28 $mL^*min^{-1}*kg^{-1}$) of liver plasma flow.

Compounds are ordered based on predicted $CL_{b,\,app}$.

Predictions outside the twofold error of observed *in vivo* CL_b are highlighted in bold.

AAFE, average absolute fold error; CL_b, biliary clearance; $CL_{b,\,app}$, apparent biliary clearance; $CL'_{b,app,}$ apparent biliary clearance scaled to kg body weight; f_u, unbound fraction in plasma; N.A., not available.

DOI 10.1002/jps

Accumulation Studies and Analysis

Accumulation studies were conducted as described previously.[10] Hepatocytes were rinsed twice and then preincubated for 10 min at 37°C with 0.6 mL of warmed Hank's balanced salt solution (HBSS) either containing Ca^{2+} or not containing Ca^{2+} to maintain or disrupt the tight junctions sealing bile canalicular networks, respectively. Subsequently, hepatocytes were incubated with the test compound (1 μM for [^{3}H]taurocholate and 10 μM for the other compounds) in standard HBSS for 10 min at 37°C. After incubation, the dosing solution was aspirated from the cells, and uptake was stopped by washing the cells three times with ice-cold standard HBSS. For radiolabeled compound, cells were lysed with 0.5 mL of 0.5% Triton X-100 in phosphate-buffered saline (Sigma-Aldrich, St. Louis, Missouri). The samples were analyzed for compound concentrations by liquid scintillation counting. For other compounds, cells were lysed with 0.3 mL of 90% (v/v) methanol containing 100 ng/mL of rosuvastatin-d6 (internal standard) and sonicated for 30 s with a sonic dismembrator (model 100; Thermo Fisher Scientific, Waltham, Massachusetts) and then transferred to a 96-well centrifugation filterplate (Corning Inc., Acton, Massachusetts). After centrifugation (2095*g* × 20 min), the filtrates were transferred to a 96-well plate and sealed for LC–MS/MS analysis. Substrate accumulation was corrected for nonspecific binding by using Matrigel-precoated 24-well plates without cells. Because of incompatibility of the protein assay with methanol, the average protein concentration for standard HBSS or Ca^{2+}-free HBSS incubations in the same liver preparation was used to normalize accumulation. Lysates were quantified by the bicinchoninic acid protein assay (Pierce Biotechnology, Inc., Rockford, Illinois) using bovine serum albumin as the reference standard, and accumulation was normalized to protein concentration.

LC–MS/MS Analysis

An Agilent 1100 HPLC system (Agilent Inc., Santa Clara, California) connected to an Applied Biosystems API 3000 triple quadrupole mass spectrometer with an electrospray ion source (Foster City, California) was used for sample analysis. A total of 20 μL of sample was injected into the LC–MS/MS. The flow rate of the mobile phases [aqueous phase, water with 0.1% formic acid (v/v) and 10 mM ammonium formate; organic phase, acetonitrile with 0.1% formic acid (v/v)] was 0.3 mL/min. Isocratic or gradient elution was used to elute the various compounds from a Zorbax ODS C18 (50 × 2.1 mm^2, 3 μm) column (Agilent Inc.). Rosuvastatin-d6 was used as an internal standard. The standard curve for each compound was individually established by using Matrigel-precoated 24-well plates with hepatocytes.

Data Analysis

In vitro apparent CL_b ($CL_{b,\,app,\,in\,vitro}$; mL*min^{-1}*mg^{-1} of protein), based on compound concentration in the medium, and *in vitro* intrinsic CL_b ($CL_{b,\,int,\,in\,vitro}$; mL*min^{-1}*mg^{-1} of protein), based on intracellular compound concentration in SCRHs, were calculated using B-CLEAR® technology (Qualyst, Inc.) by the following equations[9,10,60]:

$$CL_{b,\,app,\,in\,vitro} = \frac{accumulatation_{cells+bile} - accumulatation_{cells}}{concentration_{(medium)} \times 10\ min} \quad (2)$$

$$CL_{b,\,app,\,in\,vitro} = \frac{accumulatation_{cells+bile} - accumulatation_{cells}}{(accumulatation_{cells}/intracellular\,volume) \times 10\ min}. \quad (3)$$

The concentration of compound in the medium was defined as the initial substrate concentration (1 or 10 μM) in the incubation medium. Intracellular volume was assumed to be 5.2 μL/mg protein.[60] $CL_{b,\,app,\,in\,vitro}$ and $CL_{b,\,int,\,in\,vitro}$ (mL*min^{-1}*mg^{-1} of protein) were scaled to kilograms of body weight ($CL'_{b,\,app}$ and $CL'_{b,\,int}$) assuming 200 mg protein/g rat liver tissue and 40 g rat liver tissue/kg body weight. The predicted *in vivo* $CL_{b,\,app}$ values were estimated according to Eq. 4 given below, based on the well-stirred model of hepatic disposition, assuming that the red blood cell partitioning of test compounds was minimal.

$$In\,vivo\,CL_{b,app} = (Q_p \times f_u \times CL'_{b,app}) / (Q_p + f_u \times CL'_{b,app}) \quad (4)$$

where Q_p represents the rat hepatic plasma flow rate (40 mL*min^{-1}*kg^{-1})[8] and f_u represents the unbound fraction in rat plasma.

Average absolute fold error (AAFE)[1] was used to assess the prediction precision of various correction approaches. AAFE was calculated as following:

$$AAFE = 10^{1/n} \sum \left| \log \left(\frac{predicted\,CL}{observed\,CL} \right) \right| \quad (5)$$

Data Set

Forty-one compounds, which were reported to undergo biliary excretion, were randomly assigned to a training set (n = 21) and a test set (n = 20). The *in vitro* biliary excretion parameters of 21 compounds in the training set were experimentally determined from the SCRH model. The SCRH biliary excretion

parameters of 20 compounds in the independent test set, the values of f_u in rat plasma and the *in vivo* CL_b values for the 41 compounds from the training and test sets were all collected from the literature. When *in vivo* CL_b was not available in the literature, it was calculated by the following equation: $CL_b = CL_{total} \times \%$ of dose as parent compound in bile. Compounds in the training and test sets were divided into low, moderate, and high CL_b categories on the basis of predicted or observed *in vivo* CL_b values. Compounds with an *in vivo* CL_b of 30% ($12\,mL*min^{-1}*kg^{-1}$) or less of liver plasma flow were assigned to the low CL_b category, and compounds with an *in vivo* CL_b of 70% ($28\,mL*min^{-1}*kg^{-1}$) or more of liver plasma flow were assigned to the high CL_b category. All other compounds were assigned to the moderate CL_b category. Information regarding the major uptake and efflux transporters of each compound was collected from the literature (Tables 1 and 2) and UCSF-FDA TransPortal (http://bts.ucsf.edu/fdatransportal/index/).

RESULTS

Selection of Probe Substrates

The selection criteria for probe substrates in the training set included: rat *in vivo* CL_b and rat plasma f_u values available in literature; active hepatic uptake and/or biliary excretion; and limited hepatic metabolism. To ensure limited hepatic metabolism, the metabolic stability of 18 compounds in the training set were assessed in suspended rat cryopreserved hepatocytes. As shown in Table S1, IVIVE from cryopreserved rat hepatocyte incubation showed that all the 18 compounds were metabolically stable. The other three compounds (rosuvastatin, fexofenadine, and olmesartan) in the training set have been previously reported to be stable in rat liver microsome and hepatocyte incubations.[4,98]

Determination of Universal Correction Factor

In vitro apparent CL_b values based on compound concentration in the medium were calculated using Eq. 2 and scaled to kilograms of body weight ($CL'_{b,\,app}$; $mL*min^{-1}*kg^{-1}$) (Table 1 for training set and Table 2 for test set). The predicted $CL_{b,\,app}$ was obtained from $CL'_{b,\,app}$ using Eq. 4 (Tables 1 and 2). A reasonable linear correlation ($R^2 = 0.875$) between the predicted $CL_{b,\,app}$ and observed *in vivo* CL_b was observed for 21 compounds in the training set (Fig. 1a), although the observed *in vivo* CL_b values were on average 10.2-fold higher than the predicted values. Similarly, a linear correlation ($R^2 = 0.746$) between predicted $CL_{b,\,app}$ and observed *in vivo* CL_b was observed for 20 compounds in the independent test set (Fig. 1b), and the predicted $CL_{b,\,app}$ values were underestimated by an average of 11.0-fold.

The slope (10.2) of the linear correlation equation obtained from the training set was used as a universal correction factor to correct the underestimation of CL_b. The correction factor was applied to both the training and test sets (Tables 1 and 2), and the corrected CL_b values were plotted against the observed *in vivo* CL_b for the training (squares) and test (triangles) sets (Fig. 2). A total of seven out of 21 compounds in the training set (highlighted in bold font in Table 1) and seven out of 20 compounds in the test set (highlighted in bold font in Table 2) were outside the limit of twofold error of the observed *in vivo* CL_b. Noticeably, the CL_b values of candesartan and cefmetazole were underpredicted, even after correction, by more than 30- and 20-fold, respectively. Poor prediction accuracy of absolute CL_b values was observed for compounds with predicted $CL_{b,\,app} \times 10.2 \leq 3.5\,mL*min^{-1}*kg^{-1}$ in the training set (Fig. 2, solid squares; one out of seven predictions within twofold error) and test set (solid triangles; two out of seven predictions within twofold error). By contrast, more accurate predictions (13 out of 14 predictions within twofold error for the training set and 11 out of 13 predictions within twofold error for the test set) were achieved when the predicted $CL_{b,\,app} \times 10.2$ was greater than 3.5 mL/min/kg. Overall, when corrected by a universal factor of 10.2, IVIVE from the SCRH model 10.2 provides acceptable predictability for compounds with moderate-to-high CL_b.

Although the absolute CL_b values of compounds with predicted $CL_{b,\,app} \times 10.2$ of $3.5\,mL*min^{-1}*kg^{-1}$ or less were inaccurately predicted, both predicted and observed *in vivo* CL_b consistently suggested low biliary excretion. For all seven compounds with predicted $CL_{b,\,app} \times 10.2$ of $3.5\,mL*min^{-1}*kg^{-1}$ or less in the test set (Table 2), both predicted and observed values consistently indicated that the compounds were in the low CL_b category ($CL_b < 12\,mL*min^{-1}*kg^{-1}$). Similar results were found in the training set (Table 1). These observations suggest that the SCRH model combined with a correction factor can be utilized to predict whether an unknown compound has low, moderate, or high CL_b.

As a comparison, *in vivo* CL_b was tentatively extrapolated from $CL_{b,\,int,\,in\,vitro}$, which is based on the intracellular drug concentration in the SCRH model (Eq. 3).[99] $CL_{b,\,int,\,in\,vitro}$ ($mL*min^{-1}*mg^{-1}$ of protein) was scaled to *in vivo* CL_b ($mL*min^{-1}*kg^{-1}$) using physiological parameters; however, no linear correlation between the predicted CL_b and observed *in vivo* CL_b was observed in the training set (Table S2 and Fig. S1). The linear regression R^2 value was 0.235.

Table 2. Independent Test Set ($n = 20$)–Observed and Predicted CL_b (Extrapolated from $CL_{b,\,app,\,in\ vitro}$)

Compounds	Major Influx Transporters	Major Efflux Transporters	$CL'_{b,app}$ ($mL^*min^{-1}*kg^{-1}$)[a]	f_u	$CL'_{b,\,app} \times f_u$	Predicted $CL_{b,\,app}$ (Eq. 4) ($mL^*min^{-1}*kg^{-1}$)	CL_b Corrected by $\times$ 10.2 ($mL^*min^{-1}*kg^{-1}$)	Observed *In Vivo* CL_b ($mL^*min^{-1}*kg^{-1}$)	Category Predicted/ Observed[b]
$E_2$17βG	Oatps (Ref. 61)	Mrp2 (Ref. 62)	45.6 (Ref. 4)	0.069 (Ref. 4)	3.15	2.92	29.7	32.1 Ref. 4)	H/H
Digoxin	Diffusion (Ref. 63)	P-gp (Ref. 64)	4.9 (Ref. 64)	0.61 (Ref. 65)	2.99	2.78	28.3	21 (Ref. 66)	H/M
5-CFDA[c]	Diffusion (Ref. 67)	Mrp2 (Ref. 67)	39.05 (Ref. 68)	0.074 (Ref. 69)	2.89	2.70	27.5	44.78 (Ref. 68)	M/H
ICG[d]	Oatps(Ntcp) (Ref. 70)	Mrp2 (Ref. 70)	25.2 (Ref. 71)	0.1 (Ref. 72)	2.52	2.37	24.2	32 (Ref. 71)	M/H
Ouabain	N.A.	N.A.	3.12 (Ref. 71)	0.8	2.50	2.35	23.9	14.7 (Ref. 71)	M/M
Rhodamine123	Diffusion (Ref. 73)	P-gp (Ref. 64)	8 (Ref. 64)	0.3 (Ref. 74)	2.40	2.26	23.1	18.3 (Ref. 64)	M/M
Pravastatin	Oatps (Ref. 75)	Mrp2 (Ref. 75)	4.43 (Ref. 10)	0.472 (Ref. 10)	2.09	1.99	20.3	27.9 (Ref. 10)	M/M
Methotrexate	Oatps (Ref. 76)	Mrp2(Bcrp) (Ref. 77)	4.1 (Ref. 9)	0.34 (Ref. 78)	1.39	1.35	13.7	12.1 (Ref. 9)	M/M
Doxorubicin	Diffusion (Ref. 79)	Mrp2(P-gp) (Ref. 80)	2.48 (Ref. 71)	0.344 (Ref. 81)	0.853	0.835	8.51	16.6 (Ref. 71)	L/M
Cimetidine	Oct1 (Ref. 82)	Mate1 (Ref. 83)	0.8 (Ref. 68)	0.81 (Ref. 68)	0.648	0.638	**6.50**	2.58 (Refs. 84,85)	L/L
Cefpiramide	Oatps (Ref. 86)	Mrp2 (Ref. 87)	4.72 (Ref. 4)	0.099 (Ref. 4)	0.467	0.462	4.71	5.47 (Ref. 4)	L/L
Topotecan	N.A.	Bcrp(P-gp) (Ref. 88)	0.487 (Ref. 60)	0.747 (Ref. 60)	0.364	0.361	3.67	5.3 (Ref. 60)	L/L
Rifampicin	Oatps (Ref. 89)	N.A.	1.99 (Ref. 68)	0.18 (Ref. 68)	0.358	0.355	**3.62**	1.01 (Ref. 68)	L/L
AAFE ($n = 13$)								1.55	
Pitavastatin	Oatps (Ref. 90)	Bcrp (Ref. 91)	34.6 (Ref. 10)	0.008 (Ref. 10)	0.277	0.275	2.80	4.55 (Ref. 10)	L/L
Cefoperazone	Oatps (Ref. 92)	Mrp2 (Ref. 87)	0.56 (Ref. 4)	0.429 (Ref. 4)	0.240	0.239	**2.43**	11 (Ref. 4)	L/L
Dexamethasone	N.A.	P-gp (Ref. 93)	0.9 (Ref. 68)	0.2 (Ref. 68)	0.180	0.179	**1.83**	0.43 (Ref. 68)	L/L
Valsartan	Oatps (Ref. 94)	Mrp2 (Ref. 94)	28 (Ref. 11)	0.004 (Ref. 11)	0.112	0.112	**1.14**	3.5 (Ref. 11)	L/L
Cephradine	N.A.	Mrp2(Bcrp) (Ref. 87)	0.08 (Ref. 68)	0.94 (Ref. 95)	0.0752	0.0751	**0.765**	3.1 (Ref. 68)	L/L
Tolbutamide	Oatps (Ref. 96)	N.A.	0.16 (Ref. 68)	0.24 (Ref. 68)	0.0384	0.0384	0.391	0.28 (Ref. 68)	L/L
Cefmetazole	Oatps (Refs. 92,97)	Mrp2 (Ref. 87)	0.04 (Ref. 11)	0.738 (Ref. 11)	0.0295	0.0295	**0.301**	6.5 (Ref. 11)	L/L
AAFE of total test set ($n = 20$)								2.14	

[a] $CL'_{b,app}$ (mL/min/kg) was derived by scaling *in vitro* apparent CL_b ($mL^*min^{-1}*mg^{-1}$ of protein) with physiological parameters: 200 mg protein/g rat liver tissue and 40 g rat liver tissue/kg body weight.

[b] CL_b catagories: low, *in vivo* CL_b ≤30% (12 $mL^*min^{-1}*kg^{-1}$) of liver plasma flow; moderate, 30%–70% (12—28 $mL^*min^{-1}*kg^{-1}$) of liver plasma flow; and high, ≥70% (28 $mL^*min^{-1}*kg^{-1}$) of liver plasma flow.

[c] 5-CFDA: 5-carboxyfluorescein diacetate

[d] ICG: Indocyanine green

Compounds are ordered based on predicted $CL_{b,\,app}$.

Predictions outside the twofold error of observed *in vivo* CL_b are highlighted in bold.

AAFE, average absolute fold error; CL_b, biliary clearance; $CL_{b,\,app}$, apparent biliary clearance; $CL'_{b,\,app,}$ apparent biliary clearance scaled to kg body weight; *f*u, unbound fraction in plasma; N.A., not available.

DOI 10.1002/jps

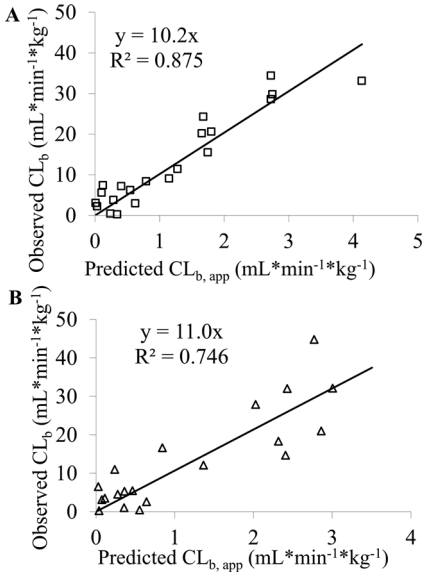

Figure 1. Linear correlations between observed *in vivo* CL_b and predicted $CL_{b,\,app}$ in the (a) training set ($n = 21$) and (b) test set ($n = 20$). Predicted $CL_{b,\,app}$ was based on compound concentration in medium (Eq. 2) and obtained from Eq. 4. Squares, compounds in the training set; triangles, compounds in the test set.

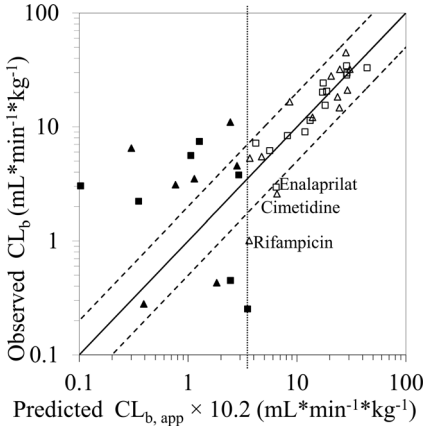

Figure 2. Plot between observed *in vivo* CL_b and predicted $CL_{b,\,app} \times 10.2$ ($n = 41$). Dashed lines represent the limit of twofold error of observed values. The dotted line represents the boundary (predicted $CL_{b,\,app} \times 10.2 = 3.5\,mL*min^{-1}*kg^{-1}$) between inaccurate predictions (left) and accurate predictions (right). Squares, compounds in the training set; triangles, compounds in the test set; solid squares or triangles, compounds with predicted $CL_{b,\,app} \times 10.2 \leq 3.5\ mL*min^{-1}*kg^{-1}$.

Determination of Transporter-Specific Correction Factors

As the variable (*in vitro* vs. *in vivo*) expression levels of hepatic influx and efflux transporters in the SCRH model lead to inaccuracies in predictions, transporter-specific correction factors were introduced into IVIVE to determine whether these corrections would improve the model. The major influx and efflux transporters involved in the active transport of substrates are listed in Tables 1 and 2. The transporters in parentheses are minor transporters responsible for uptake and efflux. Compounds in the training set were selected for linear regression analysis by the following criteria: predicted $CL_{b,\,app} \times 10.2$ was greater than $3.5\,mL*min^{-1}*kg^{-1}$; both uptake and efflux mechanisms are known; and at least two compounds were available in each group for linear regression. Hence, seven compounds in the training set were divided into an Oatps/Mrp2 group ($n = 5$) and a diffusion/P-gp group ($n = 2$). Linear correlation analysis among the five Oatps/Mrp2 substrates (Fig. 3a, squares) and the two diffusion/P-gp substrates (Fig. 3b, squares) in the training set gave Oatps/Mrp2-specific and diffusion/P-gp-specific correction factors (11.8 and 8.0, respectively).

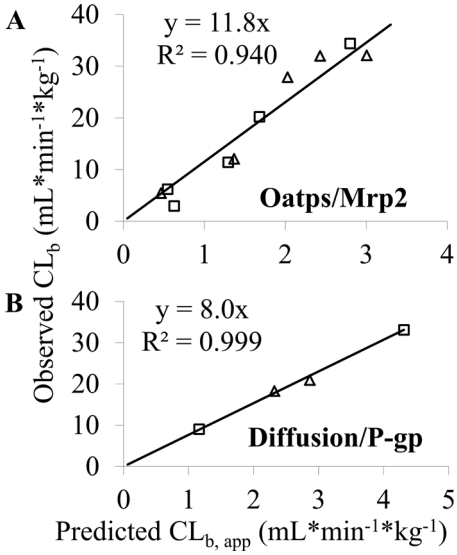

Figure 3. Linear correlations between observed *in vivo* CL_b and predicted $CL_{b,\,app}$. (a) Oatps/Mrp2 substrates ($n = 5$). (b) Diffusion/P-gp substrates ($n = 2$). Squares, compounds in the training set; triangles, compounds in the test set. The linear correlation analysis was based on compounds in the training set.

Application of Transporter-Specific Correction Factors to Test and Training Sets

The two transporter-specific correction factors were appropriately applied to five Oatps/Mrp2 substrates and two diffusion/P-gp substrates in the test set (Table 3; Fig. 3, triangles). The CL_b values of the other 13 compounds in the test set were not corrected because either the corresponding correction factors were not available or the predicted $CL_{b,\,app} \times 10.2$ was $3.5\,mL*min^{-1}*kg^{-1}$ or less. The observed *in vivo* CL_b values were plotted against the predicted CL_b values based on transporter-specific

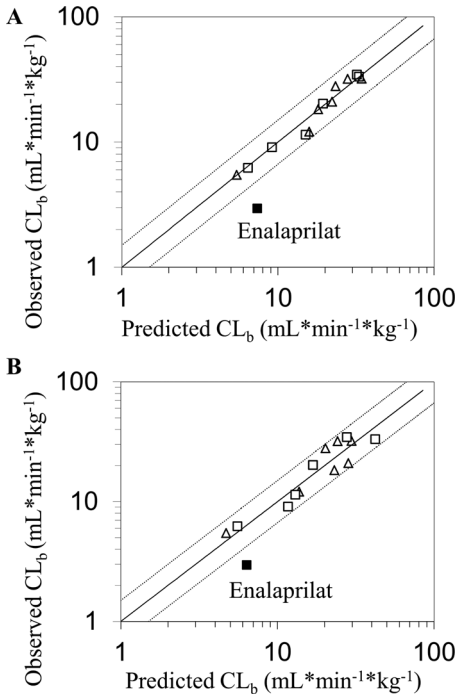

Figure 4. Plot between observed *in vivo* CL_b and predicted CL_b ($n = 14$) after correction with (a) transporter activity-based factors and (b) a single universal factor (10.2). Dotted lines represent the limits of 1.5-fold error of observed values. Squares, compounds in the training set; triangles, compounds in the test set; solid squares or triangles, predictions outside 1.5-fold error.

corrections (Fig. 4a, triangles). All seven predictions for the test set compounds were within 1.5-fold error of the observed *in vivo* CL_b. When the transporter-specific correction factors were appropriately applied to seven compounds in the training set (five Oatps/Mrp2 substrates and two diffusion/P-gp substrates), six predictions fell within 1.5-fold error, whereas the predicted CL_b of enalaprilat (7.35 mL*min^{-1}*kg^{-1}) was more than twofold of the observed CL_b (2.96 ± 0.32 mL*min^{-1}*kg^{-1})[18] (Fig. 4a, squares).

As a comparison, the general correction factor (10.2) was applied to the seven test set compounds and seven training set compounds (Table 3). The observed *in vivo* CL_b values were plotted against the predicted CL_b values based on the correction factor of 10.2 (Fig. 4b, triangles for the test set and squares for the training set). The results show that the transporter-specific corrections improve prediction precision compared with the universal correction. For the seven compounds in the test set, the AAFE of predictions decreased from 1.24 (universal correction factor 10.2) to 1.11 (transporter-specific correction factors). For the seven compounds in the training set, the AAFE of predictions decreased from 1.31 (universal correction factor 10.2) to 1.21 (transporter-specific correction factors).

DISCUSSION

Biliary excretion is an important elimination mechanism of xenobiotics and their metabolites. Although SCH models have been utilized to estimate human and rat CL_b for more than a decade,[99,100] the accurate prediction of CL_b is still a challenging task. For drugs that undergo similar uptake and efflux mechanisms, such as angiotensin II receptor blockers, HMG-CoA reductase inhibitors, and β-lactam antibiotics, the CL_b values predicted by SCHs are linearly correlated with observed *in vivo* CL_b.[9,11,100,97] However, CL_b can be underestimated by 10–100-fold because of limited dynamic bile and blood flow and time-dependent alterations in the protein concentration of influx and efflux transporters.[3,4] To address this underestimation, Li et al.[4] incorporated ratios of efflux transporter protein levels in rat liver to those in SCRHs into IVIVE as correction factors. Similarly, in the present study, compounds were divided into subgroups based on uptake and efflux mechanisms. We utilized the ratio of the active transport activity in rat liver and to that in SCRH as a transporter-specific correction factor for each subgroup. Transporter-based correction factors were obtained by linear correlation analysis between the observed *in vivo* CL_b and predicted $CL_{b,\,app}$ of training set compounds that undergo the same influx and efflux mechanisms. Compared with correction factors based on the protein amount of efflux transporters only, the transporter-based correction factors in this study address the variable activities of both influx and efflux transporters in the SCRH model. The predictability of the correction factors was examined using an independent test set in which the *in vitro* and *in vivo* data were collected from the literature. The results showed that both the prediction accuracy and precision were improved by incorporating the transporter-based correction factors into IVIVE.

In this study, a linear correlation between predicted $CL_{b,\,app}$ from the SCRH model and *in vivo* CL_b was observed in both a training and an independent test set (Figs. 1a and 1b), although different uptake and efflux mechanisms were involved in the biliary excretion of these compounds (Tables 1 and 2). The lower coefficient of determination in the literature-based test set ($R^2 = 0.746$) than in the training set ($R^2 = 0.875$) is likely because of interbatch and interlaboratory variations. The underestimation of $CL_{b,\,app}$ between the training set (10.2-fold) and the independent test set (11.0-fold) was consistent, suggesting that the underestimation of CL_b can be corrected by an empirical correction factor. Consistent with the previous reports,[11,60,101] normalization with f_u in rat plasma in Eq. 4 significantly improved the linear correlation between the predicted $CL_{b,\,app}$ and observed *in vivo* CL_b but caused further underestimation of CL_b. When f_u

Table 3. Comparison of CL_b Predictions for Seven Compounds in the Training Set and Seven Compounds in the Test Set Using Transporter-Specific Corrections Versus a Universal Correction

Compounds	Transporter-Based Subgroup	Predicted $CL_{b,\ app}$ (Eq. 4) ($mL*min^{-1}*kg^{-1}$)	Predicted CL_b ($mL*min^{-1}*kg^{-1}$)		Observed *In Vivo* CL_b ($mL*min^{-1}*kg^{-1}$)
			With Transporter-Based Correction[a]	With Universal Correction Factor[b] 10.2	
Training Set					
Atorvastatin	Oatps/Mrp2 (11.8)	0.546	6.43	5.56	6.2
Temocaprilat	Oatps/Mrp2 (11.8)	2.72	32.1	27.8	34.4
Enalaprilat	Oatps/Mrp2 (11.8)	0.624	**7.35**	**6.36**	2.96
Benazeprilat	Oatps/Mrp2 (11.8)	1.65	19.5	16.9	20.2
Fexofenadine	Oatps/Mrp2 (11.8)	1.28	15.1	13.0	11.4
Vincristine	Diffusion/P-gp (8.0)	4.13	33.1	42.1	33.1
Colchicine	Diffusion/P-gp (8.0)	1.15	9.19	11.7	9.05
AAFE of training set ($n = 7$)			1.21	1.31	
Test Set					
Pravastatin	Oatps/Mrp2 (11.8)	1.99	23.4	20.3	27.9
Methotrexate	Oatps/Mrp2 (11.8)	1.35	15.9	13.7	12.1
$E_2$17βG	Oatps/Mrp2 (11.8)	2.92	34.4	29.7	32.1
Cefpiramide	Oatps/Mrp2 (11.8)	0.462	5.44	4.71	5.47
ICG	Oatps/Mrp2 (11.8)	2.37	27.9	24.2	32
Rhodamine123	Diffusion/P-gp (8.0)	2.26	18.1	23.1	18.3
Digoxin	Diffusion/P-gp (8.0)	2.78	22.3	28.3	21
AAFE of test set ($n = 7$)			1.11	1.24	
AAFE of both training set and test set ($n = 14$)			1.16	1.27	

[a] A correction of 11.8 was applied to compounds in the Oatps/Mrp2 subgroup, and a correction of 8.0 was applied to compounds in the diffusion/P-gp subgroup.

[b] A universal correction factor of 10.2 was used.

Predictions outside the 1.5-fold error of observed *in vivo* CL_b are highlighted in bold.

AAFE, average absolute fold error; CL_b, biliary clearance; $CL_{b,\ app}$, apparent biliary clearances.

was not incorporated into Eq. 4, the predicted CL_b was closer to *in vivo* CL_b but the correlation coefficient of determination was much lower (data not shown).

The value of 3.5 mL/min/kg for predicted $CL_{b,\ app} \times 10.2$ was found to be a cutoff level for accurate prediction of CL_b in the SCRH model. The CL_b of compounds with high $CL_{b,\ app,\ in\ vitro}$ and/or low plasma protein binding are more likely to be accurately predicted by the SCRH model. For compounds with low $CL_{b,\ app,\ in\ vitro}$ and/or high plasma protein binding, CL_b might be underestimated by up to 30-fold (e.g., candesartan) or overestimated by up to 14-fold (e.g., lomefloxacin), although both predicted and observed CL_b consistently suggested low biliary excretion for these compounds ($CL_b < 12\ mL*min^{-1}*kg^{-1}$). The poor predictions could be a result of many factors. First, a measurement error in *in vitro* and *in vivo* CL_b may affect the prediction accuracy, especially for compounds with low biliary excretion. Second, prediction errors might be caused by variable transporter activities in the SCRH model. For example, decreased sinusoidal influx transporter activities in SCRH might have caused the underprediction of CL_b of octreotide, cefoperazone, cephradine, and cefmetazole. A previous report showed that CL_b values of cefoperazone and cefmetazole were consistently underestimated by more than 10-fold, even though the Mrp2 protein concentration ratio between rat liver and SCRH was incorporated into IVIVE as a correction factor.[4] On the contrary, increased or relatively unchanged canalicular efflux transporter activities likely resulted in the overprediction of CL_b for lomefloxacin, rifampicin, and dexamethasone, especially when the universal correction factor 10.2 was incorporated into IVIVE. Third, compounds with high plasma protein binding, not surprisingly, exhibit poor predictions of CL_b. When f_u is 0.01 or less (e.g., indomethacin, deferasirox, olmesartan, candesartan, and valsartan), normalization with f_u (Eq. 4) is expected to cause significant prediction errors because of potential measurement errors in f_u. Fourth, to achieve good responses on LC–MS/MS, most compounds, especially those with low hepatocyte uptake, were incubated at 10 μM in protein-free medium. For high-protein-binding compounds, the free concentration in the medium was much higher than the plasma-free drug concentrations in rats used for *in vivo* CL_b measurement. The high concentration in the medium might partially saturate influx and/or efflux transporters in SCRHs, especially for compounds with low $CL_{b,\ app,\ in\ vitro}$ and a low Michaelis constant (K_m), thus resulting in underprediction of CL_b (e.g., deferasirox, olmesartan, candesartan, and valsartan). If a very sensitive assay is available for compound quantitation, the prediction errors caused by high protein binding might be reduced by adding plasma protein

to the incubation medium[35] or preparing the dosing solution in rat serum[102] for which normalization with f_u is not required. It is noteworthy to mention that the addition of serum proteins to incubation medium did not remarkably change hepatocyte uptake of some lipophilic compounds with very high protein binding.[35] For these lipophilic compounds, serum proteins in culture medium serve as a drug solubilizer. The protein-bound drug might be transferred to hepatocyte cell membranes during protein–hepatocyte interaction, and the serum proteins facilitate drug uptake. This observation might explain the underestimation of CL_b for very high-protein-binding drugs such as olmesartan and candesartan in the current study where f_u correction was incorporated (Eq. 4).

Most SCRH studies are designed to determine $CL_{b,\,app,\,in\,vitro}$ on the basis of compound concentration in medium (Eq. 2), and then the predicted $CL_{b,\,app}$ is correlated with the observed *in vivo* $CL_{b,\,app}$ on the basis of compound concentration in rat plasma. Recently, Nakakariya et al.[60] reported IVIVE between $CL_{b,\,int,\,in\,vitro}$ and *in vivo* intrinsic CL_b, which are based on compound concentrations in hepatocytes in the SCRH model (Eq. 3) and in liver tissue, respectively. In the SCRH model, the expression levels of sinusoidal influx transporters considerably decrease, whereas the protein expression of canalicular efflux transporters is relatively maintained during culture. Theoretically, IVIVE from $CL_{b,\,int,\,in\,vitro}$ values that reflect only canalicular efflux processes, should, therefore, be less susceptible to the variable expression of influx transporters.[60] Unfortunately, compound concentrations in rat liver were not available, and so *in vivo* intrinsic CL_b values were unable to be determined in the current study. We, therefore, tentatively plotted the CL_b extrapolated from $CL_{b,\,int,\,in\,vitro}$ and the observed *in vivo* CL_b. A very poor correlation between *in vivo* $CL_{b,\,app}$ and $CL_{b,\,int,\,in\,vitro}$ was observed (Table S2 and Fig. S1). The $CL_{b,\,int,\,in\,vitro}$ only takes into account the canalicular efflux of a compound. However, *in vivo* CL_b is a function of both the basolateral uptake and canalicular efflux. Therefore, a correlation would be expected only in cases where the canalicular efflux was the rate-limiting step.

Two uptake and efflux mechanism-based factors were obtained by linear correlation analysis between predicted $CL_{b,\,app}$ and observed *in vivo* CL_b. The Oatps/Mrp2-specific factor (11.8) was higher than the universal factor (10.2), which might be explained by the decreased expression of Oatps in the SCRH model. The low value of the diffusion/P-gp-specific factor (8.0) was likely due to the unchanged or increased expression of P-gp in the SCRH model. Transporter expression variability between batches of SCRHs might limit the successful application of transporter-based corrections. Previous transporter quantitation studies[7,8] consistently revealed that the changes in transporter expression and activities were mainly dependent on SCRH culture time. A dramatic decrease or increase in transporter expression occurred during 0–72 h after cell seeding. On day 4 and day 5 after cell seeding, further changes in transporter expression were relatively limited. In this study and the studies in the literature, the incubation of compounds with SCRHs was usually conducted on day 4 and day 5 after cell seeding, which might explain the comparable transporter activities among SCRH models used for the training set and test set compounds. Because of the time-sensitive changes in transporter expression in the SCRH model, a consistent culture time is highly recommended.

The transporter-based correction approach for CL_b predictions requires an understanding of the major uptake and efflux mechanisms undergone by the drug candidate, which are usually not available at the early stage of drug development. In the absence of this information, the universal correction factor gave reasonable CL_b predictions when the predicted CL_b was greater than 3.5 mL/min/kg. For compounds with low *in vivo* CL_b, the SCRH model combined with the universal correction factor could not quantitatively estimate absolute values of CL_b, but it could qualitatively assign compounds to low, moderate, and high CL_b categories.

CONCLUSIONS

In this study, we demonstrated that both the universal correction factor and transporter-based correction factors provide reasonable corrections of CL_b, which are usually underestimated by the SCRH model. Transporter-based correction factors improved predictions compared with a universal correction factor. Considering the complicated hepatobiliary clearance processes of hepatic uptake, sinusoidal efflux, metabolism, and canalicular efflux, this transporter-based corrected IVIVE method provides excellent predictions of rat CL_b.

ACKNOWLEDGMENTS

We thank Juli Teague, Bilin Chou, and Dr. Brian Dean for their technical assistance and the DMPK department of Genentech, Inc. for their support.

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