Research Article

Predicting when Biliary Excretion of Parent Drug is a Major Route of Elimination in Humans

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Received 29 January 2014; accepted 27 May 2014; published online 9 July 2014

Abstract. Biliary excretion is an important route of elimination for many drugs, yet measuring the extent of biliary elimination is difficult, invasive, and variable. Biliary elimination has been quantified for few drugs with a limited number of subjects, who are often diseased patients. An accurate prediction of which drugs or new molecular entities are significantly eliminated in the bile may predict potential drug-drug interactions, pharmacokinetics, and toxicities. The Biopharmaceutics Drug Disposition Classification System (BDDCS) characterizes significant routes of drug elimination, identifies potential transporter effects, and is useful in understanding drug-drug interactions. Class 1 and 2 drugs are primarily eliminated in humans *via* metabolism and will not exhibit significant biliary excretion of parent compound. In contrast, class 3 and 4 drugs are primarily excreted unchanged in the urine or bile. Here, we characterize the significant elimination route of 105 orally administered class 3 and 4 drugs. We introduce and validate a novel model, predicting significant biliary elimination using a simple classification scheme. The model is accurate for 83% of 30 drugs collected after model development. The model corroborates the observation that biliary eliminated drugs have high molecular weights, while demonstrating the necessity of considering route of administration and extent of metabolism when predicting biliary excretion. Interestingly, a predictor of potential metabolism significantly improves predictions of major elimination routes of poorly metabolized drugs. This model successfully predicts the major elimination route for poorly permeable/poorly metabolized drugs and may be applied prior to human dosing.

KEY WORDS: biliary excretion; drug disposition; metabolism; orally administered; renal excretion.

INTRODUCTION

Drugs are primarily eliminated *via* metabolism, biliary excretion, or renal clearance of unchanged drug in the urine. During development, predicting how a drug will be eliminated from the body can help to assess potential toxicities, drug-drug interactions (DDIs), and pharmacokinetics, including possible exposure to the target site. Extent of metabolism and urinary excretion are readily quantifiable. However, biliary excretion is difficult to quantify in humans, and is often predicted in preclinical animal models, which perform poorly, especially when hepatic uptake transporters mediate biliary clearance (1). It would therefore be ideal to model when biliary excretion will be a primary elimination route prior to human dosing.

Electronic supplementary material The online version of this article (doi:10.1208/s12248-014-9636-1) contains supplementary material, which is available to authorized users.

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Transporter-mediated drug interactions can alter the exposure of drugs, resulting in toxicity or lack of efficacy. For example, cyclosporine inhibits the uptake of rosuvastatin, a biliary eliminated drug, by OATP1B1, resulting in a sevenfold increase in AUC (2), which may result in life-threatening rhabdomyolysis. It is now considered essential to determine possible transporter-mediated drug interactions and develop respective guidances during drug development (3). The Biopharmaceutics Drug Disposition Classification System (BDDCS) predicts when drug-drug interactions may be a concern utilizing extent of metabolism, which is qualitatively correlated with passive intestinal permeability rate, and solubility (4).

Biliary elimination is a vectorial process mediated by transport on the basolateral and apical membranes of hepatocytes, which may both cause interactions and affect disposition. To access the liver, drugs in the portal vein must traverse the hepatic basolateral membrane, requiring active transport for biliary eliminated drugs, which are poorly permeable. Notably, Varma *et al.* (5) observed a large overlap in the physicochemical space between human OATP substrates and drugs where biliary excretion accounts for $\geq 10\%$ of the administered dose in rats. Human OATP substrates and biliary excreted compounds both tended toward $MW \geq 400$ Da, $cLogD_{7.4} < 2.0$, and RPSA (polar surface area normalized by molecular mass) $\geq 20\%$. Subsequent to

hepatic uptake, biliary eliminated drugs are actively effluxed across the canalicular membrane to the highly concentrated bile by transporters such as P-gp, BCRP, MRP2, MDR3, BSEP, or MATE1. Drugs that are not eliminated in the bile can reenter the systemic circulation by permeation back across the basolateral membrane or be metabolized. As the most promiscuous efflux transporter, biliary eliminated drugs are frequently P-gp substrates and therefore might be expected to exhibit physicochemical properties that overlap with those of P-gp substrates. Recently, Broccatelli determined that P-gp nonsubstrates have a calculated surface area (S) < 400 Å² (6). Transport efficiency can be inhibited by xenobiotics, endogenous substrates, disease states, or genetic polymorphisms, resulting in decreased clearance of drugs and endogenous compounds such as bilirubin and bile salts and may result in unpredictable, possibly toxic exposure.

The major route of elimination can dictate a drug's observed pharmacokinetics and therefore may be targeted or avoided. For instance, drugs that are excreted into the bile may be subject to enterohepatic circulation, resulting in variable plasma concentrations with multiple peaks (7), and a longer half-life. Drugs eliminated in the bile may not be appropriate or may require extra pharmacokinetic monitoring for patients with certain diseases or genetic polymorphisms, such as those with Dubin-Johnson syndrome, where a mutation in MRP2 results in poor biliary elimination of bilirubin glucuronides and drug substrates. Biliary elimination, because of enterohepatic circulation, could be usefully targeted to treat diseases in the enterohepatic system, such as Crohn's disease or liver cancers. Alternatively, renal elimination should be targeted for drugs that need to reach the systemic circulation. Overall, understanding the major routes of elimination can help predict drug-drug interactions, toxicities and pharmacokinetics during development, and may be useful in predicting substrates of efflux transporters in other tissues.

While human liver microsomes generally provide reliable predictions of human metabolic clearance for extensively metabolized drugs (8–13) and renal clearance is not difficult to determine, predictions of human biliary clearance are difficult and scarce, despite ongoing efforts (14). Clinical methods include bile duct cannulation during surgery, collection of duodenal fluid in healthy volunteers, or fecal collections. These procedures are difficult, uncommon, and variable. *In vitro* predictions can be carried out in sandwich-cultured rat or human hepatocytes, which preserve cell polarity and bile canaliculi (15,16), but uptake transporter expression is not well preserved in sandwich-cultured rat hepatocytes (17) and biliary clearance can be rate limited by uptake (18). Several studies have demonstrated that *in vitro* measures correlate with, but underpredict, *in vivo* biliary clearance (19–21). Bile duct cannulation in rats is often performed, but may not scale to humans, especially as rats have greater rates of bile flow (22) and canalicular efflux transport (23), as well as increased expression (24,25) of canalicular efflux transporters.

Historically, a molecular weight cut-off of 500–600 Da was proposed for minimizing biliary clearance in humans (26). Recently, Yang *et al.* published a model including 97 drugs, which as a part predicted that anionic drugs with molecular weights greater than 475 Da are likely to be significantly (>10% of parent dose) excreted in the bile (27).

While we initially consider only poorly metabolized drugs and drugs that can be administered orally, their dataset also included extensively metabolized drugs, as well as drugs that cannot be orally administered. Their study and others implicated hydrogen bond interactions (5,27), charge state (5,27,28), the presence of polar groups or a large polar surface area (5,27–30), and the presence of dipole or quadrupole moments (27,28), while others have implicated hydrophilicity (5,28,30), carboxylic acid groups (28,29), and rotatable bonds (5,29).

Since our objective was to predict when biliary excretion is a major route of elimination (>35% of parent dose), we initially did not consider extensively metabolized drugs, as their disposition is unlikely to be greatly affected by changes in biliary excretion. Wu and Benet (4) proposed the Biopharmaceutical Drug Disposition Classification System (BDDCS), which segregates highly metabolized (class 1 and 2) drugs in humans from those that are cleared primarily *via* renal or biliary routes (class 3 or 4). They noted that relatively few drugs have an extent of metabolism between 30% and 70%, and that high permeability drugs were extensively metabolized in humans, while low permeability drugs were primarily eliminated unchanged. The classification system makes predictions about transporter effects and disposition for drugs in each class, based on extent of metabolism and solubility. In particular, highly soluble and extensively metabolized class 1 drugs will not exhibit clinically relevant alterations in disposition due to transporters. Transporters may affect class 2 drugs, but their disposition changes would primarily reflect changes in metabolite formation and parent drugs are unlikely to be greatly affected by biliary excretion. Drugs that are significantly eliminated in the bile or urine fall within classes 3 and 4, and may exhibit altered disposition due to drug-drug interactions affecting transporters in the gut and/or liver. Benet *et al.* have compiled a dataset of over 900 drugs and provided the BDDCS class for each of these drugs (31).

Here, we combine BDDCS's observations about major routes of elimination with easily obtained urinary excretion data (see "METHODS") to characterize drugs significantly eliminated in the bile (>35% of available parent drug). As class 1 and 2 compounds exhibit less than 30% elimination into the bile and urine, biliary or renal excretion may only need to be evaluated for extensively metabolized drugs with a narrow therapeutic range. Indeed, Varma *et al.* (32) recently reported that drugs in their data set with MDCK permeabilities greater than 5×10^{-6} cm/s contribute less than 30% of parent drug to human renal elimination and are unlikely to be affected by renal DDIs, while rat biliary elimination and permeability were inversely related. Finally, low permeability compounds were highly represented as substrates of hepatic basolateral uptake transporters (32). Importantly, their data indicate that *in vitro* permeability rate can be used as a surrogate for extent of metabolism for new molecular entities (NMEs) when clinical data is unavailable, as has been proposed by our laboratory (31,33,34). Specifically, compounds with permeability rates equal to or exceeding a standard, *e.g.*, metoprolol, are likely extensively metabolized *in vivo* in humans, while those with permeability rates lower than the standard are likely eliminated primarily as unchanged drug in either the bile or the urine.

Lipinski *et al.* (35) published guidelines for predicting which drugs are likely to be absorbed upon oral dosing. However, these rules do not apply when transporters mediate the intestinal uptake of drugs, *i.e.*, class 3 and 4 drugs that are eliminated in the bile or the urine.

Here, we initially evaluate the molecular properties associated with significant biliary elimination of orally administered drugs. We then evaluate the importance of considering routes of administration (oral *versus* non-oral) and elimination when developing predictive models and discuss the interesting relationship between absorbed drugs that are eliminated in the bile and non-orally administered drugs, presumed as poorly absorbed, eliminated in either the bile or urine. We discuss a surprising and novel observation that poorly metabolized drugs can be classified by qualitative *in silico* predictions of CYP3A4 metabolism, and discuss the overlap in molecular properties of hepatically cleared compounds. The classification model outlined here can be applied to predict the major route of elimination of poorly metabolized drugs and provides guidelines to determine if a drug predicted to be poorly absorbed should be evaluated for active intestinal uptake.

METHODS

Dataset

BDDCS classification of 927 drugs was assigned by Benet *et al.* as previously described (31). Briefly, compounds were classified as highly soluble if the highest dose strength was soluble in 250 mL of water over the pH range of 1–7.5 at 37 °C. Compounds with greater than 70% metabolism in humans were classified as highly metabolized. From this dataset, we selected orally administered BDDCS class 3 and 4 drugs. Two clear outliers, tenofovir disoproxil, a prodrug, and vancomycin, which is rarely administered orally, were removed. Finally, drugs that fell into the primary excretion route criteria outlined below were selected for analysis, leaving a dataset of 105 drugs. An external dataset of 6 biliary eliminated and 24 renally eliminated drugs was developed by considering clinical data of orally administered BDDCS class 3 and 4 or poorly metabolized drugs that did not meet the initial criteria based on fraction excreted unchanged, but had clinical data supporting biliary or renal elimination.

Class 3 and 4 drugs were classified as primarily excreted renally, with no significant biliary contribution, or significantly excreted in the bile as follows:

$$\text{Total absorbed dose} = 100 = f_e + f_b + f_m$$

$$f_m < 30$$

$$70 < f_e + f_b$$

Here, f_e represents percentage of absorbed dose excreted unchanged in the urine, f_b represents percentage of absorbed dose excreted unchanged in the bile, and f_m represents percentage of absorbed dose eliminated *via* metabolism. Assuming less than 30% of the absorbed dose is metabolized for class 3 and 4 drugs allows calculation of the minimum amount of drug excreted in the bile. Therefore, class 3 and 4

drugs with 35% or less of the parent drug excreted unchanged in the urine are presumed to be significantly excreted in the bile ($\geq 35\%$ dose), while drugs with 65% or greater of the dose excreted unchanged in the urine are primarily excreted renally, with biliary elimination presumed to be insignificant. Drugs with $35 < f_e < 65$ were removed due to mathematical uncertainty of the significance of biliary excretion, since we wanted to initially operate on a set of drugs where preferential biliary or renal elimination were well differentiated. Drugs excreted in the bile were considered the positive class.

Model Creation

Using VolSurf+ (36–38) at pH=7.5 and default options, 128 descriptors and charge state at pH=7.5 were calculated for the dataset. Physicochemical properties were calculated in ADMET Predictor™ (39) with default settings at pH=7.4.

The open software R (40) was used for principal component analysis, partial least squares analysis (41), logistic regression (40), and receiver operating characteristic curves (42). The open machine-learning software Orange (43) was used for variable selection.

Principal component analysis of the VolSurf+ features was performed using the *stats* package in R. The data were scaled and centered. Scores for each component were obtained and compared between classification groups with the *t* test.

The number of variables was minimized to avoid overfitting the data and to physiologically interpret the results. Variables were ranked according to information gain, which is an algorithm that assesses the entropy a variable provides to the dataset, and the top 15 variables were selected for analysis. The classification accuracy, specificity, and sensitivity of variable combinations were assessed for Naïve Bayes, k-Nearest Neighbors (kNN), and logistic regression models with fivefold cross-validation by adding variables in order of information gain. Variables were left in the model if one or more of the evaluations (classification accuracy, specificity, or sensitivity) increased for one or more of the models. Optimal variable combinations were assessed with the VizRank tool in Orange with the following settings: six attributes, tenfold cross-validation of 100% of the dataset, and were evaluated by average accuracy in kNN. Variable selection data are not shown, as the methods were used only for variable reduction, and not model development and validation.

Partial least squares discriminant with scaling of all variables and six selected variables was performed using the *ppls* package in R (41). Models were validated by 10×5-fold cross-validation. Cross-validation training and test sets were randomized and stratified.

Logistic regression models were developed and used to define a decision boundary to predict drugs excreted in the bile from drugs excreted in the urine using the *stats* package in R (40). The default fitting characterized by iteratively reweighted least squares was employed. Models were validated by 10×5-fold cross-validation. Cross-validation training and test sets were randomized and stratified. An external dataset was collected, selected from compounds expressed in the literature as having significant biliary or renal elimination, but which were not available in the BDDCS classified compounds, or did not meet our initial ($f_e \leq 35$ or $f_e \geq 65$), which was developed to instill certainty in our classifications.

Receiver operating characteristic (ROC) plots were created in the ROCR (42) package in R. The true positive rate was plotted against the false positive rate and an area under the ROC curve (AUC) obtained. Thresholds of each model depicting optimal separation between classes were defined at the minimum distance to the ROC curve from (0, 1) where sensitivity and specificity were each greater than 0.8. Drugs were assigned class predictions by considering the value of the feature or model evaluator of a drug in relation to the threshold, and sensitivity

$\frac{TP}{TP+FN}$, specificity $\frac{TN}{TN+FP}$, positive predictive value (PPV) $\frac{TP}{TP+FP}$, negative predictive value (NPV) $\frac{TN}{TN+FN}$, and accuracy $\frac{TP+TN}{TP+TN+FP+FN}$ were calculated, where TP represents true positives, FP represents false positives, TN represents true negatives, and FN represents false negatives.

Additional Considerations

Models trained initially on orally administered drugs were tested with non-orally administered drugs. A model encompassing all routes of administration was created. Differences in physicochemical properties between orally administered and non-orally administered drugs were detected with principal component analysis.

P-gp substrate data were collected from Broccatelli's dataset (4) and compared with renally and biliary eliminated drugs. The search was extended to other sources for biliary eliminated drugs (44–51). Drugs were considered nonsubstrates for efflux ratios <1.8 and substrates if the efflux ratio was >2.2.

RESULTS

From the dataset, 105 of 188 orally administered class 3 and 4 drugs met the primary excretion class criteria. Of these, 27 were significantly excreted in the bile and 78 were primarily excreted in the urine. Categorized by charge at pH 7.5, 29 drugs were anionic, 26 were cationic, 33 were neutral, and 17 were zwitterionic. It was noted during analysis that ranitidine was listed in the database with a fraction excreted unchanged in the urine of 30, but the correct value is 69, and this adjustment was made (52).

Principal component analysis including all features revealed a clear segregation between the excretion classes and there was a significant difference between biliary and renally eliminated drugs along the first component ($p < 1e^{-9}$) (Fig. 1).

Feature Selection

The following features from VolSurf+ were selected for evaluation: molecular weight (MW); metabolic stability (MetStab), a calculated prediction of the percent of parent drug remaining after metabolism in CYP3A4 supersomes; intrinsic solubility (SOLY); polarizability (POL), which describes the extent to which a molecule can form an induced dipole in an electric field (53); hydrophobic surface area (HSA); and rugosity (RUG), a ratio of molecular volume to surface area. The parameter values for MW, SOLY, POL, HSA, and RUG were the generally accepted units as follows: MW: Da, SOLY: mol/L at 25°C, POL: Å²s⁴/kg, HSA: Å², and RUG: Å, while MetStab

Fig. 1. First two principal components including information from all features calculated by VolSurf+. The first two components contributed 0.457 cumulative variance and the first-component scores were significantly different between elimination routes ($p < 1e^{-09}$)

ranges from 0 to 100%. All of the features except MetStab were highly correlated with each other (Pearson's R values >0.8) (Supplementary Table I). The following features were selected in ADMET Predictor: natural population analysis partial charge on hydrogens (NPAh), number of CYP Sites (NCYPSites), and LogD (pH=7.4).

Models

Partial Least Squares Discriminant

MetStab+POL was 92.5±0.1% accurate in 10×5-fold cross-validation and was more accurate than other models ($p < 0.01$). Table I highlights the performance of the model testing sets.

Logistic Regression

Logistic regression resulted in slightly lower performance, but is more appropriate for classification problems and was selected as the primary model. Sensitivity, specificity, and accuracy exceeded 0.8 for all models, except NCYPSites+POL (designated as PolG in ADMET Predictor), which was developed as a comparative model (Table I). Logistic regression, when molecular polarizability and calculated metabolic stability are considered, predicts the probability of biliary elimination, given by:

$$\Pi(x) = \frac{1}{e^{-(0.217POL - 0.0745MetStab - 2.28)} + 1}$$

and the optimal threshold predicts biliary elimination when $\Pi(x) > 0.237$. This can be transformed into the linear equation depicted as a decision boundary in Fig. 2: $0 = 0.344 \times MetStab - POL + 5.14$. When the combination of MetStab and POL gives a result <0, the compound is predicted to be eliminated in the bile. From the external dataset collected after review, 6/6

Table I. Performance Statistics of Top Logistic Regression or Partial Least Squares Models Developed Using VolSurf+ or ADMET Predictor™ Parameters Validated by 10×5-Fold Cross-Validation

Model ^a	Sensitivity	Specificity	PPV ^b	NPV ^b	Accuracy	AUC
MetStab+POL (PLS)	0.90±0.10	0.93±0.06	0.84±0.12	0.97±0.04	0.92±0.05	0.95±0.04
MetStab+POL (LogReg)	0.85±0.14	0.87±0.09 +++	0.73±0.15 +++	0.95±0.05	0.87±0.08 +++	0.94±0.05
MetStab+MW (PLS)	0.86±0.15	0.88±0.08 +++	0.72±0.14 +++	0.95±0.05	0.87±0.07 +++	0.94±0.04
MetStab+MW (LogReg)	0.81±0.15 +++	0.86±0.08 +++	0.68±0.13 +++	0.93±0.05 +++	0.85±0.07 +++	0.93±0.05
NCYPSites+PolG	0.75±0.17 +++	0.84±0.11 +++	0.65±0.17 +++	0.91±0.07 +++	0.82±0.10 +++	0.90±0.08 +++
NPAh+LogD	0.81±0.18 +++	0.90±0.08	0.77±0.17	0.93±0.06 +++	0.88±0.08 +++	0.90±0.08 +++
NPAh+LogD+NCYPSites	0.80±0.17 +++	0.91±0.08	0.79±0.17	0.93±0.06 +++	0.88±0.08 ++	0.90±0.09 +++

Compared to MetStab+POL (PLS): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

^a MetStab metabolic stability, POL or PolG polarizability, MW molecular weight, NCYPSites number of CYP sites on the molecule, LogD distribution coefficient at pH=7.4, NPAh natural population analysis partial charge on hydrogens

^b PPV positive predictive value, NPV negative predictive value. The threshold represents where optimal average between sensitivity and specificity occurs

biliarily eliminated compounds (100% sensitivity) and 19/24 renally eliminated compounds (79.2% specificity) were correctly predicted, resulting in 83% accuracy overall (Supplementary Table III).

The predictive ability of individual variables was assessed with ROC plot analysis (Supplementary Table II and Supplementary Fig. 1). Boxplots assessed the distribution of descriptors within each excretion class (Fig. 3). The minimum predicted metabolic stability observed in a renally eliminated drug was 71.8% (levocetirizine), while norfloxacin and leucovorin, drugs eliminated in the bile, were predicted to be 100% metabolically stable. The median weight of drugs excreted in the bile was 434 Da, with a lower limit of 288 Da. The median weight of drugs excreted in the urine was 282 Da, with an upper limit of 461 Da.

Although historically biliary excretion was predicted for high molecular weight anionic drugs (26,27), segregating drugs into ionization classes provided somewhat better

performance of MW as a predictor of excretion class for cationic, neutral and zwitterionic compounds compared to anionic compounds (Table II).

Models Including Non-orally Administered Drugs

Using the same methods outlined for orally administered drugs, PLS models were developed that included non-orally administered drugs only or all administration routes, but satisfactory performance was not achieved. Significant differences were observed between the PCA first-component scores of orally and non-orally administered drugs ($p < 1e-06$). The distributions of the variables selected to represent the differences, largely indicative of hydrophilicity/lipophilicity, size/shape, or permeability, are depicted in Supplementary Figure 2.

Eight of 27 orally administered, biliarily cleared and 1/78 renally cleared (methotrexate) drugs violate Lipinski's Rule of Five. Alternatively, 4/11 biliarily eliminated compounds and 17/49 renally eliminated drugs given *via* the intravenous route violated the Rule of Five.

Clinical Validation of the Classification Scheme and Transporter Effect

Our classification system was compared to clinical data from Yang *et al.* (27) Ten of the 11 drugs falling within our selection criteria (BDDCS Class 3 or 4, orally administered, with $f_e \leq 35$ or $f_e \geq 65$ depicting biliary or renal elimination, respectively) were in agreement with the clinical classifications. To further validate that BDDCS classification and low f_e indicate biliary elimination, we extended the search for clinical data of biliary elimination. In total, there were 18 drugs that we classified as biliarily excreted for which clinical information provided some indication of presence or lack of biliary elimination. Fifteen of these drugs (83%) indicated likely biliary excretion from clinical data.

Fig. 2. Calculated metabolic stability and polarizability of 105 orally administered, poorly metabolized drugs. The decision boundary represents the average threshold ($\Pi(x) = 0.237$) of best average sensitivity and specificity of the training group that predicts the probability of biliary excretion. It is represented by: $0 = 0.344 \times \text{MetStab} - \text{POL} + 5.14$

Fig. 3. Boxplots of the selected variables, model, and external validation of the model such that the box represents the values between the 25th and 75th percentile and the median. Tukey-defined extremes are represented by the whiskers and outliers are represented as individual datapoints

Efflux data were found for 16 of the 27 biliary eliminated, orally administered compounds. Twelve of these 16 drugs were P-gp substrates. Of the remaining four, two were MRP2 substrates, and one was a BCRP substrate. Six of 15 renally eliminated drugs present in the Broccatelli dataset were P-gp substrates and nine were nonsubstrates. Thirty-seven compounds in our orally administered

dataset had $S < 400 \text{ \AA}^2$ and were all excreted renally. Calculated metabolic stability is significantly lower ($p < 0.0001$) for P-gp substrates in this subset (Fig. 4).

Additional Considerations

Projecting Non-orally Administered Drugs on the Model

Non-orally dosed drugs tested on the MetStab+POL logistic regression model developed for orally administered drugs yielded AUC=0.659, sensitivity=0.889, specificity=0.429, and accuracy=0.541. AUC determined for POL was 0.818 when all administration routes were considered, but drops to 0.671 when only non-orally dosed drugs are considered. The AUC for MetStab of orally and non-orally dosed compounds is 0.806, while the AUC of non-orally dosed drugs is 0.673. Figure 5 depicts the metabolic stability and molecular weight of orally and non-orally administered drugs.

Table II. AUC of ROC Curve Representing Molecular Weight When Orally Administered Compounds Were Segregated into Ionization State at pH 7.5

	AUC	Accuracy
Anion	0.858	0.759
Cation	0.917	0.846
Neutral	0.957	0.970
Zwitter	0.808	0.824

Predicting Extensively Metabolized Drugs on the Biliary and Renal Excretion Discriminating Model

When projected on the MetStab+POL logistic regression and PLS models, respectively, $70.0 \pm 5.6\%$ and $73.2 \pm 0.4\%$ of extensively metabolized parent drugs were predicted as eliminated in the bile. The AUC of MetStab as an indicator of biliary or metabolic elimination was 0.478 and the p value of the t test was 0.710. Figure 5 depicts the metabolic stability and molecular weight of drugs by major routes of elimination.

Applicability to Other Software

ADMET Predictor™ has metabolic features including intrinsic clearance for various CYP isoforms, as well as “number of CYP atoms” and “number of CYP sites”. The AUC for the number of CYP sites (NCYPsites) predicting primary route of elimination on the subset of poorly metabolized drugs was 0.858. The number of CYP atoms and CYP sites correlated with MetStab (Pearson's $R=0.725$ and 0.712, respectively). Polarizability calculations were reproducible in ADMET Predictor (Pearson's $R>0.99$). The number of CYP sites is correlated with MW (Pearson's $R=0.821$). Table I depicts the models' performance.

DISCUSSION

Classification Scheme

We classified the major route of elimination of drugs using easily obtained and reliable urinary excretion data (f_e), initially filtered by removing highly permeable/extensively metabolized BDDCS class 1 and 2 drugs. Our classification scheme reliably identifies which poorly permeable/poorly metabolized drugs are eliminated in the bile, independent of biliary excretion data. In fact, of the 11 drugs for which direct comparison (orally administered, BDDCS class 3 and 4, $35 \leq f_e$ or $f_e \geq 65$) with the human dataset compiled by Yang *et al.* (27) was possible, all classifications except methotrexate were in agreement, but this one discordant classification is expected. While we considered methotrexate's primary route of elimination as renal ($f_e=81$), Yang *et al.* (27) classified this drug as having significant biliary elimination, since this group utilized $>10\%$ biliary elimination as the criteria for significant elimination by this route. However, measurements of parent

methotrexate eliminated in the bile range from 3 to 26%, so variations in classification are expected (27,54–57). Of the drugs we defined as eliminated in the bile using BDDCS class and $f_e \leq 35$, 83% agreed with available clinical data (Supplementary Table III). We expected agreement with clinical data, as BDDCS class 3 and 4 drugs attribute $<30\%$ of their disposition to metabolism, the fraction excreted as unchanged drug in the urine was known, and other routes of elimination only impact a small number of drugs. This demonstrates the utility of BDDCS to characterize the major routes of elimination when permeability/extent of metabolism and the fraction excreted unchanged in the urine are known.

Application to New Molecular Entities

Prior to any studies in animals or humans for an NME, *in vitro* permeability data, as demonstrated by Varma *et al.* (32) and initially proposed by our laboratory (4,33,34), can identify which drugs are primarily eliminated by metabolic or non-metabolic (biliary, renal) routes. Highly permeable drugs are likely extensively metabolized *in vivo*. Of the poorly permeable, poorly metabolized drugs, consideration of metabolic stability and polarizability may be applied to predict the primary route of excretion (biliary or renal), using the relationship defined below and depicted in Fig. 2, such that (MetStab, POL) combinations above the line are predicted as eliminated in the bile, while combinations below the line are predicted as eliminated in the urine. The probability of biliary elimination is given by $\Pi(x) = \frac{1}{e^{-(0.217\text{POL} - 0.0745\text{MetStab} - 2.28)} + 1}$ ($p < 1 \times 10^{-11}$) and predicts biliary elimination when $\Pi(x) > 0.237$. This equation can be transposed into a linear equation depicted as a decision boundary in Fig. 2 using the optimal threshold of $\Pi(x) = 0.237$. Our external dataset was collected and tested with 83% accuracy, indicating that this model can be applied to compounds that were not included in the dataset and may perform well on NMEs.

Importance of Metabolic Stability

The VolSurf+ calculated metabolic stability model was created from a PLS of 94 parameters calculated in VolSurf+ and was initially created using *in vitro* data from 1800 compounds incubated with CYP3A4 human cDNA microsomes. The value of calculated MetStab represents the fraction of a drug predicted to remain unmetabolized by CYP3A4 *in vitro*. These calculations have been validated, and accurately predict the *in vitro* metabolic stability of 85% of the tested drugs (58). With a large number of parameters contributing to the model, individual descriptors do not contribute greatly, but trends of lipophilicity and size appear to drive the MetStab model. Metabolically unstable compounds tend to have wide hydrophobic interactions, high amphiphilicity, as well as high polarizability and size, and hydrogen bond acceptor groups. Stable compounds, on the other hand, tend to have dense polar regions, large polar surfaces, and high hydrophilic-lipophilic balances, descriptors that largely indicate that stable compounds are more hydrophilic than unstable compounds (58). With no significant difference in MetStab between metabolized and biliary eliminated compounds (Fig. 5b), we believe that this term

Fig. 4. Boxplots of calculated metabolic stability of BDDCS Class 3 and 4 P-gp substrates and nonsubstrates. The box represents the values between the 25th and 75th percentile and the median. Tukey-defined extremes are represented by the whiskers and outliers are represented as individual datapoints

Fig. 5. Distribution of calculated molecular weight and metabolic stability of orally administered drugs by major route of elimination (a and b) or by route of administration (c and d)

reflects hepatic access. Specifically, we expect that this finding could be explained by one or more of the following phenomena: 1) biliary eliminated compounds with low MetStab may be substrates of metabolizing enzymes *in vitro*, but are stronger substrates of transporters *in vivo* and therefore are eliminated unchanged in the bile, 2) as 70% of metabolized compounds were predicted as eliminated in the bile, we expect many metabolized compounds are in fact partially biliary eliminated *in vivo*, but a third condition, high permeability, allows the drug to be reabsorbed into hepatocytes, such that hepatic metabolism is the ultimate method of elimination, and/or 3) low metabolic stability predicts which class 3 and 4 drugs are P-gp substrates.

Predictions of extensively metabolized drugs in a naïve dataset were heavily skewed toward biliary elimination, partially due to overlapping MetStab (Fig. 5b). Some studies have identified compounds that are metabolically unstable *in vitro*, but are primarily eliminated as unchanged drug *in vivo* (59,60). Our data indicate that this may be because biliary eliminated drugs may be metabolically unstable CYP substrates *in vitro*, but competition with transporters on the canalicular membrane *in vivo* partially determines the molecule's fate.

Permeability of hepatically available compounds likely plays a great role in the observed disposition of a drug. Highly permeable/extensively metabolized drugs may be capable of being partially eliminated in the bile initially, but are sufficiently permeable to be reabsorbed into hepatocytes, resulting in low excretion of unchanged drug, but extensive metabolism *in vivo*. Gustafson and Benet showed that 46% of a phenolphthalein glucuronide dose administered directly to the bile duct in cannulated rats was available in the plasma,

indicating the possibility of molecular reabsorption from the bile duct (61). The same phenomenon is predicted with highly permeable drugs initially filtered or secreted in the urine (33), following the observation that extensively metabolized drugs show high permeability rates.

The final hypothesis is based on the well-known substrate overlap between CYP3A4 and P-gp (62). Although the MetStab model was developed to predict CYP3A4 substrates, considering the intrinsic overlap of substrates, low MetStab may also tend to predict affinity for P-gp. Correspondingly, we saw that class 3 and class 4 P-gp substrates had low calculated MetStab (predicted *in vitro* CYP3A4 substrate) (Fig. 4) while nonsubstrates had significantly higher MetStab ($p < 1 \times 10^{-4}$). To point, compounds that were incorrectly predicted by the model in part due to a MetStab value uncharacteristic of the compound's excretion route were either substrates of hepatic canalicular efflux transporters besides P-gp (norfloxacin is a substrate of BCRP) or were P-gp substrates and renally eliminated (acrivastine, levocetirizine). Renally eliminated compounds that are substrates for P-gp, which is present in both the liver and kidney among many other tissues, might not be substrates for hepatic uptake transporters, and are therefore not biliary accessible. As others have hypothesized, hepatic uptake, particularly by OATPs, may be a rate-limiting step in biliary excretion (5,8). Indeed, acrivastine and levocetirizine were not found to be substrates of major hepatic uptake transporters in the literature.

We believe that each of these previously noted observations play a role in the surprising finding that biliary eliminated compounds are predicted to be metabolically unstable.

Polarizability and MW

Polarizability, which is highly correlated with MW, was the best secondary predictor, and may describe the physiological recognition of biliary eliminated compounds better than the conventional molecular weight (Supplementary Table II). This simple property quantifies the ability of the molecule to distort its own electron density when interacting with other molecular entities; polarizability is therefore a measure of the non-specific weak intramolecular dispersion forces and has been shown to correlate with a number of biological properties by Hansch and Kurup (63), and has been found to contribute to biliary excretion by others (28,30). Polarizability could account for non-specific weak interactions between a drug molecule and transporter proteins (64). Highly polarizable molecules may be more apt to interact with hepatic uptake and efflux transporters on hepatic membranes.

Historical and Current Relevance of Molecular Weight and Correlated Features

Drugs eliminated in the bile tend to have a high POL, MW, RUG, and HSA, or a low SOLY compared to renally cleared compounds (Fig. 3). Physiologically, these descriptors may predict that hydrophilic molecules are more likely to remain in blood and less likely to partition into the basolateral membranes of hepatocytes. Small hydrophilic molecules may bypass hepatic elimination and be filtered through the glomerulus at the kidney.

As historically proposed, molecular weight may be a reliable surrogate for physiological processes that contribute to the excretion route of a poorly metabolized/poorly permeable drugs (Fig. 3 and Supplementary Table II). In our dataset, this occurs regardless of charge state (Table II). Predicting that poorly metabolized, orally administered drugs with a MW > 380 Da are significantly eliminated in the bile, while those with a MW < 380 Da are renally eliminated will correctly predict the excretion route greater than 80% of the time. Poorly permeable, orally administered drugs with a MW < 288 Da will almost certainly be eliminated in the urine, while those with MW > 462 Da will almost certainly be eliminated in the bile. This is similar to the proposed cut-off for anions at 475 Da by Yang *et al.* (27).

We believe we are the first to emphasize that these molecular weight cutoffs only apply for orally administered,

poorly metabolized drugs (*i.e.*, BDDCS classes 3 and 4). As we and others (27) have demonstrated, low molecular weight compounds will largely be eliminated by extra-biliary routes no matter the route of administration or BDDCS class. However, there are many high molecular weight compounds that are extensively metabolized (Fig. 5a) or non-orally administered, renally eliminated drugs (Fig. 5c). These compounds represent false positives that overwhelm the true positives when using high MW as a basis of predicting biliary elimination (Table III). Demonstrating this principle, tiotropium bromide, a renally eliminated compound, was incorrectly classified as biliary eliminated by the model, but upon investigation, was incorrectly classified as orally administered (31) and is actually an inhaled drug. Additionally, metabolized drugs account for 73% of drugs on the market and 72% of NMEs (33), and the proportion of high molecular weight drugs is skewed toward metabolism. While the MW of extensively metabolized drugs slightly increases over the years, as defined by CAS number (Pearson's $R=0.38$), the MW of biliary excreted drugs is not changing over time (Pearson's $R=0.05$).

Additional Considerations

Active Efflux in Biliary Excretion

We hypothesize that active transport results from both necessity (highly protein-bound compounds cannot be passively filtered) and convenience (unbound drugs with high POL may be good *in vivo* transporter substrates). P-gp is a promiscuous transporter that is expressed on the bile canalicular membrane and in various other tissues. Due to its promiscuity and a relative deficit of known substrates for other ABC transporters, we hypothesize that many biliary eliminated drugs must be substrates of P-gp. Available data of P-gp substrates indicate that this is true, and that active efflux is a mandatory process of biliary elimination, excepting saxagliptin. Further investigation indicated that the f_e may have been incorrectly reported for saxagliptin, as the bioavailability was not reported (65). Unsurprisingly, P-gp efflux does not overwhelm the transport of renally eliminated drugs. Poorly permeable, poorly metabolized drugs that are not substrates for P-gp or other hepatic efflux transporters are likely eliminated renally. As biliary eliminated compounds are poorly permeable, as predicted by BDDCS, and observed by others (32), we expect that hepatic uptake must be an active process. Varma *et al.* showed the overlap in physicochemical properties between drugs that were biliary eliminated in rats and drugs that are substrates for human OATP, including high MW (5). As biliary cleared drugs have high MW/POL, they may be substrates for OATPs, while many renally cleared drugs may not be. While a number of the drugs that we classified as biliary eliminated are P-gp substrates and there are many biliary eliminated compounds that are substrates for OATP, other transporters can also play a role, and, although they were not investigated, may correlate with properties such as high polarizability and low metabolic stability.

Administration Route

Physicochemical differences exist between orally and non-orally dosed drugs and considering the groups together

Table III. Population of Compounds in Molecular Weight Groups by Route of Administration and Elimination

	Molecular weight (Da)			
	>380	<380	>475	<475
Major elimination route	<i>Oral administration</i>			
Biliary	22	5	13	14
Renal	13	65	0	78
Metabolism	153	345	53	445
	<i>Non-oral administration</i>			
Biliary	11	1	7	5
Renal	42	21	21	42
Metabolism	42	50	29	63

can confound model predictions. Specifically, orally administered drugs are more permeable and lipophilic, while non-orally administered drugs tend to be more polar, hydrophilic, and larger (Supplementary Fig. 2). Specifically, high MW/high POL appears necessary for biliary elimination, but low MW/low POL is not necessarily indicative of renal elimination, particularly when a drug is not orally administered. The glomerulus begins filtering out molecules when MW > 10,000 Da (66) and thus, molecular size of unbound small molecule drugs may be unimportant at the kidney. Instead, protein binding may be a key deciding factor of elimination route at the kidney. Protein-bound compounds cannot be filtered through the glomerulus (albumin MW = 67,000 Da) and passively eliminated by the kidney, so will require active transport into an eliminating organ. We hypothesize that, by requiring an active process, many of these highly protein-bound compounds would be eliminated in the bile. We noticed that biliary eliminated compounds were indeed more highly bound to plasma proteins than renally cleared compounds, for both orally and non-orally administered compounds (Supplementary Figure 3).

Almost 1/3 of biliary excreted drugs that are orally administered, and therefore presumed to be reasonably well-absorbed, violate Lipinski's Rule of Five. These rules do not predict oral absorption when transporters mediate absorption, which is presumed to always be true for poorly permeable drugs, including those eliminated in the bile (33). Our model suggests that prior to dosing a NME to animals or humans, the major unknown in predicting whether biliary or renal excretion will be the major route of excretion of the NME, will be knowledge of whether the compound can achieve its desired effects following oral dosing. If oral dosing is feasible, the accuracy of the prediction of biliary *versus* renal excretion should be quite good. There would be less confidence in this prediction if oral dosing is not feasible. However, as noted above in characterizing drug disposition in humans, probably the most difficult mechanism to define is the differentiation between poor absorption and biliary excretion of parent drug. This is, however, the issue addressed here. Therefore, we recommend that drugs predicted to be poorly absorbed using Lipinski's Rule of Five be evaluated for intestinal uptake.

Charge Status

Although other groups have suggested that charged groups play a role in biliary elimination (5,27,28), our data indicate that charge is a relatively unimportant factor distinguishing primary modes of elimination, perhaps because transporters exist for each charged state in both the kidney and the liver and charge is not a limiting factor for active transport. For instance, OATPs can transport anions, amphipathic compounds, and some cations, while OCTs, OCTNs, and MATEs specifically transport cations and OATs specifically transport anions. The efflux transporter MDR1 can transport both cationic and amphipathic compounds, while MDR3, MRPs, and BCRP are responsible for transporting anions. Interestingly, cations exhibit the greatest range of molecular weights, including the lowest and highest MW for drugs eliminated in the bile. This may be a result of P-gp

efflux into the bile, which is well known to be a promiscuous transporter.

CONCLUSIONS

- We have developed a novel classification scheme and model predicting significant biliary excretion. This model does not rely on unreliable animal models and is not limited by scantily available human biliary excretion data. This model is supported by analyses developed from *in vivo* data.
- The model proposed here takes advantage of the BDDCS system, which allows identification and classification of highly metabolized (high permeability rate) drugs *versus* poorly metabolized (low permeability rate) drugs. Biliary and renal elimination of unchanged drug will not be significant for high permeability compounds. Thus, the methodology here is useful for differentiating biliary *versus* renal elimination for poorly metabolized/poorly permeable BDDCS class 3 and 4 drugs.
- We show that *in silico* determinations of metabolic stability may provide a simple mechanism for predicting significant biliary elimination, especially when co-employed with polarizability.
- This model, utilizing polarizability and metabolic stability, can be applied to new molecular entities to predict the major route of elimination when the extent of metabolism is known or predicted from *in vitro* permeability data, but its accuracy will be poorer for NMEs that cannot be dosed orally.
- Compounds that violate Lipinski's Rule of Five should be evaluated for intestinal uptake, as these compounds may be well-absorbed and eliminated in the bile.

ACKNOWLEDGMENTS

We would like to thank Dr. Julian Blagg at the Institute of Cancer Research for critical review of the manuscript, Dr. Gabriele Cruciani and Molecular Discovery Ltd. for generously supplying the VolSurf+ license, and Dr. Michael Bolger and Simulations Plus for generously supplying the ADMET Predictor™ license. Chelsea Hosey was supported in part by NIH Training Grant T32 GM007175.

REFERENCES

1. Grime K, Paine SW. Species differences in biliary clearance and possible relevance of hepatic uptake and efflux transporters involvement. *Drug Metab Dispos.* 2013;41:372–8. doi:10.1124/dmd.112.049312.
2. Simonson SG, Raza A, Martin PD, Mitchell PD, Jarcho JA, Brown CD, *et al.* Rosuvastatin pharmacokinetics in heart transplant recipients administered an antirejection regimen including cyclosporine. *Clin Pharmacol Ther.* 2004;76:167–77. doi:10.1016/j.clpt.2004.03.010.
3. Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, *et al.* Membrane transporters in drug development. *Nat Rev Drug Discov.* 2010;9:215–36. doi:10.1038/nrd3028.

4. Wu CY, Benet LZ. Predicting drug disposition via application of BCS: transport/absorption/elimination interplay and development of a biopharmaceutics drug disposition classification system. *Pharm Res.* 2005;22:11–23. doi:10.1007/s1095-004-9004-4.
5. Varma MVS, Chang G, Lai Y, Feng B, El-Kattan AF, Litchfield J, *et al.* Physicochemical property space of hepatobiliary transport and computational models for predicting rat biliary excretion. *Drug Metab Dispos.* 2012;40:1527–37. doi:10.1124/dmd.112.044628.
6. Broccatelli F. QSAR models for P-glycoprotein transport based on a highly consistent data set. *J Chem Inf Model.* 2012;52:2462–70. doi:10.1021/ci3002809.
7. Roberts MS, Magnusson BM, Burczynski FJ, Weiss M. Enterohepatic circulation: physiological, pharmacokinetic and clinical implications. *Clin Pharmacokinet.* 2002;41:751–90.
8. Watanabe T, Kusuhara H, Maeda K, Kanamaru H, Saito Y, Hu Z, *et al.* Investigation of the rate-determining process in the hepatic elimination of HMG-CoA reductase inhibitors in rats and humans. *Drug Metab Dispos.* 2010;38:215–22. doi:10.1124/dmd.109.030254.
9. Iwatsubo T, Hirota N, Ooie T, Suzuki H, Shimada N, Chiba K, *et al.* Prediction of *in vivo* drug metabolism in the human liver from *in vitro* metabolism data. *Pharmacol Ther.* 1997;73:147–71. doi:10.1016/S0163-7258(96)00184-2.
10. Obach RS. Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: an examination of *in vitro* half-life approach and nonspecific binding to microsomes. *Drug Metab Dispos.* 1999;27:1350–9.
11. Naritomi Y, Terashita S, Kimura S, Suzuki A, Kagayama A, Sugiyama Y. Prediction of human hepatic clearance from *in vivo* animal experiments and *in vitro* metabolic studies with liver microsomes from animals and humans. *Drug Metab Dispos.* 2001;29:1316–24.
12. Stringer R, Nicklin PL, Houston JB. Reliability of human cryopreserved hepatocytes and liver microsomes as *in vitro* systems to predict metabolic clearance. *Xenobiotica.* 2008;38:1313–29. doi:10.1080/00498250802446286.
13. Kilford PJ, Stringer R, Sohal B, Houston JB, Galetin A. Prediction of drug clearance by glucuronidation from *in vitro* data: use of combined cytochrome P450 and UDP-glucuronosyl-transferase cofactors in alamethicin-activated human liver microsomes. *Drug Metab Dispos.* 2009;37:82–9. doi:10.1124/dmd.108.023853.
14. Ghibellini G, Leslie EM, Brouwer KLR. Methods to evaluate biliary excretion of drugs in humans: an updated review. *Mol Pharm.* 2006;3:198–211. doi:10.1021/mp060011k.
15. LeCluyse EL, Audus KL, Hochman JH. Formation of extensive canalicular networks by rat hepatocytes cultured in collagen-sandwich configuration. *Am J Physiol Cell Physiol.* 1994;266:C1764–6.
16. Liu X, Brouwer KLR, Gan LSL, Brouwer KR, Stieger B, Meier P, *et al.* Partial maintenance of taurocholate uptake by adult rat hepatocytes cultured in a collagen sandwich configuration. *Pharm Res.* 1998;15:1533–9.
17. Kotani N, Maeda K, Watanabe T, Hiramatsu M, Gong LK, Bi YA, *et al.* Culture period-dependent changes in the uptake of transporter substrates in sandwich-cultured rat and human hepatocytes. *Drug Metab Dispos.* 2011;39:1503–10. doi:10.1124/dmd.111.038968.
18. Kusuhara H, Sugiyama Y. Pharmacokinetic modeling of the hepatobiliary transport mediated by cooperation of uptake and efflux transporters. *Drug Metab Rev.* 2010;42:539–50. doi:10.3109/03602530903491824.
19. Abe K, Bridges AS, Yue W, Brouwer KL. *In vitro* biliary clearance of angiotensin II receptor blockers and 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors in sandwich-cultured rat hepatocytes: comparison with *in vivo* biliary clearance. *J Pharmacol Exp Ther.* 2008;326:983–90. doi:10.1124/jpet.108.138073.
20. Abe K, Bridges AS, Brouwer KL. Use of sandwich-cultured human hepatocytes to predict biliary clearance of angiotensin II receptor blockers and HMG-CoA reductase inhibitors. *Drug Metab Dispos.* 2009;37:447–52. doi:10.1124/dmd.108.023465.
21. Fukuda H, Ohashi R, Tsuda-Tsukimoto M, Tamai I. Effect of plasma protein binding on *in vitro-in vivo* correlation of biliary excretion of drugs evaluated by sandwich-cultured rat hepatocytes. *Drug Metab Dispos.* 2008;36:1275–82. doi:10.1124/dmd.107.019026.
22. Davies B, Morris T. Physiological parameters in laboratory animals and humans. *Pharm Res.* 1993;10:1093–6.
23. Li M, Yuan H, Lin N, Song G, Zheng Y, Baratta M, *et al.* Identification of interspecies difference in efflux transporters of hepatocytes from dog, rat, monkey, and human. *Eur J Pharm Sci.* 2008;35:114–26. doi:10.1016/j.ejps.2008.06.008.
24. Li N, Zhang Y, Hua F, Lai Y. Absolute difference of hepatobiliary transporter multidrug resistance-associated protein (MRP2/MRP2) in liver tissues and isolated hepatocytes from rat, dog, monkey, and human. *Drug Metab Dispos.* 2009;31:66–73. doi:10.1124/dmd.108.023234.
25. Li N, Palandra J, Nemirowskiy O, Lai Y. LC-MS/MS mediated absolute quantification and comparison of bile salt export pump and breast cancer resistance protein in livers and hepatocytes across species. *Anal Chem.* 2009;81:2251–9. doi:10.1021/ac8024009.
26. Millburn P. Factors in the biliary excretion of organic compounds. In: Fishman WH, editor. *Metabolic conjugation and metabolic hydrolysis.* New York: Academic; 1970. p. 1–70.
27. Yang X, Gandhi YA, Duignan DB, Morris ME. Prediction of biliary excretion in rats and humans using molecular weight and quantitative structure–pharmacokinetic relationships. *AAPS J.* 2009;11:511–25. doi:10.1208/s12248-009-9124-1.
28. Sharifi M, Ghafourian T. Estimation of biliary excretion of foreign compounds using properties of molecular structure. *AAPS J.* 2014;16:65–77. doi:10.1208/s12248-013-9541-z.
29. Luo G, Johnson S, Hsueh MM, Zheng J, Cai H, Xin B, *et al.* In silico prediction of biliary excretion of drugs based on physicochemical properties. *Drug Metab Dispos.* 2010;38:422–30. doi:10.1124/dmd.108.026260.
30. Chen Y, Cameron K, Guzman-Perez A, Perry D, Li D, Gao H. Structure-pharmacokinetic relationship of *in vivo* rat biliary excretion. *Biopharm Drug Dispos.* 2010;31:82–90. doi:10.1002/bdd.692.
31. Benet LZ, Broccatelli F, Oprea TI. BDDCS applied to over 900 drugs. *AAPS J.* 2011;13:519–47. doi:10.1208/s12248-011-9290-9.
32. Varma MV, Gardner I, Steyn SJ, Nkansah P, Rotter CJ, Whitney-Pickett C, *et al.* pH-dependent solubility and permeability criteria for provisional biopharmaceutics classification (BCS and BDDCS) in early drug discovery. *Mol Pharm.* 2012;9:1199–212. doi:10.1021/mp2004912.
33. Benet LZ. The role of BCS (biopharmaceutics classification system) and BDDCS (biopharmaceutics drug disposition classification system) in drug development. *J Pharm Sci.* 2013;102:34–42. doi:10.1002/jps.23359.
34. Larregieu CA, Benet LZ. Drug discovery and regulatory considerations for improving *in silico* and *in vitro* predictions that use Caco-2 as a surrogate for human intestinal permeability measurements. *AAPS J.* 2013;15:483–97. doi:10.1208/s12248-013-9456-8.
35. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev.* 1997;23:3–25. doi:10.1016/S0169-409X(96)00423-1.
36. VolSurf+ manual (<http://www.moldiscovery.com>)
37. Cruciani G, Crivori P, Carrupt P-A, Testa B. Molecular fields in quantitative structure-permeability relationships: the VolSurf approach. *J Mol Struct.* 2000;503:17–30. doi:10.1016/S0166-1280(99)00360-7.
38. Cruciani G, Crivori P, Carrupt P-A, Testa B. Predicting blood-brain barrier permeation from three-dimensional molecular structure. *J Med Chem.* 2000;43:2204–16. doi:10.1021/jm990968+.
39. Simulations Plus, Inc. ADMET Predictor (Version 6) [software] Retrieved from <http://www.simulations-plus.com>.
40. R Core Team. R: A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria. 2013. Available from: <http://www.R-project.org/>.
41. Mevik B-H, Wehrens R, Liland KH. pls: partial least squares and principal component regression. 2013. R package version 2.3-0. Available from: <http://CRAN.R-project.org/package=pls>.

42. Sing T, Sander O, Beerenwinkel N, Lengauer T. ROCr: visualizing classifier performance in R. *Bioinformatics*. 2005;21:7881. Available from: <http://rocr.bioinf.mpi-sb.mpg.de>.
43. Demšar J, Zupan B. Orange: from experimental machine learning to interactive data mining. In: Boulicaut JF, Esposito F, Giannotti F, Pedreschi D, editors. *Knowledge discovery in databases: PKDD 2004*. Berlin: Springer; 2004. p. 537–9.
44. Zhou L, Chen X, Gu Y, Liang J. Transport characteristics of candesartan in human intestinal Caco-2 cell line. *Biopharm Drug Dispos*. 2009;30:259–64. doi:10.1002/bdd.664.
45. Salix Pharmaceuticals, Inc. Xifaxan® (rifaximin) Tablets, 550 mg. NDA 22–554. 2010. Available from: <http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/GastrointestinalDrugsAdvisoryCommittee/UCM201081.pdf>.
46. Yamashiro W, Maeda K, Hirouchi M, Adachi Y, Hu Z, Sugiyama Y. Involvement of transporters in the hepatic uptake and biliary excretion of valsartan, a selective antagonist of the angiotensin II AT1-receptor, in humans. *Drug Metab Dispos*. 2006;34:1247–54. doi:10.1124/dmd.105.008938.
47. Kato Y, Miyazaki T, Kano T, Sugiura T, Kubo Y, Tsuji A. Involvement of influx and efflux transport systems in gastrointestinal absorption of cefiprolol. *J Pharm Sci*. 2009;98:2529–39. doi:10.1002/jps.21618.
48. Brillault J, De Castro WV, Harnois T, Kitzis A, Olivier JC, Couet W. P-glycoprotein-mediated transport of moxifloxacin in a Calu-3 lung epithelial cell model. *Antimicrob Agents Chemother*. 2009;53:1457–62. doi:10.1128/AC.01253-08.
49. Haritova AM, Schrickx J, Lashev L, Fink-Gremmels J. ABC efflux transporters: P-gp, MRP2, and BCRP—the 3rd dimension in kinetics not only of fluoroquinolones. *Br J Vet Med*. 2006;9:223–42.
50. He XJ, Zhao LM, Qiu F, Sun YX, Li-Ling J. Influence of ABCB1 gene polymorphisms on the pharmacokinetics of azithromycin among healthy Chinese Han ethnic subjects. *Pharmacol Rep*. 2009;61:843–50.
51. Deacon CF. Dipeptidyl peptidase-4 inhibitors in the treatment of type 2 diabetes: a comparative review. *Diabetes Obes Metab*. 2011;13:7–18. doi:10.1111/j.1463-1326.2010.01306.x.
52. Gladziwa U, Klotz U. Pharmacokinetics and pharmacodynamics of H₂-receptor antagonists in patients with renal insufficiency. *Clin Pharmacokinet*. 1993;24:319–32. doi:10.2165/00003088-199324040-00005.
53. Hansch C, Steinmetz WE, Leo AJ, Mekapati SB, Kurup A, Hoekman D. On the role of polarizability in chemical-biological interactions. *J Chem Inf Comput Sci*. 2003;43:120–5. doi:10.1021/ci020378b.
54. Shen DD, Azarnoff DL. Clinical pharmacokinetics of methotrexate. *Clin Pharmacokinet*. 1978;3:1–13.
55. Calvert AH, Bondy PK, Harrap KR. Some observations on the human pharmacology of methotrexate. *Cancer Treat Rep*. 1977;61:1647–56.
56. Suda Y, Akazawa S. Biliary and pancreatic excretion of methotrexate (MTX) and 5-FU on the MTX/5-FU sequential therapy. *Gan To Kagaku Ryoho*. 1990;17:1357–63.
57. Nuernberg B, Koehnke R, Solsky M, Hoffman J, Furst DE. Biliary elimination of low-dose methotrexate in humans. *Arthritis Rheum*. 1990;33:898–902. doi:10.1002/art.1780330620.
58. Crivori P, Zamora I, Speed B, Orrenius C, Poggesi I. Model based on GRID-derived descriptors for estimating CYP3A4 enzyme stability of potential drug candidates. *J Comput Aided Mol Des*. 2004;18:155–66. doi:10.1023/B:JCAM.0000035184.11906.c2.
59. Masimirembwa CM, Bredberg U, Andersson TB. Metabolic stability for drug discovery and development: pharmacokinetic and biochemical challenges. *Clin Pharmacokinet*. 2003;42:515–28. doi:10.2165/00003088-200342060-00002.
60. Clarke SE, Jeffrey P. Utility of metabolic stability screening: comparison of *in vitro* and *in vivo* clearance. *Xenobiotica*. 2001;31:591–8. doi:10.1080/00498250110057350.
61. Gustafson JH, Benet LZ. Biliary excretion kinetics of phenolphthalein glucuronide after intravenous and retrograde biliary administration. *J Pharm Pharmacol*. 1974;26:937–44. doi:10.1111/j.2042-7158.1974.tb09212.x.
62. Wachter VJ, Wu C-Y, Benet LZ. Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: implications for drug delivery and activity in cancer chemotherapy. *Mol Carcinog*. 1995;13:129–34. doi:10.1002/mc.2940130302.
63. Hansch C, Kurup A. QSAR of chemical polarizability and nerve toxicity. 2. *J Chem Inf Comput Sci*. 2003;43:1647–51.
64. Quillin ML, Breyer WA, Griswold IJ, Matthews BW. Size versus polarizability in protein-ligand interactions: binding of noble gases within engineered cavities in phage T4 lysozyme. *J Mol Biol*. 2000;302:955–77. doi:10.1006/jmbi.2000.4063.
65. Chacra AR. Saxagliptin for type 2 diabetes. *Diabetes Metab Syndr Obes*. 2010;3:325–35. doi:10.2147/DMSOTT.S12241.
66. Renkin EM, Robinson RR. Glomerular filtration. *N Engl J Med*. 1974;290:785–92.