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AN EVALUATION OF THE IN VITRO METABOLISM DATA FOR PREDICTING THE CLEARANCE AND DRUG-DRUG INTERACTION POTENTIAL OF CYP2C9 SUBSTRATES

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ABSTRACT:

In the early drug discovery process, metabolic stability and cytochrome P450 inhibition are often used as an early selection tool to identify useful compounds for further development. The reliability of the data in this process is therefore crucial. In the present study, in vitro enzyme kinetic data were used to predict the in vivo clearance and drug-drug interaction potential of four well known CYP2C9 substrates (tolbutamide, fluvastatin, ibuprofen and diclofenac) that are frequently used as benchmark substances in screening programs. Quantitative predictions of hepatic clearance using the well stirred prediction model and CL_{int} calculated from enzyme kinetic measurements were not useful. Including and excluding protein binding resulted in under- and overestimation, respectively, of in vivo clearance. The only predicted in vivo clearance that fell into the range of reported measured values was for fluvastatin when protein binding was not included. In an open, randomized, seven-armed, crossover study in healthy volunteers, tolbutamide, ibuprofen, and fluvastatin were investigated as inhibitors of the metabolism of diclofenac, and vice versa. None of the combinations was found to interact with each other in vivo. The in vitro drug-drug interaction potential was investigated by K_i determinations of the same combinations. In contrast to clearance predictions, the interaction potential in vivo was best predicted when plasma protein binding was included in the various models used. This study points to the uncertainty in calculating in vivo kinetics from in vitro enzyme kinetic data. The in vitro metabolic screening can thus be questioned as a compound selection tool without a proven in vitro-in vivo correlation.

Because of the major investment of time and money in a drug development program, it is desirable to select good drug candidates as early as possible. The recognition that many compounds fail because of inappropriate drug metabolism and pharmacokinetic (DMPK) properties has front-loaded screening for metabolic properties in the early drug discovery process. Predictions of in vivo clearance and drug-drug interaction properties, made on the basis of in vitro intrinsic clearance, and cytochrome P450 (P450) inhibition properties have become an important compound selection tool. Retrospective studies have shown, however, that quantitative predictions of in vivo clearance from in vitro data have been poor for many compounds (Clarke and Jeffrey, 2001; Masimirembwa et al., 2003). Despite this outcome, metabolism in human liver microsomes and whole cell systems is routinely used to assess the metabolic clearance of drugs in early drug discovery. In addition, the P450 inhibition potency of new compounds is used to select compounds without an in vivo drug-drug interaction potential. The reliability of these assessments is therefore crucial in selecting compounds with appropriate DMPK characteristics.

Concomitant medications causing drug-drug interactions have led to serious adverse effects during treatment, resulting in restrictions in prescribing drugs and even withdrawal of drugs from the market (Yuan et al., 1999). In clinical development programs, drug candidates with a serious drug interaction profile have encountered early termination or refusal of approval. The majority of drug-drug interactions are caused by the inhibition of metabolism of one drug by a concomitantly administered drug. Clinically relevant drug-drug interactions are caused mainly by an inhibition of P450-dependent reactions. Regulatory bodies such as the Food and Drug Administration have also noted that an increasing number of submissions also include in vitro P450 inhibition data (Yuan et al., 1999).

The present study investigated four marketed drugs: diclofenac, tolbutamide, ibuprofen, and fluvastatin. They all represent CYP2C9 substrates, which exhibit a broad range of affinities to CYP2C9 as indicated by published K_m values. Diclofenac and tolbutamide are also recommended as probe substrates for in vitro metabolism studies (Anonymous, 2001; Bjornsson et al., 2003). The in vitro kinetics of diclofenac and tolbutamide are thus especially important, since many pharmaceutical companies will use them as benchmark compounds to classify new chemical entities. Tolbutamide is considered to be a low affinity compound (K_m 97–200 μM) (Komatsu et al., 2000), ibuprofen and diclofenac, intermediate compounds (K_m 38 μM and 5–9 μM, respectively) (Leeman et al., 1993; Hamman et al., 1997; Fischer et al., 1999), and fluvastatin a high affinity compound (K_m <1 μM) (Transon et al., 1995; Fischer et al., 1999). The principal metabolites of ibuprofen are 3- and 2- hydroxyibuprofen and that of diclofenac is the 4-hydroxy derivative, all of which are formed by CYP2C9 (Davies and Anderson, 1997). A search of the literature yields little information about drug-drug interactions for combinations of these CYP2C9 substrates (Jorga et al., 2000; Scripture and Pieper, 2001).

The aim of the present study was to evaluate the usefulness of in

ABBREVIATIONS: DMPK, drug metabolism and pharmacokinetic(s); P450, cytochrome P450; CL_{int}, intrinsic clearance; HPLC, high-performance liquid chromatography; AUC, area under the plasma concentration-time curve.

vitro enzyme kinetic data as predictors for the in vivo human kinetics of four well known CYP2C9 drug substances. The CL_{int} and P450 inhibition profiles were studied in vitro, and the drug-drug interaction was investigated in vivo in an open, randomized, seven-armed, crossover study in healthy males.

Materials and Methods

Chemicals. β-NADPH tetrasodium salt, diclofenac (2[(2′, 6′-dichlorophenyl)amino]benzene acetic acid sodium salt), tolbutamide (1-butyl-3-*p*-tolylsulfonylurea), and potassium phosphate (USP, dibasic) were purchased from Sigma-Aldrich (St. Louis, MO). 4′-Hydroxydiclofenac (2[(2′,6′-dichloro, 4′-hydroxyphenyl)amino]-benzene acetic acid) was purchased from BD Gentest (Woburn, MA). Acetonitrile (LiChrosolv, gradient grade) and potassium dihydrogen phosphate (analytical grade) were purchased from Merck (Darmstadt, Germany), and methanol (HPLC grade) was obtained from Rathburn Chemicals Ltd. (Walkerburn, Scotland, UK).

Ibuprofen [2-(4-isobutylphenyl)propionic acid] was kindly provided by AstraZeneca AB (Södertälje, Sweden), and fluvastatin (7-[3-(4-fluorophenyl)-1-(methylethyl)-1H-indol-2-yl]-3,5-dihydroxy-6-heptenoic acid monosodium salt) was synthesized at the Department of Medicinal Chemistry at AstraZeneca R&D Mölndal. 4-Methylhydroxytolbutamide was obtained from Ultrafine (Manchester, UK). Ibuprofen metabolites were a kind gift from Professor Brian Houston, University of Manchester (Manchester, UK). All other chemicals were of the highest purity available.

Human Liver Microsomes. Human liver samples were obtained from Sahlgrenska University Hospital (Göteborg, Sweden). Small biopsies were frozen in liquid nitrogen and stored at −80°C until preparation of liver microsomes. Microsomes were then prepared according to the method of Ernster et al. (1962). Microsomes from five individuals were pooled. The microsomal protein concentration was determined according to the method of Lowry et al. (1951), using bovine serum albumin as a standard. The method of Omura and Sato (1964) was used to estimate the total P450 content. The microsomal preparation was stored at −80°C until use.

Enzyme Assays. The incubations were done at 37°C in 96-well microtiter plates at a total volume of 150 μl or 200 μl. A 0.1 M potassium phosphate buffer was used to dilute the microsomes to the desired concentration. Each assay was optimized for linearity with time and protein concentration over the concentration range used. For the saturation curves, 9 to 11 concentrations were used for each substrate. For determination of K_i, three substrate concentrations, 1/3 K_m, K_m, and 3 K_m, were used. Seven concentrations of the inhibitor were incubated for each substrate concentration.

Analytical systems. The analytical system used was a Hewlett Packard 1100 HPLC with a precolumn, NewGuard cyano, 3.2 × 15 mm, 7 μm, and a separation column, Zorbax SB C_{18}, 4.6 × 150 mm. Diclofenac and tolbutamide and their metabolites were analyzed as described by Masimirembwa et al. (1999). Ibuprofen and 3-hydroxyibuprofen were separated by using the mobile phases: A, 26% acetonitrile, 74% potassium phosphate, pH 2.9; B, 50% acetonitrile, 50% potassium phosphate, pH 2.9, in a gradient: 0 to 6 min 0% B, 6 to 13 min 0 to 100% B, 13 to 20 min 100% B. The peaks were detected at 220 nm. Since no reference metabolites were available for fluvastatin, the kinetics was studied by substrate disappearance. Fluvastatin was separated from the metabolites by using the mobile phases: A, 25:75 (v/v) acetonitrile/10 mM NH_4Ac, pH 7.6; B, acetonitrile in a gradient: 0 to 7 min 0% B, 7.1 to 16 min 0 to 35% B, 16 to 17 min 35 to 90% B, 17 to 20 min 90% B, 20 to 20.5 min 90 to 0% B, 20.5 to 23 min 0% B. The peaks were detected at 235 nm.

Human plasma samples. The plasma concentrations of diclofenac, ibuprofen, and tolbutamide were measured by HPLC. The concentrations of fluvastatin were measured by liquid chromatography-tandem mass spectrometry. The calibration curves for the different methods were shown to be linear within the range studied (diclofenac, 10–1000 ng/ml; ibuprofen, 10–1000 ng/ml; tolbutamide, 50–5000 ng/ml; fluvastatin, 0.5–500 ng/ml). The precision (expressed as the coefficient of variation) and the accuracy (expressed as the percentage difference) never exceeded 17% regardless of method. The limit of quantification was set at 10 ng/ml for diclofenac and ibuprofen. The corresponding values for tolbutamide and fluvastatin were 50 and 0.5 ng/ml, respectively.

Microsomal protein binding. Human liver microsomes were diluted to 0.4 mg/ml in 0.1 M potassium phosphate buffer, pH 7.4, and the compound under study was added to reach a final concentration of 10 μM. The mixture was centrifuged at 1500*g* for 15 min in Millipore (Bedford, MA) Centrifree YM-30 (30,000 molecular weight cutoff) tubes. To investigate possible unspecific binding, the experiment was also done using only buffer. All steps were run at 37°C. The fraction unbound (f_u) was calculated as concentration found in filtrate/total concentration.

In Vivo Study. *Subjects and study design.* Twelve healthy males were enrolled in the open, randomized, seven-arm, crossover study. The following inclusion criteria were used: males 20 to 50 years old, body mass index 19 to 30 kg/m^2, weight 50 to 100 kg, written informed consent to participate in the study, and clinically normal physical findings and laboratory values, as judged by the investigator. The subjects observed the following restrictions: no intake of alcohol in the 2 days before and during the pre-entry visit and the study days; no intense physical exertion on the morning of each study day, no new physical exercise activities or increase in the intensity of their usual physical exercise during the study period, and no blood donation or potentially hazardous work or activities during the study period. The study was conducted in accordance with Good Clinical Practice guidelines and carried out according to the principles of the Declaration of Helsinki. The Gothenburg University Ethics Committee and the Medical Products Agency of Sweden approved the protocol before the start of the study. The study site was Sahlgrenska University Hospital, Gothenburg, Sweden.

The subjects were randomly assigned to receive single oral doses of the drugs in the following dosage regimens on each study day: 50 mg of diclofenac (Voltaren), 40 mg of fluvastatin (Canef), 500 mg of tolbutamide (Tolbutamide), 400 mg of ibuprofen (Ipren), 50 mg of diclofenac + 500 mg of tolbutamide, 50 mg of diclofenac + 40 mg of fluvastatin, and 50 mg of diclofenac + 400 mg of ibuprofen. After a washout period of at least 7 days, the subjects were switched to another alternative dosage regimen. The subjects were to arrive after an overnight fast (no food after 10:00 PM) and were given the study drug at 8:00 AM. Standardized lunches and dinners were served during the study days, and lunch was served 4 h after dosing. Serial blood samples for the analysis of the drug levels were taken: predose, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, and 24 h after administration of the study drug on each study day.

Prediction of in Vivo from in Vitro Data. In the case of competitive or noncompetitive inhibition, when the substrate concentration is much lower than K_m, the degree of inhibition (AUC ratio) in the presence and absence of the inhibitor can be described as follows (Ito et al., 1998):

$$\text{AUC ratio} = \frac{v(+I)}{v(\text{control})} = \frac{1}{1 + \frac{I_u}{K_i}} \quad (1)$$

where $v(+I)$ and v(control) are the initial metabolic velocity in the presence and absence of the inhibitor, respectively, I_u is the unbound concentration of the inhibitor, and K_i is the inhibition constant of the inhibitor determined from in vitro inhibition studies (Ito et al., 1998). To avoid false negative predictions due to underestimation of I_u, I_u was calculated by eq. 2.

$$I_u = \left(I_{max} + \frac{k_a \cdot F_a \cdot D}{Q_h}\right) \cdot f_{up} \quad (2)$$

where I_{max} is the maximal concentration observed (C_{max}), k_a is the first order rate constant for gastrointestinal absorption, F_a is the fraction absorbed from the gastrointestinal tract into the portal vein, D is the dose, Q_h is the hepatic flow rate (1500 ml/min), and f_{up} is the unbound fraction in plasma. The values of k_a and F_a were unknown; the theoretical maximum value of 0.1 min^{-1} was thus used for k_a (12). The theoretical maximum value of 1 was consistently used for F_a, to avoid false negative predictions.

The predictive AUC ratio after oral administration in the presence and absence of inhibitor can be calculated from the following equations. In the case of a high clearance drug such as fluvastatin, eq. 3 can be used.

$$AUC_{ratio} = \frac{1}{\frac{f_m}{1 + \frac{I_u}{K_i}} + 1 - f_m} \quad (3)$$

TABLE 1

Values of the parameters used in the prediction of in vivo clearance or drug-drug interactions

Fraction unbound in microsomes and enzyme kinetics of diclofenac 4-hydroxylation, tolbutamide 4-hydroxylation, ibuprofen 3-hydroxylation, and fluvastatin disappearance were measured in this study.[a]

Substance	f_m	f_h	f_{up}	f_{umic}	I_u	C_{max}	$C_{max,u}$	K_m	V_{max}
					μM	μM	μM	μM	*nmol/min/mg protein*
Diclofenac	0.75	0.9	0.003	0.86	0.05	4	0.012	1.8 ± 0.22	0.92 ± 0.02
Tolbutamide	0.80	0.8	0.02	1	6.44	165	3.3	67 ± 9.9	0.13 ± 0.005
Ibuprofen	0.9	0.85	0.01	1	2.75	114	1.1	27.9 ± 3.02	0.61 ± 0.02
Fluvastatin	0.5–0.8	0.80	0.02	0.59	0.14	1.0	0.02	K_m1: 0.54 ± 1.5	V_{max}1: 0.073 ± 0.10
								K_m2: 18.9 ± 21.2	V_{max}2: 0.353 ± 0.07

f_m, fraction metabolized by CYP2C9; f_h, fraction cleared by hepatic metabolism; f_{up}, fraction unbound in plasma; f_{umic}, fraction unbound in microsomes (measured in this study); I_u, the unbound concentration of the inhibitor as calculated by eq. 2; C_{max}, plasma C_{max} values from this study; $C_{max,u}$, unbound plasma C_{max}, calculated.

[a] References: Bertz and Granneman (1997), Apple et al. (1995), Apple and Dingemans (1996), Davies (1998), Davies and Anderson (1997), Fischer et al. (1999).

In the case of a low clearance drug, such as diclofenac, tolbutamide, and ibuprofen, the AUC ratio after oral administration can be calculated by eq. 4,

$$AUC_{ratio} = \frac{1}{\frac{f_h \cdot f_m}{1 + \frac{I_u}{K_i}} + 1 - f_h \cdot f_m} \quad (4)$$

where f_m is the fraction of the substance eliminated by CYP2C9 metabolism, I_u is the value of I_u calculated by eq. 2, f_h is the fraction of drug being metabolized, and K_i is $K_{i,\,total} \cdot f_{u,mic}$. The values of $f_{u,mic}$ represent the fraction unbound of the substance in human microsomes.

Data Analysis and Statistics. SigmaPlot 2001 (version 7) and Enzyme Kinetics (version 1.1) from SPSS Science Software UK Ltd. (Birmingham, UK) were used to estimate enzyme kinetic parameters K_m, V_{max}, and K_i by nonlinear fit of the Michaelis-Menten equation and the Dixon equation to the formation rates.

Pharmacokinetic Variables. Pharmacokinetic variables in vivo of diclofenac, fluvastatin, tolbutamide, and ibuprofen were estimated after each dose intake and up to 24 h postdose. Actual sampling times were used in the pharmacokinetic calculations. The pharmacokinetic parameters were estimated by noncompartmental methods using WinNonlin Pro (ver 3.1; Pharsight, Mountain View, CA). The maximum plasma concentrations (C_{max}) and the total area under the plasma concentration-time curve (AUC) were estimated. The AUC and C_{max} were calculated for all individuals after the various treatments. A three-factor analysis of variance with treatment, subject, and period as factors, was made on the log-transformed variables. Least-squares means were used to estimate the effect of the different treatments. A two-sided 90% confidence interval for the relative effect was calculated from the least-squares estimates of the analysis of variance and its covariance matrix.

Results

In addition to enzyme kinetics, several parameters were used as scaling factors for the in vivo predictions (Table 1). The values are from the literature except for the microsomal binding data, which were measured in this study, and the calculation of the concentration of each drug (I_u) in the portal vein (calculated according to eq. 2).

Michaelis-Menten Kinetics. Saturation curves for the formation of diclofenac 4-hydroxylation, tolbutamide 4-hydroxylation, ibuprofen 3-hydroxylation, and the disappearance of fluvastatin were used to calculate enzyme kinetic parameters K_m and V_{max}. Nonlinear regression fitting of the Michaelis-Menten equation to the experimental data were done using the SigmaPlot enzyme kinetic module. The results are listed in Table 1. Fluvastatin disappearance was the only substance that showed a good fit to a two-enzyme system exhibiting two K_m and V_{max} values, whereas the enzyme kinetics of the other substances fitted into a single-enzyme system. All values are in the expected range according to earlier published data (Transon et al., 1995; Fischer et al., 1999; Komatsu et al., 2000).

The hepatic plasma clearance was estimated from the enzyme kinetic determinations of the four drugs. In Table 2 the reported in vivo blood clearance values (calculated from published plasma levels and the blood/plasma ratio) and the calculated clearance using the well stirred model are listed with or without plasma and/or microsomal protein binding data. The C_{max} for diclofenac and fluvastatin is about double the measured K_m, which could substantially affect the CL_{int} measurements. The CL_{int} was therefore calculated using the equations V_{max}/K_m ratio and $V_{max}/K_m + C_{max}$ for diclofenac and fluvastatin, since C_{max} values reach K_m values. However, the inclusion of the C_{max} did not dramatically change the calculated hepatic clearance values.

Inhibition Experiments. Tolbutamide showed a mixed, predominantly competitive inhibition of the 4-hydroxylation of diclofenac, whereas ibuprofen was purely competitive. Fluvastatin, on the other hand, exhibited a noncompetitive inhibition.

Diclofenac was a competitive inhibitor of tolbutamide 4-methylhydroxylation, ibuprofen 3-hydroxylation, and fluvastatin hydroxylation. The type of inhibition and the K_i values are shown in Table 3, both when total concentration was used and when the free fraction was taken into account.

The potential in vivo effects using simple I/K_i ratios are listed in Table 4. The C_{max} values were used for the inhibitor concentration. As seen in Table 4, the ratio is markedly higher when plasma and microsomal protein binding is not included. The criteria for likely drug-drug interaction in vivo, as discussed by the EUFEPS conference in Basel in 2000 (Anonymous, 2001) or by the author of the article prepared by the Pharmaceutical Research and Manufacturers of American Drug Metabolism and Clinical Pharmacology Technical Working Group (Bjornsson et al., 2003), were estimated to be likely if the C_{max}/K_i value >1, possible if the C_{max}/K_i value is between 1 and 0.1, and remote if the value is <0.1. These articles did not discuss whether or not protein binding should be included. In this study the ratios of C_{max}/K_i indicate a likely potential for drug-drug interactions for tolbutamide, ibuprofen, and fluvastatin, whereas diclofenac is estimated to show possible drug-drug interactions if protein binding is not taken into account. If protein binding were taken into account, the interaction risk would be classified as possible for fluvastatin only.

In Table 5, the effect of AUC is predicted by using the Michaelis-Menten equations for noninhibited and inhibited reactions. Protein binding has a dramatic effect on the results. Equation 1A (in Table 5; no protein binding) estimates that all combinations will affect AUC. When protein binding is taken into account, fluvastatin is estimated to increase diclofenac AUC 2-fold and diclofenac to increase tolbutamide and ibuprofen AUC 1.6- and 1.4-fold, respectively. A third case was used, where the concentration was estimated in the portal vein and used as the inhibitor concentration (Fig. 1). Using this calculation,

TABLE 2

Clearance in vivo and calculated hepatic clearance values from CL_{int} (V_{max}/K_m measurements or, for diclofenac and fluvastatin, also $V_{max}/K_m + C_{max}$; values in parentheses)

The predicted hepatic CL values for fluvastatin are the sum of low and high affinity enzyme kinetic terms. Values are ml/min/70 kg. The equations for hepatic CL_{int} and hepatic clearance are shown below.

Clearance	Protein Binding	Diclofenac	Tolbutamide	Ibuprofen	Fluvastatin
			ml/min		
Blood CL in vivo[a]		424–667[b]	31[c]	64–127[b]	329–648[b]
Predicted $CL_{hepatic}$	No protein binding included	1340 (1240)	120	718	1227 (1061)
	Plasma protein binding included	96 (32)	2.6	15	175 (84)
	Plasma and microsomal protein binding included	112 (38)	2.6	15	272 (134)

Equations: $CL_{int} = V_{max} \cdot 1500$ g liver weight $\cdot$ 45 mg microsomal protein/g liver/K_m; $CL_{hepatic} = Q \cdot f_u \cdot CL_{int}/(Q + f_u \cdot CL_{int})$.

[a] Blood clearance was calculated from published plasma clearance values. The blood vs. plasma ratio is 0.55 for diclofenac, tolbutamide, and ibuprofen (Obach, 1999) and 1.8 for fluvastatin (Lennernäs and Fager, 1997).

[b] Bertz and Granneman (1997).

[c] Lee et al. (2003).

TABLE 3

Inhibition assay results

Values of the present study are mean ± S.E.

Substrate	Inhibitor	Mode of Inhibition	K_i Total	K_i Unbound
			μM	
Diclofenac	Tolbutamide	Competitive	59 ± 13	59
Diclofenac	Ibuprofen	Competitive	30 ± 2.3	30
Diclofenac	Fluvastatin	Noncompetitive	0.20 ± 0.015	0.12
Tolbutamide	Diclofenac	Competitive	6.0 ± 2.0	5.2
Ibuprofen	Diclofenac	Competitive	3.0 ± 0.2	2.6
Fluvastatin	Diclofenac	Competitive	16.0 ± 5.4	13.8

TABLE 4

The I/K_i ratio for the CYP2C9 substrates

The C_{max} values were used as inhibitor concentrations. The ratios are expressed both in terms of total and unbound concentrations (values from Tables 1 and 2).

Inhibitor	$I/K_{i,total}$	$I_u/K_{i,u}$
Tolbutamide	2.8	0.056
Ibuprofen	3.8	0.032
Fluvastatin	5	0.17
Diclofenac	0.67	0.023

no combinations were estimated to interact except for fluvastatin, which was estimated to increase diclofenac AUC 1.7-fold.

In Vivo Results. No change in C_{max} or AUC for diclofenac was seen when coadministered with tolbutamide, ibuprofen, or fluvastatin (Table 5; Fig. 2). Neither the AUC nor C_{max} of tolbutamide, ibuprofen, and fluvastatin was affected by coadministration of diclofenac (Table 5). The subjects were not genotyped for CYP2C9 polymorphisms. However, the AUC and the interindividual variation exhibited normal ranges, which indicates that the study used subjects with wild-type phenotype or allelic variants with little or no influence on the pharmacokinetics.

Discussion

The prediction of metabolic clearance using in vitro metabolic data is widely applied in the pharmaceutical industry. One of the simplest methods used is "metabolic stability," where depletion of the substance in liver microsomal incubations fortified with NADPH is measured. To be able to calculate in vitro clearance, the substrate concentration must be well below the apparent K_m, which is usually not known in early drug discovery. However, Obach et al. (1997) compared the prediction of 16 Pfizer proprietary compounds and found that simple depletion assays were as good as more elaborate enzyme kinetic (V_{max}/K_m) determinations for the prediction of human clearance. One major finding in the study by Obach et al. (1997) was the severe underprediction of highly protein-bound compounds when plasma protein binding was used in the clearance prediction models. The problem of underpredicting human clearance using metabolism data from in vitro microsomal incubations has been described by several research groups (e.g., Carlile et al., 1999; Naritomi et al., 2001). The inclusion of protein binding is a cornerstone in scaling, assuming that only the protein-unbound substance in plasma is accessible for metabolism (Pang and Rowland, 1977). However, as can be seen in this study, the clearance estimates made using the well stirred model seem either to overpredict or underpredict the in vivo clearance, depending on whether the protein binding in plasma and microsomes is or is not included in the model. Another possible confounding factor is that the concentrations used in the in vitro assays do not correspond to the concentrations in the hepatocytes in vivo. The possibility that a hepatocyte's plasma membrane is a barrier or that various transporters concentrate or efflux the compound in the cell may represent confounding factors. The use of hepatocyte suspensions for metabolic studies may only partly circumvent this issue since the transporter function and membrane integrity may be impaired in isolated cell systems. This points to a major problem in early drug discovery programs in the pharmaceutical industry concerning how to use in vitro metabolism data as a predictive tool for selecting compounds with acceptable metabolic clearance. Clarke and Jeffrey (2001) retrospectively evaluated 1163 compounds from different chemistry programs where information was available on both in vitro and in vivo clearances in rats. Only 64% of the compounds were classified correctly as high or low clearance drugs. The most worrisome part of the study was that 22% of the compounds showed high

TABLE 5

The change in AUC as estimated by calculating the effect of the respective inhibitor on the rate of metabolism as derived from the Michaelis-Menten equations

Equations 1a and 1b use C_{max} as inhibitor concentrations. Equation 2 uses an estimated portal vein concentration as inhibitor concentration (from Table 1). All p values for the observed AUC ratio were >0.5.

Substrate	Inhibitor	Eq. 1a[a]	Eq. 1b[b]	Eq. 3 or 4[c]	Observed AUC Ratio (90% CI)
Diclofenac	Tolbutamide	3.8	1.0	1.1	0.93 (0.7–1.24)
Diclofenac	Ibuprofen	2.2	1.0	1.1	0.99 (0.77–1.26)
Diclofenac	Fluvastatin	6.3	2.0	1.7	1.07 (0.82–1.41)
Tolbutamide	Diclofenac	1.7	1.6	1.0	1.01 (0.85–1.20)
Ibuprofen	Diclofenac	1.6	1.4	1.0	1.03 (0.84–1.26)
Fluvastatin	Diclofenac	2.6	1.0	1.0	1.08 (0.81–1.45)

CI, confidence interval.
[a] Equation 1a: AUC ratio = $v(I)/v(\text{control}) = 1/(1 + I/K_i)$, $I = C_{max}$ total; K_i total.
[b] Equation 1b. The free fractions for the inhibitor (I_u) and K_i ($K_{i,u}$) are used.
[c] Equation 3: AUC ratio = $1/((f_m/1 + I_u/K_i) + 1 - f_m)$ (fluvastatin); eq. 4: AUC ratio = $1/(f_h \cdot f_m(1/1 + I_u/K_i) + (1 - f_h \cdot f_m))$ (diclofenac, tolbutamide, ibuprofen).

clearance in vitro but had low clearance in vivo. These substances would be rejected if in vivo data were not available. These results also indicate that the scaling of in vitro results is a general problem, unrelated to any specific P450 enzyme or chemical substance class. In vitro metabolism data should therefore not be used to select compounds in the belief that these data can predict acceptable in vivo clearance without a proven in vitro-in vivo correlation. Instead, the data can give insight into the chemical basis of metabolic stability. Naritomi et al. (2003) recently showed that the ratio intrinsic clearance in vivo/intrinsic clearance in vitro (isolated liver cells), calculated from the well stirred model, varied from 0.5 to 73. These results imply that there are other compound-specific factor(s) than those used in the model that are important for correct calculation of in vivo pharmacokinetics. The ratio obtained in the rat study was used to correct the human intrinsic clearance estimates from in vitro data. This gave a much better prediction, which indicates that the same factor in rats and humans influences the clearance of drugs in vivo. Understanding these additional factors, which are important for scaling in vitro metabolic data to in vivo pharmacokinetics, should be a high priority for all researchers in the field of drug metabolism.

Prediction of the potential for drug-drug interactions is another exercise that attempts to scale the extent of inhibition measured in vitro to the inhibition potential in vivo. Several excellent reviews of this topic have been published (Ito et al., 1998; Weaver, 2001). Drug-drug interactions may cause serious and even fatal adverse events. For this reason, pharmaceutical companies evaluate drug interaction potentials before candidate drugs are selected or in the screening phase to select compounds with acceptable inhibition profiles. The reliability of these screens is therefore essential to the selection of drugs with acceptable inhibition profiles and to not screen out good drug candidates because of erroneous scaling procedures. Regulatory authorities such as the Food and Drug Administration and the Committee for Proprietary Medicinal Products have published a guidance on how to study and interpret in vitro interaction data (www.FDA.gov; www.eudra.org). An attempt was made to reach consensus on how to conduct in vitro and in vivo studies to assess drug-drug interaction potential at a EUFEPS conference (Anonymous, 2001) and by the authors of the article prepared by the Pharmaceutical Research and Manufacturers of American Drug Metabolism and Clinical Pharmacology Technical Working Group (Bjornsson et al., 2003). The recommended evaluation of drug-drug interaction potential by using C_{max}/K_i values gives two different answers depending on whether protein binding is used or not. In this study, we know that the selected drugs do not inhibit CYP2C9 metabolism in vivo, and the equation using protein binding was therefore the best predictor. There are a number of reports in the area that make conflicting conclusions, i.e., that the best prediction was obtained when protein binding was used and that the best prediction was obtained when protein binding was omitted (Obach et al., 1997; von Moltke et al., 1998; Venkatakrishnan et al., 2001;). Many pharmaceutical companies seem to take a cautious attitude and do not take protein binding into account. The articles from the European Federation for Pharmaceutical Sciences conference (Anonymous, 2001) and by the American Pharmaceutical Research and Manufacturers of America (Bjornsson et al., 2003) do not give recommendations as to whether protein binding should be used or not in prediction calculations.

Ito et al. (1998) described the possibility of quantitatively predicting drug-drug interactions in vivo from in vitro data. They identify the correct estimation of I_u/K_i as the key factor in accurate scaling of interaction risks. An interesting finding is that those drug-drug interactions in vivo that have been reported not to be successfully predicted from in vitro data were all underpredicted; i.e., the in vitro data suggested no interaction potential, whereas a significant drug-drug interaction was shown in vivo. In the present study, the estimates of portal vein concentrations as described by Ito et al. (1998) gave the best estimates, i.e., no drug-drug interactions by the studied compounds.

Two allelic variants of CYP2C9 are known to be important among white people, the CYP2C9*2 and CYP2C9*3 types, with allelic frequencies of 0.08 to 0.014 and 0.04 to 0.16, respectively (Schwarz, 2003). The genetic polymorphism of CYP2C9 may affect the pharmacokinetics and the risk for drug-drug interactions differently for the drugs used in this study. Diclofenac does not seem to be affected by the genotype, whereas a decreased clearance has been observed for tolbutamide, ibuprofen, and fluvastatin in subjects genotyped for CYP2C9*2 and CYP2C9*3 (Kirschheiner et al., 2003; Schwarz, 2003). Furthermore, ibuprofen is a racemate and CYP2C9 favors *S*-ibuprofen, whereas CYP2C8 favors *R*-ibuprofen. However, CYP2C9 is the overall most important enzyme for ibuprofen metabolism in white people (Hamman et al., 1997). Even though the genetic disposition of the subjects was not determined, the variations in AUCs measured in this study are in the normal ranges, which suggests that any CYP2C9 variants present in the subjects would have little importance for the outcome of the study.

With the increasing number of compounds requiring screening, most pharmaceutical companies have adopted high-throughput

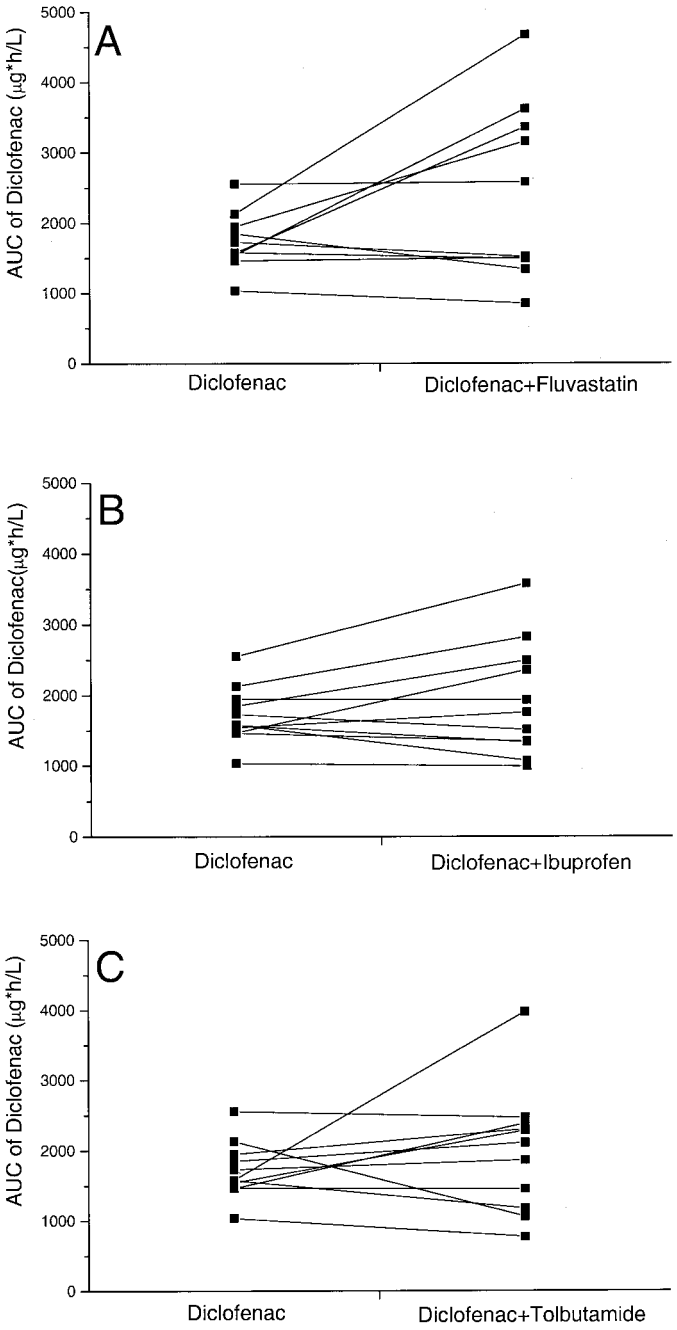

FIG. 1. Individual AUC values of diclofenac following administration alone and in combination with fluvastatin (A), ibuprofen (B), and tolbutamide (C).

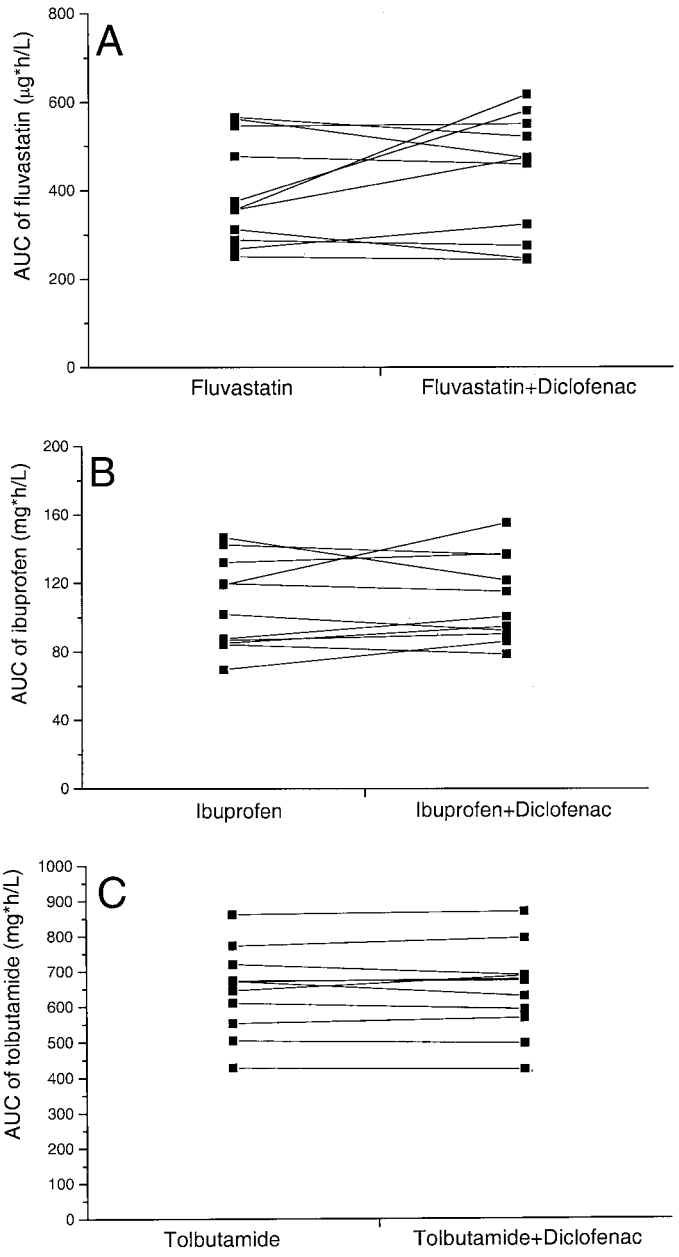

FIG. 2. Individual AUC values of fluvastatin (A), ibuprofen (B), and tolbutamide (C) following administration alone and in combination with diclofenac.

screening approaches to aid in the rapid discovery of new chemical entities. The front-loading of studies of DMPK properties has resulted in high-throughput screening methods for metabolic stability and P450 inhibitions, and more thorough enzyme kinetic studies are made in fewer compounds later in the discovery process. Often, cutoff numbers from the in vitro screens are used to discard compounds that are thought to exhibit unacceptable in vivo clearance or inhibition profiles. Accurate predictions are therefore critical in order not to screen out compounds that could be developed into good drug substances. This study and many others clearly show that there are still too many caveats before we can reliably use in vitro screen data or in vivo estimates of clearance and inhibition (Clarke and Jeffrey, 2001; Masimirembwa et al., 2003). The importance of checking predictions of in vivo clearance to identify whether the screens are valid must be emphasized, and this is probably an underestimated problem in many pharmaceutical companies since the discarded compounds will never be tested in vivo, and the reliability of the methods will therefore not be evaluated. Screening programs for metabolic stability or drug-drug interactions use model or benchmark substances, most often drugs on the market. In most cases, these model substances have chemical properties different from those of the new chemical entities under investigation, which makes the usefulness of the scaling exercise questionable.

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