Microsomal prediction of *in vivo* clearance of CYP2C9 substrates in humans

David J. Carlile,[1,2] Nancy Hakooz,[2] Martin K. Bayliss[3] & J. Brian Houston[2]

[1]*Centre for Applied Pharmacokinetic Research,* [2]*School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, UK,*[3]*Biomet, GlaxoWellcome, Ware, Herts, UK*

Aims To assess the utility of human hepatic microsomes for predicting *in vivo* intrinsic clearance (CL_{int}) via the use of four cytochrome P450 2C9 substrates: phenytoin, tolbutamide (S)-ibuprofen (two pathways) and diclofenac, and to examine the role of exogenous albumin within the microsomal incubation.

Methods V_{max}, K_m and CL_{int} (defined as V_{max}/K_m ratio) were estimated under initial rate conditions for five pathways of metabolism in a bank of 15 human hepatic microsomal samples and were scaled to *in vivo* units using the microsomal protein index. Non-metabolic related binding in microsomes was measured for phenytoin and tolbutamide in the presence and absence of albumin.

Results Microsomal CL_{int} values differed by over two orders of magnitude, with the means ranging from 0.18 (phenytoin) to 40.70 (diclofenac) μl min^{-1} mg^{-1} microsomal protein. When these data were scaled and compared with published *in vivo* studies a similar rank order was obtained, however, the actual CL_{int} tended to be underpredicted. While the *in vivo* unbound K_m for phenytoin, 1–5 μM is substantially lower than the value determined in microsomes based on total concentrations (56 μM), correction for the *in vitro* binding reduces this value to 20 μM and 6 μM in the absence and presence of albumin, respectively. Similar trends were seen with tolbutamide K_m.

Conclusions An appreciation of the utility of *in vitro* prediction can be best achieved when the range of CL_{int} values predicted from the individual hepatic microsomal samples are compared with the range of individual *in vivo* CL_{int} values reported in the literature. The degree of underprediction is less evident using the range than the mean data and no consistent advantage in adding albumin to the incubation media is apparent.

Keywords: CYP2C9, human microsomes, intrinsic clearance, *in vitro* predictions

Introduction

A general strategy to quantitatively predict *in vivo* drug clearance from CL_{int} values estimated from *in vitro* drug metabolising systems has recently been explored and evaluated in the rat using a data base of 35 drugs which show a range of turnovers covering four orders of magnitude [1–3]. The ultimate application of such a prediction strategy is with human tissue, and although there have been many studies with human *in vitro* tissue, often sufficient methodological detail is lacking from the original report to allow a systematic analysis. Iwatsubo and coworkers [3] have compiled literature data on the *in vitro* and *in vivo* clearances of 25 drugs and were able to demonstrate a significant correlation despite the numerous sources of these data and the low sample numbers upon which the parameters were based (see later). There remains a need for more indepth examinations of the utility of human *in vitro* kinetic data for the prediction of the *in vivo* situation.

As there is a shortage of good quality human liver material from which hepatocytes can be successfully isolated, the vast majority of researchers employ the hepatic microsomal fraction as their enzyme source due to its ease of preparation from tissue of varying quality and its long storage time. One of the main confounding issues with the use of human material is the large degree of interindividual variability in terms of the cytochrome P450 (CYP) complement. CYP2C9, a human CYP isoform of major importance [4], has been reported to

Correspondence: Dr J. B. Houston, School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester M13 9PL, UK.
Received 6 August 1998, accepted 27 January 1999.

consistently display the least interindividual variability, both in terms of immunoblotted content (four-fold range) [5, 6] and probe substrate kinetic properties (four-fold range) [6, 7] and contrasts with CYP3A4 (60-and 15 fold for immunoblotted content and substrate kinetics) [5–7] and CYP2D6 (16-and 14-fold range) [5–7]. However, an *in vivo* study [8], using tolbutamide as a CYP2C9 probe and the ratio of metabolite to parent drug as the index of enzyme activity, demonstrated a 10-fold range in a group of 106 healthy subjects and speculated on a possible incidence of 1 in 500 for the poor metaboliser phenotype.

As part of a systematic study to assess the utility of human microsomes to predict *in vivo* clearance, four CYP2C9 substrates were selected for study: phenytoin, tolbutamide (S)-ibuprofen and diclofenac. These compounds show a wide range of CL_{int} covering $0.28–44\ l\ min^{-1}\ 70\ kg^{-1}$ (see Table 1), are all extensively metabolized by CYP2C9 and show considerable plasma protein binding. Phenytoin, an anticonvulsive agent, is metabolized via 4′-hydroxylation to hydroxyphenytoin, which is eliminated mainly in the urine as the glucuronide conjugate [9]. Interestingly phenytoin displays nonlinear kinetics *in vivo*, consistent with saturation of the metabolic enzyme, and the parameters V_{max} and K_m have been well documented due to the need for plasma concentration monitoring in therapy. Tolbutamide is a compound of low hepatic extraction which is also metabolized to one metabolite by hydroxylation of the 4-benzyl group. However, most of an administered dose is eliminated in urine as carboxytolbutamide, formed by oxidation of the primary hydroxy metabolite [10]. (S)-Ibuprofen, a nonsteroidal anti-inflammatory drug, has been less extensively studied than the other CYP2C9 substrates detailed above, but it is known that there are two main metabolites, both hydroxylated on the propyl group to form 2-hydroxy-and 3-hydroxyibuprofen. The latter metabolite undergoes further oxidation to form 2-carboxyibuprofen, the fraction of each metabolite formed (fm) from the two primary metabolites are 0.3 and 0.5, for the 2-and 3-hydroxy forms, respectively [10]. Diclofenac is also a nonsteroidal anti-inflammatory agent, it is metabolized in several positions, although the main metabolite, with an fm of 0.5, is 4′-hydroxydiclofenac [12, 13].

Recently it was reported that the addition of bovine albumin (BSA) to human hepatic microsomal incubations, presumably to aid solubility, results in a reduction of the K_m for phenytoin hydroxylation *in vitro* to a value more comparable with the *in vivo* value [14]; both parameters were based on unbound concentrations. Therefore studies were undertaken to investigate this phenomenon as tolbutamide (S)-ibuprofen and diclofenac are also extensively bound to plasma proteins, with unbound fractions in plasma of 0.05 for the former two compounds [15, 16] and 0.004 for the latter [17, 18].

The aims of the present study were twofold; first to assess the utility of hepatic microsomes in predicting the *in vivo* CL_{int} of four CYP2C9 substrates, and secondly to examine the role of exogenous albumin on kinetic parameters obtained from *in vitro* incubations.

Methods

Chemicals

[^{14}C]-phenytoin (specific activity $53.1\ mCi\ mmol^{-1}$, radiochemical purity $>99\%$) was purchased from New Life Science Products (Stevenage, Herts, UK), [^{14}C]-tolbutamide (specific activity $54.0\ mCi\ mmol^{-1}$, radiochemical purity $>99\%$ was purchased from Amersham Life Sciences (Bucks, UK). Phenytoin and tolbutamide and their hydroxy metabolites were obtained from Sigma Chemical Co (Poole, Dorset, UK) (S)-ibuprofen and 3-hydroxy (S)-ibuprofen were gifts from Boots

Table 1 *In vivo* intrinsic clearance estimates for phenytoin, tolbutamide (S)-ibuprofen and diclofenac from literature reports[a].

Drug	*Number of studies*[b]	*Number of individuals*[c]	*Mean*	CL_{int}[d] *Low*	*High*
Phenytoin[e]	5	48	0.28	0.057	0.378
Tolbutamide[f]	8	78	0.14	0.089	0.313
(S)-Ibuprofen[g] (2-hydroxy metabolite)	2	12	2.17	1.43	3.03
(S)-Ibuprofen[g] (3-hydroxy metabolite)	2	12	3.62	2.38	5.05
Diclofenac[h]	4	34	43.90	18.81	61.83

[a]CL_{int} values are reported as a mean, calculated from the mean data in each literature report, the values in parentheses represent the range of individual CL_{int} values. [b]Number of studies refers to separate literature reports. [c]Refers to the total number of individuals assessed in the literature reports. [d]CL_{int} values are reported as $l\ min^{-1}\ 70\ kg^{-1}$ man. [e]Phenytoin data from references; 15, 35, 36, 37, 38, 39, 40, 41. [f]Tolbutamide data from references; 15, 40, 41, 42, 43, 44, 45, 46. [g](S)-Ibuprofen data from references; 11, 47. [h]Diclofenac data from references; 48, 49, 50, 51.

 © 1999 Blackwell Science Ltd

(Nottingham, UK), diclofenac and 4-hydroxydiclofenac were gifts from Novartis (Basel, Switzerland). BSA and other general laboratory reagents were also purchased from Sigma Chemical Co and BDH (Lutterworth, Leics, UK).

Source and preparation of human liver microsomes

Human liver samples ($n=9$) were acquired from the International Institute for the Advancement of Medicine (Exeter, PA, USA) and ($n=6$) from nontransplantable material from Addenbrookes Hospital (Cambridge, UK). All human liver samples were stored on arrival at $-80°$ C until microsomes were prepared using the ultracentrifugation method described previously [19] with the exception of the homogenizing and resuspension buffer (0.25 M sucrose, 50 mM Tris, pH 7.4) and the storage buffer (0.1 M phosphate, pH 7.4). CYP and protein contents were determined in microsomes using established methods [20, 21].

Microsomal incubations

In all cases experiments were undertaken to identify the initial rate conditions with respect to time and microsomal protein concentration. All further experiments were within the appropriate linear conditions and were carried out in duplicate. In all cases microsomes ($n=15$, 1 mg ml^{-1}, except for tolbutamide which was 0.75 mg ml^{-1}) were preincubated with substrate for 2 min in a shaking water bath at 37° C. Reactions were started by the addition of 100 µl of an NADPH regenerating system (1 mM $NADP^{+}$, 7.5 mM isocitric acid, 1 U isocitrate dehydrogenase, 0.015 M magnesium chloride, 0.1 M phosphate buffer, pH 7.4). In order to minimize the effects of organic solvent on enzyme activity, the final concentration of solvent in the incubation was kept between 0.5 and 1% (v/v). Preliminary data indicates that the solvents used had only small effects on CYP2C9 activity.

Phenytoin Microsomes were incubated with ^{14}C and unlabelled phenytoin (both added in methanol) to yield final drug concentrations of 5, 30, 100 and 200 µM (methanol volume of 10 µl), the final incubation volume was 1 ml. Reactions were terminated after 120min by the addition of 5 ml of tert-butyl-methylether and samples were immediately tumble mixed for 1 h. The organic layer was transferred to clean tubes and evaporated to dryness under oxygen-free nitrogen. Samples were resuspended in 150 µl of h.p.l.c. mobile phase. In addition, incubations were also carried out as described in the presence of 2% BSA ($n=8$).

Tolbutamide Microsomes were incubated with tolbutamide (0.05, 0.10, 0.50 and 1.0 mM, added in 3.75 µl methanol), the incubation volume was 0.75 ml. Reactions were terminated after 30 min by the addition of 50 µl of concentrated hydrochloric acid, samples were extracted as described elsewhere [19]. In addition, incubations were also carried out as described in the presence of 2% BSA ($n=8$).

(S)-Ibuprofen Microsomes were incubated with (S)-ibuprofen (10–500 µM, added in 5 µl dimethylformamide), the incubation volume was 500 µl. Incubations were terminated after 30 min by the addition of 50 µl of concentrated hydrochloric acid. Flurbiprofen was subsequently added (25 µl of 250 µg ml^{-1}, in methanol) as an internal standard and samples tumble mixed for 30 min with 5 ml chloroform. The aqueous layer was aspirated off and the organic layer evaporated to dryness under oxygen-free nitrogen. The residue was reconstituted in 200 µl of 0.05% phosphoric acid and acetonitrile (69 : 31, v:v).

Diclofenac Microsomes were incubated with diclofenac (5, 30 and 100 µM, added in 10 µl of water), the incubation volume was 200 µl. Reactions were terminated after 20 min by the addition of 50 µl of acetonitrile. Samples were then centrifuged in a microcentrifuge for 10 min in order to precipitate the protein.

Binding studies with phenytoin and tolbutamide

Microsomes (liver G, 1 mg ml^{-1} for phenytoin and 0.75 mg ml^{-1} for tolbutamide) were incubated in a shaking water bath at 37° C with either ^{14}C and unlabelled phenytoin (1–200 µM) or ^{14}C and unlabelled tolbutamide (50–1000 µM) in 0.2 µm microfiltration tubes (Whatman, UK) for 30 min in the absence of an NADPH regenerating system. In addition the samples were incubated in the presence and absence of 2% BSA. After the incubation the samples were immediately centrifuged for 10 min in a microcentrifuge to separate the bound and unbound drug. Aliqouts of the filtrate were mixed with liquid scintillant and counted in an LKB liquid scintillation counter. Incubations were carried out in triplicate.

H.p.l.c. analysis

Phenytoin Microsomal incubates were analysed as described previously [19] with an increased flow rate of 2 ml min^{-1} employing an on-line radioflow scintillation counter (Hewlett Packard, UK). The peak area of the hydroxyphenytoin, confirmed by coelution with spiked unlabelled metabolite and u.v. detection was measured.

Results were calculated in molar equivalents accounting for a concentration independent extraction efficiency of 97.2 ± 5.1% (1–200 μM, $n = 40$).

Tolbutamide Microsomal extracts were analysed as described previously for rat hepatic microsomal tissue [19].

(S)-Ibuprofen The method followed was a modification of that of Lockwood & Wagner [22]. 100 μl of the reconstituted samples were injected onto a Spherisorb 5ODS C8 column. Mobile phase comprising solvent A (0.05% phosphoric acid, containing 0.1% triethylamine, v : v) and solvent B (acetonitrile) was delivered (1.6 ml min^{-1}) for 0–8 min at 31% solvent B. Between 8 and 16 min solvent B concentration was increased linearly to 65%, solvent B concentration was then reduced to 31% over 5 min. The eluent was monitored at 220 nm, retention times were as follows: 2-hydroxy metabolite, 5.7 min, 3-hydroxy metabolite, 6.7 min, flurbiprofen, 15.1 min and (S)-ibuprofen, 16.1 min. A standard curve was constructed in samples incubated in the absence of a regenerating system (which was added after the acid) using 3-hydroxy (S)-ibuprofen as the standard, the 2-hydroxy metabolite could not be obtained, thus the 3-hydroxy standard was used to quantify this metabolite, as previously has been adopted [23]. Results were quantified by the peak height ratio method with respect to the internal standard.

Diclofenac The method employed was a modification of that described by Godbillon and coworkers [24]. 100 μl of the microsomal supernatant was injected onto a Hypersil 5ODS C18 column, employing a mobile phase (1 ml min^{-1}) of methanol : acetonitrile : 1% (v:v) acetic acid (55 : 7:38, v : v : v). The eluent was monitored at 282 nm, and a standard curve constructed in samples incubated in the absence of a regenerating system which was added after the organic solvent. The retention times of the 4′-hydroxy metabolite and diclofenac were 6.2 and 12.9 min, respectively.

Data analysis

In vitro *kinetic data* In order to reduce the number of drug concentrations required to accurately determine Michaelis-Menten parameters by nonlinear regression, an alternative approach was adopted for phenytoin, diclofenac and tolbutamide. There is an abundance of data which confirms that the formation of the metabolites is monophasic, characterized by V_{max} and K_m for phenytoin [14, 25], tolbutamide [25–27] and diclofenac [28, 29]. Therefore the direct-linear plot has been employed which enables V_{max} and K_m to be estimated from rates measured at only two to four substrate concentrations [30]. For the tolbutamide and phenytoin incubations conducted in the presence of albumin the free substrate concentrations were used in the estimation of K_m. An initial pilot study with tolbutamide, employed five microsomal samples and eight substrate concentrations. The data were analysed using nonlinear regression (as described below), the V_{max} and K_m values were statistically compared (Wilcoxon test) with the corresponding values obtained using the direct-linear plot with four substrate concentrations, there was no statistically significant difference between the two approaches ($P < 0.05$).

A larger substrate concentration range was employed for (S)-ibuprofen in view of the less well reported metabolite kinetics, and the discrepancy between livers which indicate monophasic or biphasic kinetics [23]. The data were then analysed using nonlinear regression software (Siphar, Cimed, Creteil, France), using both a one and two site model, the one site model was chosen based on visual inspection of the line of best fit, consideration of the randomness of the residuals, the standard error of the parameter estimates (< 10%) and the Aikaike information criteria.

The individual CL_{int} values obtained for the five metabolites were compared between the substrates using the Spearman Rank correlation analysis, to test for significant correlations between the compounds. The phenytoin and tolbutamide samples incubated in the presence of albumin were excluded from this analysis.

The V_{max} and K_m estimates obtained in the presence of 2% BSA for phenytoin and tolbutamide were statistically compared using the Wilcoxon test to the corresponding parameters determined in the absence of BSA.

Calculation of in vivo *CL_{int} from literature data* In order to assess the accuracy of microsomal predictions of pharmacokinetic behaviour it is necessary to estimate the observed *in vivo* CL_{int} values. Therefore literature reports were examined to enable CL_{int} to be calculated. However, several criteria had to be satisfied in order for a study to be included, these were; oral dosage (providing the amount absorbed was known), the dosage must be within the region of linear kinetics, the plasma protein binding should be known and preferably the blood : plasma concentration also reported. The venous equilibration model was assumed and eqn 1 used to estimate CL_{int}:

$$CL_{int} = \frac{fm.D}{fub.AUC} \qquad (1)$$

where fm is the fraction of drug converted to a metabolite, D is the dose, AUC is the area under the plasma concentration-time curve and fub is the unbound fraction of drug in the blood. If the blood : plasma

© 1999 Blackwell Science Ltd

concentration ratio is unknown then it is possible to calculate a value for this parameter if the drug binds extensively to albumin, as is the case with tolbutamide (S)-ibuprofen and diclofenac. If drug is highly bound to plasma proteins the ratio of the concentration of drug in the blood cell to the unbound drug in plasma approaches 0, therefore the blood:plasma concentration ratio reduces to (1—the haematocrit), i.e. 0.55 [31].

Scaling microsomal parameters to in vivo *CL_{int}* The CL_{int} values obtained for each of these substrates were scaled to *in vivo* units, employing a scaling factor based on the microsomal protein index, which reflects the inefficiency of the microsomal preparation procedure. There are only a handful of reported values for this scaling factor, and they range from 15 mg microsomal protein g^{-1} liver [32] to 77 mg g^{-1} [33]. This range is similar to that reported for rat microsomal protein recovery and it has been recommended that a literature average of around 45 mg g^{-1} be adopted [2, 34]. A recovery of 52.5 mg g^{-1} has been calculated based on the CYP contents of human hepatocytes (0.14 nmol CYP/10^6 cells) and microsomal tissue (0.32 nmol CYP mg^{-1} microsomal protein) and assuming a hepatocellularity of 120×10^6 cells g^{-1} liver [3]. Therefore, taking into account the range of human recovery reflecting that of rat, and the calculated recoveries for both species, a scaling factor of 50 mg g^{-1} liver has been used, together with a liver weight of 1500 g 70 kg^{-1} man.

Results

Microsomal incubations

The mean CYP content of the 15 microsomal samples employed in the present study was 0.27 nmol mg^{-1} protein (s.d.: 0.10). The metabolite formation kinetics for the five metabolites monitored are presented in Table 2 as the parameters V_{max}, K_m and CL_{int} (defined as the V_{max}/K_m ratio). The V_{max} values for each substrate displayed between four-and 10-fold interindividual variability, the K_m values showed slightly lower variability between samples, with the exception of tolbutamide. For this drug there is around 12-fold variability, resulting from one sample having a K_m (838 μM) considerably higher than the other livers (67–520 μM) although this is still within the substrate concentration range employed. The CL_{int} values reflect these interindividual variations in V_{max} and K_m, with five-to 19-fold variations between samples. The range of mean CL_{int} values for the four drugs exceeds three orders of magnitude, from 0.18 μl min^{-1} mg^{-1} protein (phenytoin) to 40.70 μl min^{-1} mg^{-1} protein (diclofenac). The individual CL_{int} values for the 15 microsomal samples are shown in Figure 1 for the five pathways monitored. Statistically significant correlations were obtained between diclofenac and tolbutamide ($P<0.001$), diclofenac and phenytoin ($P<0.01$), tolbutamide and phenytoin ($P<0.05$) and (S)-ibuprofen 2-hydroxylation and (S)-ibuprofen 3-hydroxylation ($P<0.05$), thus confirming the involvement of CYP2C9 in diclofenac, phenytoin and tolbutamide metabolism. The lack of correlations between either of the (S)-ibuprofen metabolites with any of these substrates indicates that other isoforms may be involved in their formation. However, the V_{max} values for both pathways were statistically significantly correlated with diclofenac ($P<0.01$), and the 2-hydroxy pathway also correlated with phenytoin ($P<0.05$). Recent evidence has shown correlations between the formation rates of the 2-hydroxy and 3-hydroxy metabolites and dextromethorphan *O*-demethylation, a CYP2D6 marker, and chlorzoxazone 6-hydroxylation, a CYP2E1 marker, in addition to the 6α-hydroxylation of the CYP2C8 probe taxol [23] indicating that other CYP isoforms, in particular 2C8, may contribute to (S)-ibuprofen metabolism as well as CYP2C9.

In vivo *Cl_{int} values*

Table 1 presents a summary of the *in vivo* CL_{int} data obtained for the four substrates studied, taken from literature reports. Both phenytoin and tolbutamide have been studied more extensively than the other two compounds, reflecting their long therapeutic usage and, in the former case, the need for therapeutic drug monitoring due to its nonlinear kinetics. Most ibuprofen studies have used the racemate, since this is the clinically administered form. However, two studies have investigated the (S)-isomer, the active form of this nonsteroidal anti-inflammatory drug. The mean CL_{int} values range over three orders of magnitude, with tolbutamide having the lowest value and diclofenac the highest. The interindividual range of values for each substrate varies from two-fold, for both metabolites of (S)-ibuprofen to almost seven-fold for tolbutamide. The latter range is more in keeping with the range of microsomal CL_{int} values.

Scaling microsomal data to in vivo

The microsomal CL_{int} data reported in Table 2 were scaled to *in vivo* units of l min^{-1} 70 kg^{-1} man, to enable direct comparison with the observed parameters. These scaled data, together with the predicted/observed ratio of the mean values are presented in Table 3. In general the mean predicted CL_{int} values are poor, as demonstrated by the low predicted/observed CL_{int} ratios of 0.05–0.31. The exception to this trend is shown with tolbutamide, for which a ratio of 0.72 was obtained. Figure 2 illustrates

Table 2 Metabolite formation kinetics for phenytoin, tolbutamide, (S)-ibuprofen and diclofenac metabolism in human hepatic microsomes[a].

Substrate	*BSA*	V_{max}[b]	K_m[c]	CL_{int}[d]
Phenytoin	–	0.008 ± 0.003	56 ± 30	0.18 ± 0.11
		(0.003–0.013)	(21–117)	(0.08–0.43)
Phenytoin[e]	+	0.006 ± 0.004	7 ± 4***	1.15 ± 0.61**
		(0.003–0.016)	(2–15)	(0.17–1.88)
Tolbutamide	–	0.207 ± 0.083	254 ± 212	1.35 ± 1.23
		(0.065–0.351)	(67–838)	(0.27–4.00)
Tolbutamide[e]	+	0.094 ± 0.036*	13 ± 5**	7.88 ± 3.11***
		(0.025–0.143)	(6–23)	(3.05–14.00)
(S)-Ibuprofen	–	0.171 ± 0.097	77 ± 54	3.30 ± 2.22
(2-hydroxy metabolite)		(0.042–0.389)	(13–196)	(0.40–6.62)
(S)-Ibuprofen	–	0.576 ± 0.482	110 ± 53	6.32 ± 5.51
(3-hydroxy metabolite)		(0.090–1.910)	(29–200)	(1.00–18.62)
Diclofenac	–	0.715 ± 0.363	22 ± 11	40.70 ± 26.20
		(0.339–1.423)	(6–40)	(14.00–96.30)

[a]Results as expressed as mean ± s.d., with range in parentheses, $n = 15$ for all samples except [e] $n = 8$ due to sample shortage. [b]V_{max} values expressed as nmol min^{-1} mg^{-1} microsomal protein. [c]K_m values expressed as μM. [d]CL_{int} values expressed as μl min^{-1} mg^{-1} microsomal protein. Statistical comparisons of the kinetic parameters for phenytoin and tolbutamide between the absence and presence of BSA in the incubation were not significant ($P > 0.05$), except *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

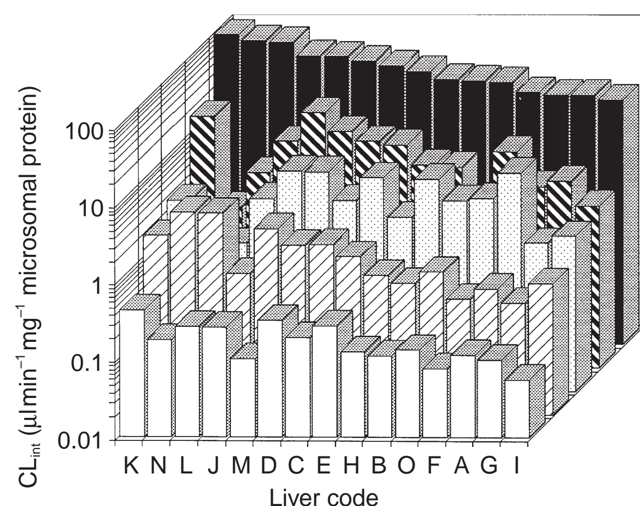

Figure 1 CL_{int} values for individual livers ($n = 15$) for phenytoin (□), tolbutamide (▨) (S)-ibuprofen 2-hydroxy metabolite (▦) (S)-ibuprofen 3-hydroxy metabolite (▧) and diclofenac (■).

these predictions and demonstrates that, in general, the same rank order of metabolic stability is observed *in vitro* as in *in vivo*. Also a similar range of CL_{int} values are obtained extending over three orders of magnitude. It is clear that the *in vitro* data are underpredicting, with the exception of tolbutamide.

There is a trend for the accuracy of the microsomal prediction to increase linearly with the *in vitro* K_m value. Thus, the lowest predicted/observed CL_{int} ratios (0.05 and 0.07) are achieved for the low K_m substrates, diclofenac (22 μM) and phenytoin (56 μM), whereas the ratio for tolbutamide (0.71) with a K_m of 254 μM, is much more accurate.

Phenytoin and tolbutamide binding studies in microsomes

Phenytoin binding in microsomes, in the absence of exogenous BSA, was concentration independent over the range 1–200 μM used for the microsomal studies, with a mean unbound fraction of 0.36 ± 0.07. In the presence of 2% albumin this value decreased further to 0.25 ± 0.06. Figure 3 presents these data, together with the corresponding tolbutamide binding data. This latter compound demonstrated concentration dependent binding in the presence of 2% BSA over the concentration range used in the microsomal studies, 50–1000 μM. However, in the absence of albumin there was no binding, the free fraction was 1.0 ± 0.05.

The metabolite formation kinetic parameters determined for these two drugs in the presence of 2% BSA are presented in Table 2. In the presence of 2% BSA there are statistically significant decreases in the K_m values for both phenytoin and tolbutamide. In contrast, only the V_{max} value for tolbutamide alters significantly with the addition of albumin, an approximate two-fold reduction. Interestingly both compounds show a statistically significant six-fold increase in CL_{int}. Figure 4 presents a comparison of the reduction in K_m, as defined by the ratio of K_m values in the absence : presence of albumin, for tolbutamide and phenytoin in each microsomal preparation. Linear regression analysis of these data yields a correlation coefficient of 0.709, which is

 © 1999 Blackwell Science Ltd

Table 3 Comparison of observed *in vivo* intrinsic clearances with predicted values from hepatic microsomes.

Substrate	*BSA*	*Predicted* in vivo CL_{int} [a] *Mean*	*Low*	*High*	*Predicted/Observed mean CL_{int} ratio*
Phenytoin	−	0.014	0.006	0.032	0.05
Phenytoin	+	0.086	0.013	0.141	0.31
Tolbutamide	−	0.101	0.020	0.300	0.72
Tolbutamide	+	0.591	0.229	1.050	4.22
(S)-Ibuprofen (2-hydroxy metabolite)	−	0.248	0.030	0.248	0.11
(S)-Ibuprofen (3-hydroxy metabolite)	−	0.474	0.075	0.474	0.13
Diclofenac	−	3.053	1.226	3.053	0.07

[a] CL_{int} values expressed as l min^{-1} 70 kg^{-1} man, values in parentheses represent the range.

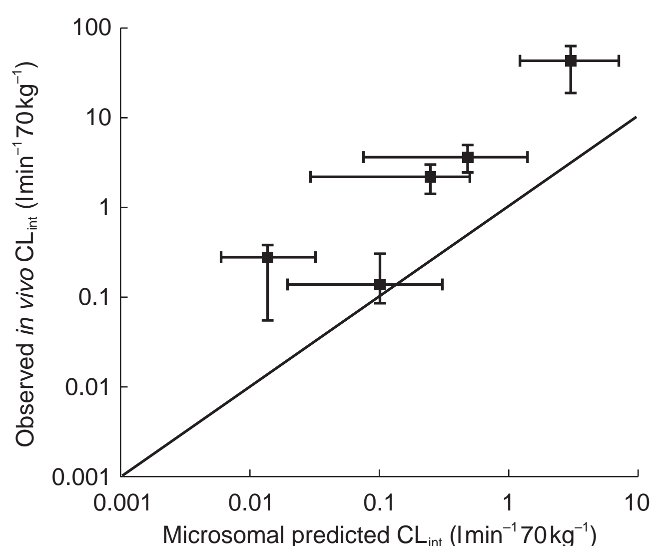

Figure 2 Comparison of observed *in vivo* CL_{int} with CL_{int} values predicted from microsomal studies. Each point represents the mean value together with the range for the observed and predicted CL_{int} values. The solid line represents the line of unity.

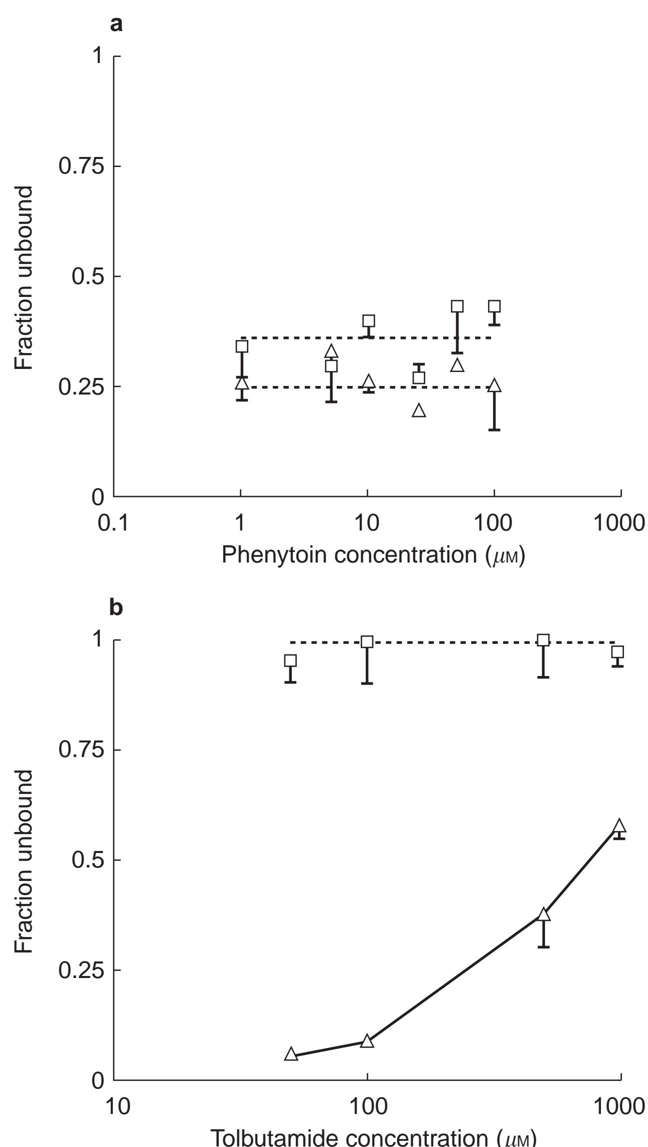

Figure 3 Microsomal binding of phenytoin (a) and tolbutamide (b) as a function of drug concentration in the absence (□) and presence (△) of 2% BSA.

statistically significant ($P<0.05$), thus there are similar decreases in K_m for each microsomal sample with both drugs.

Figure 5 illustrates the degree of overlap of the *in vitro* CL_{int} values for the 15 individual livers and the range of *in vivo* CL_{int} values reported in the literature for both phenytoin and tolbutamide. Although the addition of BSA produces an increase in both sets of *in vitro* CL_{int} values, there is a good degree of overlap between these parameters estimated from both *in vitro* systems and the corresponding *in vivo* parameters.

Discussion

To assess the utility of human microsomes in predicting *in vivo* CL_{int} values, the metabolite formation kinetics of four CYP2C9 substrates have been determined in human microsomes. These substrates were selected based on

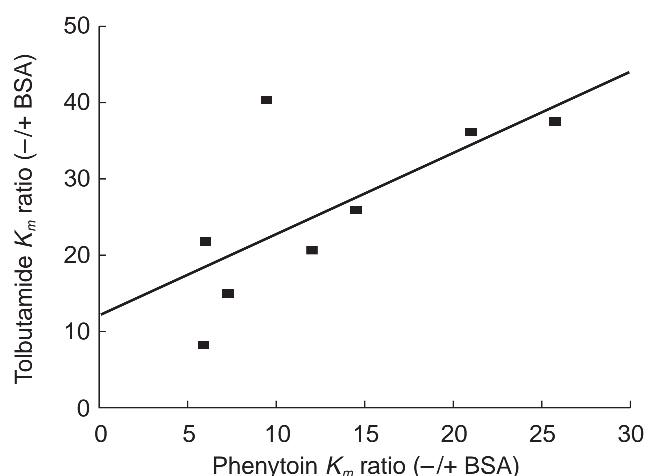

Figure 4 Relationship between change in K_m for phenytoin and tolbutamide after the addition of albumin, expressed as the -/+ BSA ratio.

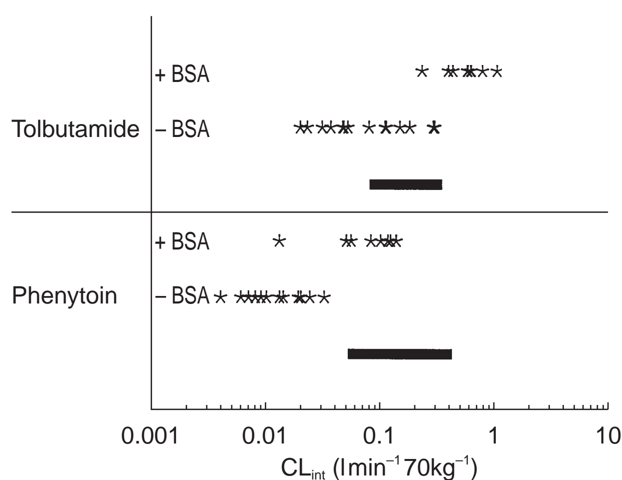

Figure 5 Range of observed *in vivo* CL_{int} values (—) for phenytoin and tolbutamide together with the corresponding range of predicted CL_{int} values from microsomes (*) in the absence and presence of BSA.

their range of *in vivo* CL_{int} values, spanning several orders of magnitude. The kinetic parameters determined *in vitro* display the expected range of activity: for V_{max} of 0.008–0.715 nmol min^{-1} mg^{-1} protein, for K_m of 7–254 μM and for CL_{int} of 0.18 –40.70 μl min^{-1} mg^{-1} protein. The rank order of the latter parameter was consistent in both *in vitro* and *in vivo* systems. However, in absolute terms, scaling the *in vitro* CL_{int} data to *in vivo* units using the microsomal protein index as the scaling factor provided predictions which were consistently lower than the reported *in vivo* data.

It has been proposed, based on animal investigations, that acceptable CL_{int} predictions should fall within the precision limits of 50–200%, i.e. between a 50% underprediction to a 100% overprediction [2]. Application of these limits to the mean data would indicate that only the prediction for tolbutamide is accurate, 72% of the observed CL_{int}. The other four other pathways are underpredicted, between 5 and 13% of the observed value, based on the mean values. Interestingly these data (phenytoin, diclofenac and both ibuprofen metabolites) appear to lie on a line parallel to the line of identity implying a consistent underprediction. One possible cause of consistent underprediction would be that the recovery of CYP2C9 during microsomal preparation differs markedly from that of other CYPs and hence this is not reflected in the total CYP measured spectrophotometrically. It is interesting that there is a linear trend between the accuracy of the predicted CL_{int} value and the K_m estimated *in vitro* whereas no such relationship exists for V_{max}, implying that there are problems associated with the determination of the former parameter. Confirmation of this is provided by considering phenytoin, a drug which exhibits nonlinear kinetics *in vivo* consistent with enzyme saturation. Estimates of V_{max} *in vivo* range from 0.8–5.6 μmol min^{-1} 70 kg^{-1}, with a mean value of ≈1.4 μmol min^{-1} 70 kg^{-1} [35–37]. Scaling the microsomal V_{max} values using the microsomal protein recovery, yields a mean estimate of 0.6 μmol min^{-1} 70 kg^{-1} with a range 0.2–1.0 μmol min^{-1} 70 kg^{-1}. Thus the mean V_{max} estimate is 43% of the observed value. However, the CL_{int} prediction is much lower, representing just 5% of the observed value, indicating that the major source of the underprediction is associated with the K_m. Observed unbound K_m values *in vivo* range between 0.06 and 11.8 with a mean of 3.4 μM [35–37] whereas the mean microsomal value is an order of magnitude larger, 56 μM. A recent study by Ludden and coworkers [14] has observed a similar discrepancy with phenytoin K_m *in vitro*. They estimated a similar K_m value to ours (31 μM, $n=7$), Doecke and coauthors [25] also estimated a K_m value of 30 μM ($n=3$). We have determined that there is an appreciable degree of phenytoin binding in standard microsomal incubations (i.e. in the absence of albumin), of ≈64% and this contradicts the assumption of Ludden *et al.* [14]. Correcting the mean K_m for this binding yields an estimate of 20 μM, which is still substantially higher than the observed *in vivo* value, and incorporation of this value raises the mean CL_{int} prediction to 0.03 l min^{-1} 70 kg^{-1} (representing 11% of the observed value). It has been demonstrated that the addition of BSA to microsomal incubations resulted in unbound K_m values more comparable to the *in vivo* situation [14]. Therefore we examined the effect of 2% BSA on the kinetic parameters for phenytoin and also on tolbutamide, since the CL_{int} of this compound was well predicted, and it is also bound to albumin.

Albumin caused substantial decreases in the unbound K_m values for both substrates, with the degree of interindividual variability being maintained. Although

 © 1999 Blackwell Science Ltd

there was a slight decrease in phenytoin V_{max} this was not statistically significant, however, the corresponding parameter for tolbutamide was reduced by more than half. The net effect of these changes is a six-fold increase in CL_{int} for both compounds. Thus the unbound K_m for phenytoin, 7 μM, is within the range reported for the plasma unbound K_m noted above.

The consequences of the addition of BSA to the scaled CL_{int} can be seen in Figure 5. For phenytoin there is a distinct improvement in the prediction of the observed CL_{int}. Moreover, there is no appreciable improvement for the prediction of tolbutamide CL_{int} using the unbound K_m. In fact if only the mean data are considered, then the change from 0.1 to 0.59 l min^{-1} 70 kg^{-1} results in an overestimation of the observed value (0.14 l min^{-1} 70 kg^{-1}). The mechanism by which BSA alters the kinetic parameters for metabolite formation is unclear. A possible explanation is that albumin acts by 'cleaning up' the microsomal incubation in some way, perhaps by binding some endogenous agent which binds to the enzyme. There is some indirect evidence for this since there is a direct relationship in the K_m changes between individual livers for these substrates. Unfortunately it was not feasible to assess the effects of BSA on the other two substrates due to the particularly extensive albumin binding that both diclofenac and ibuprofen demonstrate.

A previous study with rat hepatic microsomes also demonstrated similar results with regard to predictions of *in vivo* CL_{int} for phenytoin and tolbutamide, with the former being poorly predicted and the latter accurately predicted [19]. However, in that study, end-product inhibition was proposed as the reason for the poor microsomal prediction, this is less likely to be the case in the present study since the rate of hydroxyphenytoin formation is substantially lower in human microsomes, and hence there is much less metabolite accumulation.

Interindividual variability in CL_{int} confounds *in vitro-in vivo* comparisons due to the inability to match healthy volunteer subjects with human tissue donors; thus the direct comparison of mean data is compromised. In order to address one side of this issue we have collated data concerning the *in vivo* pharmacokinetics of our substrates using various data sources, and for phenytoin and tolbutamide there are a reasonably large number of individuals. It is preferable that for comparisons of *in vitro* and *in vivo* human data that the latter data should be obtained from more than one source. With regard to the *in vitro* studies, we have employed a microsomal liver bank prepared from 15 individuals and this provided a 10-fold range of kinetic parameters. The advantages of considering the range of individual parameters obtained in *in vitro-in vivo* studies rather than the mean values are illustrated by Figures 2 and 5.

In summary, the present study illustrates the complexities involved in the quantitative prediction of CL_{int}. Although hepatic extraction classification can be made based on the rank order of the *in vitro* data, there is a consistent underprediction of absolute CL_{int} values. When the range of parameter values are incorporated into the analysis a better appreciation of the utility of the *in vitro* prediction from human microsomes is obtained. The addition of albumin to the microsomal incubation does affect the K_m estimation of both tolbutamide and phenytoin. However, the advantages in the determination of CL_{int} were not consistent, therefore based on the current available information it is concluded that the addition of albumin to microsomal incubations does not assist in *in vitro-in vivo* comparisons.

DJC was financially supported by a GlaxoWellcome postdoctoral fellowship. The assistance of Judith Hargreaves in a portion of this work is gratefully acknowledged.

References

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

2 Houston JB, Carlile DJ. Prediction of hepatic clearance from microsomes, hepatocytes and liver slices. *Drug Met Rev* 1997; **29**: 891–922.

3 Iwatsubo T, Hirota N, Ooie T, *et al*. Prediction of *in vivo* drug metabolism in the human liver from *in vitro* metabolism data. *Pharmacol Ther* 1997; **73**: 147–171.

4 Miners JO, Birkett DJ. Cytochrome P4502C9: an enzyme of major importance in human drug metabolism. *Br J Clin Pharmacol* 1998; **45**: 525–538.

5 Guengerich FP, Turvy CG. Comparison of levels of several human microsomal cytochrome P-450 enzymes and epoxide hydrolase in normal and disease states using immunochemical analysis of surgical liver samples. *J Pharmacol Exp Ther* 1991; **256**: 1189–1195.

6 Shimada T, Yamazaki H, Mimura M, Inui Y, Guengerich FP. Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J Pharmacol Exp Ther* 1994; **270**: 414–423.

7 Forrester LM, Henderson CJ, Glancey MJ, *et al*. Relative expression of cytochrome P450 isoenzymes in human liver and associations with the metabolism of drugs and xenobiotics. *Biochem J* 1992; **281**: 359–368.

8 Veronese ME, Miners JO, Rees DLP, Birkett DJ. Tolbutamide hydroxylation in humans: lack of bimodality in 106 healthy subjects. *Pharmacogenenetics* 1993; **3**: 86–93.

9 Dickinson RG, Hooper WD, Patterson M, Eadie MJ, Maguire B. Extent of urinary excretion of p-hydroxyphenytoin in healthy subjects given phenytoin. *Ther Drug Monit* 1985; **7**: 283–289.

10 Thomas RC, Ikeda GJ. The metabolic fate of tolbutamide in man and in the rat. *J Med Chem* 1966; **9**: 507–510.

11 Rudy AC, Knight PM, Brater DC, Hall SD. Stereoselective

metabolism of ibuprofen in humans: administration of *R*-, *S*- and racemic ibuprofen. *J Pharmacol Exp Ther* 1991; **259**: 1133–1139.
12 Riess W, Stierlen H, Geiger UP, *et al*. Data on the pharmacokinetics and metabolism of diclofenac (Voltaren) in animals and man. In: *Chronic Forms of Polyarthritis*, Ed Wagenhauser FJ, Berne, Stuttgart and Vienna: Huber, 1976: 302–317.
13 Stierlin H, Faigle JW, Sallmann A, *et al*. Biotransformation of diclofenac sodium (Voltaren) in animals and in man I. Isolation and identification of principal metabolites. *Xenobiotica* 1979; **9**: 601–610.
14 Ludden LK, Ludden TM, Collins JM, Pentikis HS, Strong JM. Effect of albumin on the estimation, *in vitro*, of phenytoin V_{max} and K_m values: Implications for clinical correlation. *J Pharmacol Exp Ther* 1997; **282**: 391–396.
15 Tassaneeyakul W, Veronese ME, Birkett DJ, *et al*. Co-regulation of phenytoin and tolbutamide metabolism in humans. *Br J Clin Pharmacol* 1992; **34**: 494–498.
16 Evans AM, Nation RL, Sansom LN, Bochner F, Somogyi AA. Stereoselective plasma protein binding of ibuprofen enantiomers. *Eur J Clin Pharmacol* 1989; **36**: 283–290.
17 John VA. The pharmacokinetics and metabolism of diclofenac sodium (Voltarol) in animals and man. *Rheumatol Rehab Suppl* 1979; **2**: 22–37.
18 Chan KKH, Vyas KH, Brandt KD. *In vitro* protein binding of diclofenac sodium in plasma and synovial fluid. *J Pharm Sci* 1987; **76**: 105–108.
19 Ashforth EIL, Carlile DJ, Chenery R, Houston JB. Prediction of *in vivo* disposition from *in vitro* systems: Clearance of phenytoin and tolbutamide using rat hepatic microsomal and hepatocyte data. *J Pharmacol Exp Ther* 1995; **274**: 761–766.
20 Omura T, Sato R. The carbon monoxide binding pigment of liver microsomes. *J Biol Chem* 1964; **239**: 2370–2378.
21 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951; **193**: 265–275.
22 Lockwood GF, Wagner JG. High-performance liquid chromatographic determination of ibuprofen and its major metabolites in biological fluids. *J Chromatogr* 1982; **232**: 335–343.
23 Hamman MA, Thompson GA, Hall SD. Regioselective and stereoselective metabolism of ibuprofen by human cytochrome P450, 2C. *Biochem Pharmacol* 1997 54: 33–41.
24 Godbillon J, Gauron S, Metayer JP. High perfomance liquid chromatographic determination of diclofenac and its monohydroxylated metabolites in biological fluids. *J Chromatogr* 1985; **338**: 151–159.
25 Doecke CJ, Veronese ME, Pond SM, *et al*. Relationship between phenytoin and tolbutamide hydroxylations in human liver microsomes. *Br J Clin Pharmacol* 1991; **31**: 125–130.
26 Miners JO, Smith KJ, Robson RA, McManus ME, Veronese ME, Birkett DJ. Tolbutamide hydroxylation by human liver microsomes. Kinetic characterisation and relationship to other cytochrome P-450 dependent xenobiotic oxidations. *Biochem Pharmacol* 1988; **37**: 1137–1144.
27 Back DJ, Stevenson P, Tjia JF. Comparative effects of two antimycotic agents, ketoconazole and terbinafine on the metabolism of tolbutamide, ethinyloestradiol, cyclosporin and ethoxycoumarin by human liver microsomes *in vitro. Br J Clin Pharmacol* 1989; **28**: 166–170.
28 Leeman T, Transon C, Dayer P. Cytochrome P 450TB, (CYP2C): a major monooxygenase catalyzing diclofenac 4′hydroxylation in human liver. *Life Sci* 1992; **52**: 29–34.
29 Shen S, Marchick MR, Davis MR, Pohl LR. 5-Hydroxylation of diclofenac by human cytochrome P450 3A4: Possible role in idiosyncratic hepatotoxicity. *ISSX Proceedings*, 6th European ISSX Meeting, Gothenburg, 1997; 102.
30 Eisenthal R, Cornish-Bowden A. The direct linear plot. A new graphical procedure for estimating enzyme kinetic parameters. *Biochem J* 1974; **139**: 715–720.
31 Rowland M, Tozer N. Blood to plasma concentration ratio. In *Clinical Pharmacokinetics Concepts and Applications* 2nd edn. eds. Rowland M, Tozer TN, Lea and Febiger, Philadelphia and London, 1989: 476–478.
32 Schmucker DL, Woodhouse KW, Wang RK, *et al*. Effects of age and gender on *in vitro* properties of human liver microsomal monooxygenases. *Clin Pharmacol Ther* 1990; **48**: 365–374.
33 Baarnhielm C, Dahlback H, Skanberg I. *In vivo* pharmacokinetics of felodipine predicted from *in vitro* studies in rat, dog and man. *Acta Pharmacol Toxicol* 1986; **59**: 113–122.
34 Carlile DJ, Zomorodi K, Houston JB. Scaling factors to relate drug metabolic clearance in hepatic microsomes, isolated hepatocytes and the intact liver. Studies with induced livers involving diazepam. *Drug Metab Disp* 1997; **25**: 903–911.
35 Eadie MJ, Tyrer JH, Bochner F, Hooper WD. The elimination of phenytoin in man. *Clin Exp Pharmacol Physiol* 1976; **3**: 217–224.
36 Jusko WJ. Bioavailability and disposition kinetics of phenytoin in man. In *Quantitative Analytic Studies in Epilepsy* eds. Kellaway P, Petersen I, New York: Raven Press, 1976: 115–136.
37 Winter ME, Tozer TN. Phenytoin. In *Applied Pharmacokinetics: Principles of Theraputic Drug Monitoring* 2nd edn. eds. Evans WE, Schentag JJ, Jusko WJ, Vancouver, WA. Applied Therapeutics Inc, 1986: 493–539.
38 Svendsen TL, Kristensen MB, Hansen JM, Skovsted L. The influence of disulfiram on the half life and metabolic clearance rate of diphenylhydantoin and tolbutamide in man. *Eur J Clin Pharmacol* 1976; **9**: 439–441.
39 Gugler R, Manion CV, Azarnoff DL. Phenytoin: Pharmacokinetics and bioavailability. *Clin Pharmacol Ther* 1976; **19**: 135–142.
40 Grasela TH, Sheiner LB, Rambeck B, *et al*. Steady-state pharmacokinetics of phenytoin from routinely collected patient data. *Clin Pharmacokinet* 1983; **8**: 355–364.
41 Kurata D, Wilkinson GR. Erythrocyte uptake and plasma binding of diphenylhydantoin. *Clin Pharmacol Ther* 1974; **16**: 355–362.
42 Carulli N, De Ponz Leon M, Mauro E, Manenti F, Ferrari A. Alteration of drug metabolism in Gilbert's syndrome. *Gut* 1976; **17**: 581–587.
43 Williams RL, Blaschke TF, Meffin PJ, Melmon KL, Rowland M. Influence of acute viral hepatitis on disposition and plasma binding of tolbutamide. *Clin Pharmacol Ther* 1977; **21**: 301–309.

© 1999 Blackwell Science Ltd

44 Zilly W, Breimer DD, Richter E. Stimulation of drug metabolism by rifampicin in patients with cirrhosis or cholestasis measured by increased hexobarbital and tolbutamide clearance. *Eur J Clin Pharmacol* 1977; **11**: 287–293.

45 Wing LMH, Miners JO. Cotrimazole as an inhibitor of oxidative drug metabolism: effects of trimethoprim and sulphamethoxazole separately and combined on tolbutamide disposition. *Br J Clin Pharmacol* 1985; **20**: 482–485.

46 Back DJ, Tjia J, Monig H, Ohnhaus EE, Park BK. Selective inhibition of drug oxidation after simultaneous administration of two probe drugs, antipyrine and tolbutamide. *Eur J Clin Pharmacol* 1988; **34**: 157–163.

47 Veronese ME, Miners JO, Randles GD, Birkett DJ. Validation of the tolbutamide metabolic ratio for population screening with use of sulphaphenazole to produce model phenotypic metabolizers. *Clin Pharmacol Ther* 1990; **47**: 403–411.

48 Page MA, Boutagy JS, Shenfield GM. A screening test for slow metabolisers of tolbutamide. *Br J Clin Pharmacol* 1991; **31**: 649–654.

49 Lee EJD, Williams K, Day R, Graham G, Champion D. Stereoselective disposition of ibuprofen enantiomers in man. *Br J Clin Pharmacol* 1985; **19**: 669–674.

50 Willis JV, Kendall MJ, Flinn RM, Thornhill DP, Welling PG. The pharmacokinetics of diclofenac sodium following intravenous and oral adminstration. *Eur J Clin Pharmacol* 1976; **16**: 405–410.

51 Drenska A, Ognyanova V, Tomoy I, *et al.* Feloran (diclofenac sodium). Pharmacokinetics in experimental animals and humans after oral administration. *Med Biol Inform* 1984; **2**: 12–16.

52 Degen PH, Dieterle W, Schneider W, Theobald W, Sinterhauf U. Pharmacokinetics of diclofenac and five metabolites after single doses in healthy volunteers and after repeated doses in patients. *Xenobiotica* 1988; **18**: 1449–1455.

53 Transon C, Leemann T, Vogt N, Dayer P. *In vivo* inhibition of cytochrome $P450_{TB}$ (CYP2C9) by (±)-fluvastatin. *Clin Pharmacol Ther* 1995; **58**: 412–417.