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Research Paper

Determination of a Human Hepatic Microsomal Scaling Factor for Predicting *in Vivo* Drug Clearance

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Purpose. To determine a microsomal scaling factor for human liver suitable for prediction of *in vivo* drug clearance from *in vitro* data and to explore the role of inter-liver variability in this factor on the reported underprediction from microsomal parameters.
Methods. Cytochrome P450 (henceforth P450) content in whole homogenates and microsomes from 38 donor livers was used to determine a microsomal scaling factor. In a subset (n = 20) of these preparations, individual P450 enzymes were examined by Western blotting and selective probe activities were determined.
Results. The scaling factor from 38 livers averaged 40 mg microsomal protein per gram liver with a coefficient of variation of 31%. Western blotting experiments indicated that there was no P450 enzyme-specific trend in the distribution of individual P450 enzymes in liver microsomes relative to whole homogenate. Predictions based on an average scaling factor resulted in a satisfactory prediction of intrinsic clearance of three benzodiazepines similar to that obtained using individual factors for the same livers.
Conclusion. A value for human liver microsomal scaling of 40 mg microsomal protein per gram liver has been established. The reason for underprediction previously reported for 52 different drug substrates was not the use of an incorrect value for the scaling factor.

KEY WORDS: human hepatic microsomal scaling factor; prediction of clearance; variability *in vitro*.

INTRODUCTION

Human liver microsomes and, increasingly, hepatocytes, are used widely to assess the metabolic stability of drugs (1,2). Implicit in this practice is the ability to scale *in vitro* kinetic parameters, e.g., intrinsic clearance, to *in vivo* pharmacokinetic parameters (3,4). A recent assessment of this procedure based on a compilation of 52 drugs of varying chemical structure, but all substrates for cytochrome P450 (henceforth P450), indicated that on average there was an 8-fold underprediction (5). This observation was based on data from a number of different sources and highlights our uncertainties in this scaling procedure.

There are two components to the *in vitro–in vivo* scaling procedure (4,6). The first concerns scaling the kinetic parameters from the *in vitro* incubation conditions under which they were obtained up to a whole-liver capacity. The second involves the integration of this whole liver capacity (intrinsic clearance, CLint) with other physiological determinants, namely, hepatic blood flow, drug blood binding, and, under some circumstances, uptake by hepatic transporters. We have recently assessed the modeling component by comparing the three liver models that are widely used in the literature (7). In this paper, we consider the first component, namely, the factors associated with the scale up from *in vitro* incubations to the whole liver.

For hepatocytes the scale up process is a very straightforward procedure, as data are expressed per million cells; hence multiplication by the hepatocellularity of the entire liver [120 million cells per gram (8,9)] will achieve this step (3,4). For hepatic microsomes the procedure is more complicated due to the destructive nature of the procedure involved in isolating microsomes. However, the steps are analogous. Data from hepatic microsomes, conventionally expressed per milligram of microsomal protein, are normal-

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Table I. Previous Reports for Human Hepatic Microsomal Scaling Factors

Study	Number of donor livers	Method used	Scaling factor reported (mg protein/g liver)
Bäärnhielm *et al.* (10)	3	Spectral P450	77
Lipscomb *et al.* (11)	4	Glucose-6-phosphatase	21 (16–27)
Wilson *et al.* (8)	20	Spectral P450	33 (26–54)
	7	NADPH-cytochrome *c* reductase	
Lipscomb *et al.* (12)	20	Elisa for CYP2E1 and CYP3A enzymes	57 (27–108)
Present report	38	Spectral P450	40 (22–67)
	12	NADPH-cytochrome *c* reductase	32.5 (17–65)

ized per mole of P450, and this number in turn may be multiplied by the P450 content per gram liver (obtained from a whole-homogenate analysis). In practice, these two steps are usually carried out as one, and hence a scaling factor is employed that is the ratio of the nanomoles of P450 per gram liver divided by the nanomoles of P450 per milligram of microsomal protein with units of milligrams of microsomal protein per gram of liver. It should be remembered that this scaling factor is not a measure of microsomal recovery in the traditional sense as there are no corrections for dilution, yet it does allow correction for the inefficiency of the subcellular fractionation procedure and hence loss of microsomal P450.

The aim of the current work was to explore certain features of variability in human livers that may impact upon the scaling factor. Human scaling factors have been reported (Table I) but until recently these have only been based on small numbers of livers. In 2004, two studies were published, both employing 20 donor livers, but in each case there were some complexities associated with these studies. In one case (8), livers obtained from patients undergoing tumor resection were used and were homogenized in medium containing 30% (v/v) glycerol, whereas in the second case (12) P450 determination was based on immunoquantification of just two P450 enzymes, namely, CYP2E1 and CYP3A forms. The present work, based on data from 38 human livers, addresses the following three questions. First, is the scaling factor dependent upon the particular P450 enzyme studied, or can a standard recovery value be applied independent of the P450 under investigation? The second question is whether the scaling factor is donor specific or can a value obtained from a population be applied to specific cases? Third, can we be confident that the particular selection of livers used to obtain a scaling factor is representative of the population of livers available for *in vitro* studies? We made a number of comparisons between our donor livers and a much larger group of 199 livers to explore this to support the claim of obtaining an unequivocal human liver microsomal scaling factor.

In order to gather sufficient data to provide an appreciation of inter-liver variability; this study involved collaboration between three laboratories that resulted in data from 38 different human livers. From several of these microsomal preparations, kinetic data were available and this allowed assessment of whether there is a need to employ a scaling factor specific to the liver used for the generation of kinetic data or whether a generic scaling factor can be used. In addition, immunoblotting and specific enzyme activity assays for particular P450s were carried out to establish whether particular P450 enzymes gave trends different to those associated with the nonspecific (e.g., spectral) measurements of total P450.

MATERIALS AND METHODS

Source and Preparation of Human Microsomes

Human liver samples ($n = 17$) were acquired from Tissue Transformation Technologies (Exeter, PA, USA) and ($n = 21$) from nontransplantable material from liver donors from Addenbrookes Hospital (Cambridge, UK) and stored at −80°C until microsomes were prepared. The selection of livers was arbitrary and ethical consent was obtained from the local ethics committee for each center.

Liver whole homogenate and washed microsomal fractions were prepared as described by Lake (13). Human liver samples were thawed, trimmed of any connective tissue, weighed, and finely chopped. Whole homogenates of the individual liver samples were prepared (0.25 g tissue/mL) in ice-cold 0.25 M sucrose containing 25 mM Tris–HCl (pH 7.4) and 1 mM EDTA with a Potter-type Teflon glass, motor-driven homogenizer (A. H. Thomas Co, PA, USA). Aliquots of each whole homogenate were stored at −80°C and the remainder centrifuged at 10,000 × *g* for 20 min to obtain the

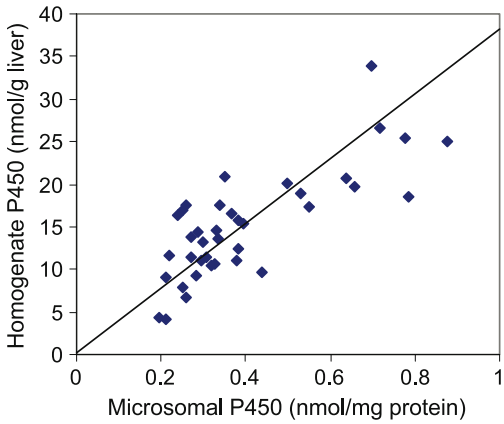

Fig. 1. Relationship between whole homogenate P450 (nanomoles per gram liver) and microsomal P450 (nanomoles per milligram protein) for 38 human livers. There is a strong ($r = 0.779$), statistically significant ($p < 0.001$) relationship between these metrics and the intercept was not significantly different from zero. The slope of the line shown corresponds to the scaling factor of 40 mg protein/g liver calculated from the mean of the 38 determinations.

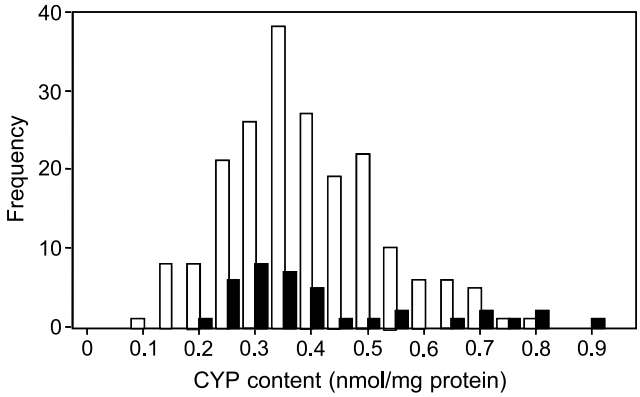

Fig. 2. Comparison of the microsomal P450 content of the 38 livers involved in this study with 199 P450 values that have been obtained from commercial suppliers. Mean ± SD, 0.399 ± 0.184 (n = 38, filled bars) and 0.366 ± 0.132 (n = 199, open bars) nmol/mg microsomal protein, respectively. Data from 199 livers obtained from the commercial literature of TCubed, Gentest, Invitrotech, and Xenotech.

postmitochondrial supernatant fraction. This was then centrifuged at 105,000 × g to separate the microsomal fraction from the cytosol. The microsomal fraction was resuspended in fresh homogenizing medium and centrifuged again at 105,000 × g. The washed microsomal fractions were resuspended in 0.25 M sucrose containing 25 mM Tris–HCl (pH 7.4) and 1 mM EDTA. Liver whole homogenate and washed microsomal fraction protein content was determined by the method of Lowry *et al.* (14) employing bovine serum albumin as standard. The washed microsomal fractions were diluted with homogenizing medium to a protein concentration of 10 mg protein/mL; aliquots were stored at −80°C.

Total P450 content (determined by carbon monoxide difference spectroscopy) and NADPH-cytochrome *c* reductase were assayed in liver whole homogenates and microsomes by established methods (15–17).

As this was a collaborative study involving three laboratories, some data were only available for a particular subset of samples. Hence, the number of livers employed varied between metrics. However, spectral P450 was determined in all livers, in both whole homogenate and microsomal fractions.

Immunoreactivity Studies

Immunoblotting of liver whole homogenate and washed microsomal fractions was performed as described previously (18). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis by use of 9% (w/v) polyacrylamide gels and electrotransferred onto nitrocellulose filters. The immunoblots were then developed for reactivity using the respective antiserum containing antibodies targeted against CYP1A2, CYP2A6, CYP2B6, CYP2C forms (CYP2C8 and CYP2C9), CYP2D6, CYP2E1, CYP3A4, and CYP3A5. All antibodies used have been previously shown to be specific for their target proteins (18). Antibody binding was detected with protein G coupled to horseradish peroxidase (12.5 ng/mL), visualized with enhanced chemiluminescence reagents, and recorded on Hyperfilm. Immunoreactive band density was quantified with a Kodak Image Station and Kodak Digital Science 1D Image analysis software (Perkin Elmer Life Sciences, Hounslow, UK). Levels of all P450 forms in whole homogenate and microsomal fraction samples were expressed in arbitrary units as amounts relative to those in a pooled microsomal preparation from six normal human livers (18).

Probe Activity Studies

Diclofenac, dextromethorphan, and testosterone were used as probes for CYP2C9, CYP2D6, and CYP3A4, respectively, via measurement of the formation of 4′-hydroxydiclofenac, dextrorphan, and 6-β-hydroxytestosterone. Incubations were performed and samples were analyzed as detailed elsewhere (19,20). Determination of the benzodiaz-

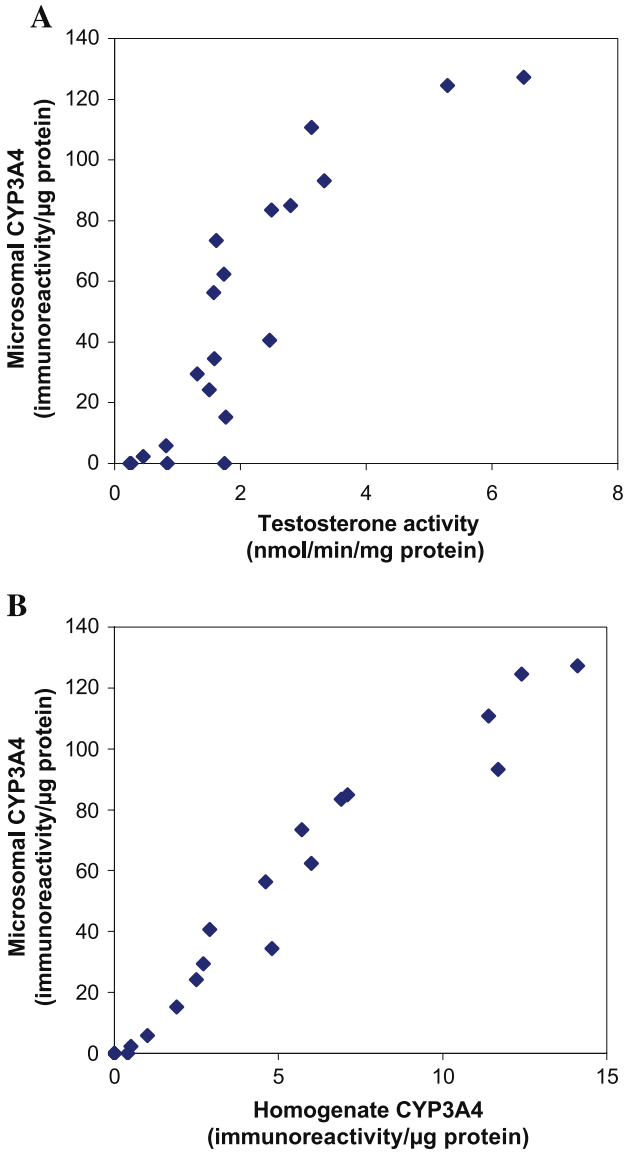

Fig. 3. (A) Relationship between catalytic activity and immunoreactivity for CYP3A4 in 19 hepatic microsomal preparations (r = 0.872) and (B) relationship between homogenate and microsomal immunoreactivity for CYP3A4 in 16 human livers (r = 0.975). CYP3A4 immnoreactivity is expressed in arbitrary units per microgram of homogenate or microsomal protein.

Table II. Comparison of Whole-Homogenate and Microsomal Immunoreactivity for Eight P450 Enzymes in 20 Human Livers

P450 enzyme	Ratio of microsomal to homogenate content[a]	Correlation coefficient[b]	*n*
1A2	11.9 ± 4.7	0.974	14
2A6	10.9 ± 6.4	0.814	20
2B6	10.2 ± 7.7	0.899	16
2C8	10.5 ± 9.1	0.633	19
2C9	8.0 ± 4.7	0.806	19
2D6	7.0 ± 6.3	0.914	19
2E1	7.1 ± 1.7	0.893	20
3A4	9.8 ± 2.6	0.975	16

[a] Mean ± SD shown for up to 20 livers. In some instances, certain P450 enzymes were not detectable in all 20 whole-homogenate samples. ANOVA showed no statistical difference between the ratios for the different enzymes.

[b] Correlation between immunoreactivity (expressed in arbitrary units per microgram of protein) in liver microsomes compared to whole homogenate.

epine kinetic parameters was carried out as described previously by Rawden *et al.* (21) for alprazolam, flunitrazepam, and triazolam. In each case CLint was determined for both oxidative pathways and summed to give the drug CLint. The parameters presented relate to particular livers used to determine the scaling factors reported here; there were additional livers in the original publication (21).

Data Analysis

The CLint values obtained for each benzodiazepine were scaled to *in vivo*, employing three different methods (A, B, and C). Method A involved the standard approach of multiplying the CLint value for each liver by its own individual scaling factor ($n = 8$). Methods B and C used the mean scaling factor from either the same livers ($n = 8$) or the bank of 38 livers, respectively. The CLint values were obtained from methods B and C by use of Monte Carlo simulations (22) involving a resampling analysis with replacement (1000 simulations in S-Plus 2000, MathSoft Inc, Cambridge, MA, USA). The procedure assumed normal distribution for the scaling factors and lognormal distribution for CLint.

RESULTS

The P450 content was determined in both whole homogenate and microsomes from 38 livers and there was a strong statistical correlation between these values ($r = 0.779$, $p < 0.001$). In both matrices there was a 5-fold range in P450 values (Fig. 1) and the ratio of the whole homogenate (nanomoles per gram liver) to the microsomal P450 content (nanomoles per milligram protein) provided a scaling factor for each liver (range 22–67 mg protein/g liver). Overall, the mean scaling factor was calculated to be 40 mg protein/g liver with a coefficient of variation of 31%. This value is at variance with some values reported previously (8,9) in studies using only a small number of human livers ($n = 3$ or 4); see Table I. However, our range of scaling factors overlaps with those reported by both Wilson *et al.* (8) and Lipscomb *et al.* (12), where 20 liver samples were used in both investigations. In the present study, a subset of liver whole homogenates and microsomes ($n = 12$) was used to determine NADPH-cytochrome *c* reductase activity. The average scaling factor of 33 mg microsomal protein/g liver (range 17–65 mg microsomal protein/g liver) by this method agreed well with the P450-derived scaling factors from the same livers (36 mg microsomal protein/g liver; not statistically different by paired *t* test).

In order to assess the representative nature of our 38 livers the microsomal P450 values for 199 livers were collated from four different sources (TCubed, Gentest, Invitrotech, and Xenotech) and compared to the values of P450 from this study (Fig. 2). The P450 values of the 38 livers show a similar mean and SD for microsomal P450 content (0.399 ± 0.184 nmol/mg protein) to the values in the larger data set (0.366 ± 0.132 nmol/mg protein).

The data from Western blotting and selective probe activity assays for P450 enzymes in human livers confirmed the trends noted for total P450 content. There was good correlation ($p < 0.001$) between liver microsomal catalytic activity and immunoreactivity (expressed in arbitrary units per unit of protein) for CYP3A4 ($r = 0.872$; see Fig. 3A), CYP2D6 ($r = 0.868$), and CYP2C9 ($r = 0.665$). There were also good correlations between liver whole homogenate and microsomal immunoreactivity for the eight P450 enzymes studied (Table II; *r* values 0.633–0.975). As an example, the data for CYP3A4 are shown in Fig. 3B. For all the P450 enzymes studied, the ratio of immunoreactivity in liver microsomes compared to whole homogenate varied from 7 to 11.9 (Table II). However, taken overall the immunoreactivity data for CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 indicate no marked enzyme-specific trends (not statistically significant by ANOVA) and suggest that the scaling factor based on total P450 content, as determined by the spectral assay, is applicable to the individual P450 enzymes.

Table III represents a summary of the clearances of three benzodiazepines used to assess the utility of the scaling factors (21). The CLint values range over an order of magnitude, with alprazolam having the lowest value and triazolam the highest. The predictions of CLint for each of the three benzodiazepines from microsomes ($n = 8$ livers) are also presented in Table III using three methods. Method A uses

Table III. Values of CLint for Three Benzodiazepines in Eight Human Liver Microsomes (20) and the Scaled Using Three Methods

	Alprazolam	Flunitrazepam	Triazolam
In vitro CLint (μL/min/mg protein, mean ± SD)	2.4 ± 2.0	5.9 ± 4.7	18.5 ± 16.8
Scaled CLint (μL/min/g liver, mean ± SD)			
Method A	125 ± 97	143 ± 159	1102 ± 1001
Method B	174 ± 164	174 ± 176	1523 ± 1511
Method C	150 ± 151	157 ± 154	1283 ± 1265

Method A uses the individual scaling factor for that particular liver. Method B uses the mean scaling factor for eight livers (same eight used for kinetic study). Method C uses the mean scaling factor for all 38 livers. ANOVA showed no statistical difference between the three methods.

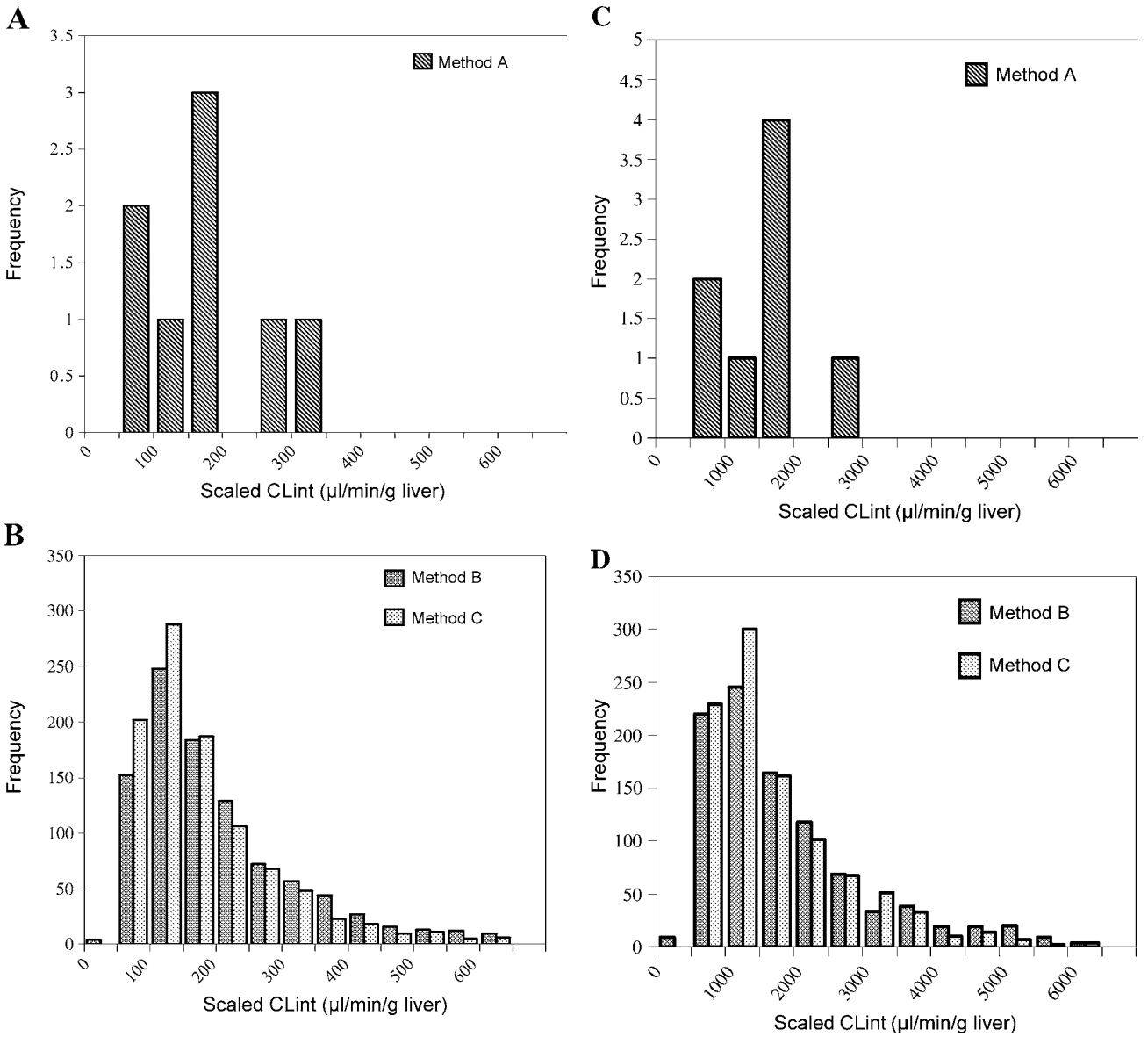

Fig. 4. Scaled CLint for alprazolam (A, B) and triazolam (C, D) from eight hepatic microsomal preparations by three different methods: method A, use of individual scaling factors; method B, use of mean scaling factors (± SD) for the same eight livers and Method C: use of a mean scaling factors (± SD) for 38 livers.

the individual scaling factors for the particular liver investigated to determine the CLint value for each liver. Methods B and C use the average scaling factor for the subset of 8 and the total 38 livers, respectively. For methods B and C, the data were generated by use of Monte Carlo simulation. Figure 4 illustrates these predictions for alprazolam and triazolam as representatives for low- and high-clearance benzodiazepines, respectively. No statistically significant differences between methods B and C are apparent and both are consistent with the data obtained using method A.

DISCUSSION

Although the prediction of drug CLint from *in vitro* kinetic parameters works well in animal experimentation (5,6), mixed success has been reported in human studies. One of the possible reasons for this apparent species difference in the confidence of *in vitro–in vivo* extrapolation lies in the selection of the scaling factor used to convert the *in vitro* clearance units from per milligram protein to per gram of whole liver. This initial step contrasts with the subsequent stages in the scale-up procedure where clearance is incorporated into a liver model and hepatic clearance is obtained with appropriate consideration of physiological factors such as blood flow and drug–protein binding and are common to all animal species scale-up. The choice of liver models has been explored (7) and a comprehensive analysis of human liver volume considerations has recently been compiled (23).

A number of practices are followed for selecting a scaling factor for human microsomes. The most common practice is to use a scaling factor derived from rat studies of

45 mg protein/g liver (3), whereas others have estimated values based on the hepatocellularity and hepatocellular P450 content (24). Until recently, there were only two reports that determined human scaling factors; they differed substantially, from 21 (11) to 77 mg protein/g liver (10), and were based on only three to four livers. With two recent studies (both using 20 livers), there were concerns regarding either the donor liver source from patients with liver cancer (8), or the use of immunoquantification of only CYP2E1 and CYP3A forms (12). Although no information on the effect of liver tumors on levels of microsomal protein is available, levels of P450 per milligram microsomal protein have been reported to be significantly lower in samples of tumorous tissue compared with those taken from noncancerous regions of the same liver (25). Therefore, although tissue was taken from sites distant to tumors, they may not reflect P450 levels comparable to that of normal tissue. Thus, in view of the critical role played by this factor we decided to determine a more unequivocal scaling factor for human liver work.

Previous experience with rat livers (20) demonstrated that microsomal recovery was dependent upon pretreatment with various drugs, indicating that the particular P450 enzyme complement of liver may be of importance. Therefore, it was necessary to establish whether there were any interindividual differences in the case of human livers. This was in fact found not to be the case. Microsomal scaling factors from human livers proved to be a robust measure and averaged a value of 40 mg of microsomal protein per gram of liver for the 38 human livers studied. In addition, for the metabolism of three benzodiazepines, it was found that it made no statistical difference whether scaling factors for individual livers were used for their respective individual kinetic parameters or whether an average scaling factor was used. Moreover, our data obtained from liver donors, from accidental deaths, were not different from the mean scaling factors obtained from cancer patient liver resections (8) or from an immunoquantification study (12). In addition, our mean value is similar to the value calculated from hepatocytes of 53 mg microsomal protein per gram of liver (24). Thus, there is general agreement between various laboratories using a variety of methodologies.

In this study 38 livers were selected for investigation; therefore it was important to establish that these were representative of a larger population. The data shown in Fig. 2 indicate that indeed this is the case. However, it cannot be assumed that this will apply to patients with compromised liver function or who are exposed to drugs or other agents in whom there may be changes in hepatic microsomal content per gram of liver, for example, in hepatitis or in patients treated with phenobarbital. It was found that there was no P450 dependency in microsomal recovery; therefore, it is not important to know the particular P450 enzymes involved in metabolism prior to scaling an *in vitro* CLint value. The data indicate that for eight P450 enzymes, there were no statistically significant differences in relative immunoquantification between whole homogenate and microsomes. Also, there was good agreement between scaling factors based on P450 content and NADPH-cytochrome *c* reductase activity, which is in agreement with an earlier report (8).

One assumption in the determination of scaling factors from P450 content and also NADPH-cytochrome *c* (P450) reductase is that the enzyme content measured in the homogenate is essentially microsomal in origin. Previously (9), we have summarized the literature to support this assumption. Similarly, Wilson *et al.* (8) have discussed the evidence that NADPH-cytochrome *c* reductase is essentially microsomal. Thus, it was not felt necessary to correct either P450 content or NADPH-cytochrome *c* reductase-based scaling factors for nonmicrosomal expression of these enzymes.

Agreement between the human microsomal scaling factor and that previously reported in another mammalian species, namely, the rat, is perhaps not surprising because a major factor in determining this value will be the procedures actually carried out in a given laboratory. Subcellular fractionation procedures are routinely performed in many laboratories and tend to be similar regardless of species employed. We suggest that other laboratories use a scaling factor of 40 mg of microsomal protein per gram of liver as a routine value for scaling microsomal data for prediction of *in vivo* pharmacokinetics. This scaling factor is equivalent to a recovery of approximately 25–50%, with an uncorrected microsomal yield thus being approximately 10–20 mg of microsomal protein per gram of liver. In our experience, this is a value that is commonly obtained and consistent with the literature (26–29). It also approximates to the mean of the two other studies (8,12) discussed above, each using 20 donor livers.

In conclusion, we have established a value for human liver microsomal scaling of 40 mg microsomal protein per gram liver. Thus, we have also demonstrated that the reason for the underprediction of CLint, previously reported for 52 different drug substrates (5), is not the use of an incorrect scaling factor. We have speculated previously (21) that the mismatch between the healthy, young volunteers (used in most *in vivo* studies) and individuals from whom liver samples are available contributed to the underprediction phenomenon. Although variations in P450 concentration exist in human livers, a scaling factor that is dependent on the microsomal protein content seems to be reproducible. In addition, there was no difference in the CLint values obtained by the use of individual scaling factors for each liver and those values obtained by the use of the mean of scaling factors for the 38 livers. Finally, based on these findings and previous reports (8,12) the recommendation of this study is to use 40 mg protein/g liver as a generic value for predicting *in vivo* drug clearance in healthy volunteers from *in vitro* human hepatic microsomal data; this can be used with a high level of confidence due to the general agreement found between a number of methodologies.

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