EFFECT OF ALBUMIN ON PHENYTOIN AND TOLBUTAMIDE METABOLISM IN HUMAN LIVER MICROSONES: AN IMPACT MORE THAN PROTEIN BINDING

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ABSTRACT:
The cytochrome P450 (P450)-dependent conversion of phenytoin (PHT) to p-hydroxy phenytoin (pHPPH), and tolbutamide (TLB) to 4-hydroxy tolbutamide (hydroxy-TLB), in human liver microsomes was studied in the presence of increasing concentrations (0–4%) of bovine serum albumin (BSA). Therefore, the free fraction \( f_u \) of PHT and TLB varied. Whereas the \( f_u \) of PHT (5 \( \mu \)M) decreased, an increase (3-fold), rather than a decrease in the pHPPH formation rate was observed when BSA (<1%) was present. The stimulation was attributed to a significant decrease in apparent \( K_m \). The change, however, was diminished as the BSA concentration reached 4% (PHT \( f_u = 0.2 \)), in which the reaction velocity remained the same as that measured in the absence of BSA. Therefore, unchanged \( K_m \) (16.2 ± 0.7 \( \mu \)M) and \( V_{max} \) (9.4 ± 0.2 pmol/min/mg of protein) values were determined based on total PHT concentra-
tions, whereas correction for \( f_u \) led to an unbound \( K_m \) (\( K_{mu} \)) of ~3.2 \( \mu \)M. Similarly, the metabolism of TLB (50 \( \mu \)M) was enhanced (~2-fold) in the presence of 0.25% BSA but remained only 35% of the control activity (no BSA) at 1% BSA. However, the remaining activity was higher (3-fold) than that determined with an equivalent free concentration of TLB (4 \( \mu \)M) calculated according to its \( f_u \) (0.08). The difference became less significant when BSA concentration was 4% \( f_u < 0.02 \). Collectively, the results suggest a 2-fold effect of BSA on PHT and TLB hydroxylation: first, facilitation of the reactions via a decrease in \( K_m \); second, a decrease in \( f_u \) leading to a drop in reaction rate. For a given P450 reaction, therefore, the effect of BSA may depend upon enzyme affinity, catalytic capacity, and the extent of protein binding.

For the longest time, it has been accepted that only unbound drug contributes to pharmacological activities. This free drug hypothesis has also found applications in drug transport, drug metabolism and disposition, receptor binding, enzyme kinetics, and inhibition processes. However, some recent findings appear to contradict this hypothesis. For example, it has been noted that during one passage through the brain, in many cases, more drug than “free drug” can penetrate through the blood-brain barrier (Spector, 2000). Similarly, the hepatic uptake of many lipophilic compounds is not necessarily restricted by protein binding (Kurz and Fichtl, 1983; Pacifici and Viani, 1992), and it has been found that the antifungal activity of itraconazole and ketoconazole is not diminished despite extensive binding albumin (Schäfer-Korting et al., 1995). In agreement, Tran et al. (1997) have reported that the \( K_v \) value of stiripentol obtained in microsomal studies is more consistent with total plasma concentration than unbound concentration. All these reports suggest that factors, other than protein binding, may be involved in a given biological process.

Recently, with the growing demand for quantitative predictions of in vivo pharmacokinetics and drug-drug interactions, this free drug hypothesis has been investigated. For instance, it has been reported that nonspecific binding in liver microsomal incubations can result in an underestimation of intrinsic clearance for highly protein binding drugs. Therefore, use of free drug fraction to correct the \( K_m \) values has been suggested (Obach, 1996, 1997). These observations have been further substantiated by others. For example, addition of heat-inactivated microsomal protein to the incubation system increased the apparent \( K_m \) with a minimal effect on \( V_{max} \) for amitriptyline metabolism, and correction for unbound fraction led to a comparable \( K_m \) to the control value (Venkataraman et al., 2000). However, under this free drug hypothesis, \( K_m \) values for phenytoin metabolism in human liver microsomes were found to decrease in the presence of bovine serum albumin (BSA1) (Ludden et al., 1997). Moreover, a better vivo-in vitro correlation can be obtained applying total concentration versus unbound fraction for some drugs metabolized in human hepatocytes and microsomes (Obach, 1999). The discrepancy suggests that simple correction of \( K_m \) or \( K_v \) value by multiplying unbound drug fraction might not apply to every enzymatic reaction. For a better understanding of the effect of protein binding on the estimation of in vitro kinetic parameters, we examined phenytoin (PHT) and tolbutamide (TLB) metabolism in human liver microsomes. These two CYP2C9 substrates are known to differ in terms of protein binding and turnover rates (Bajpai et al., 1996; Carlile et al., 1999).

Materials and Methods

Chemicals and Enzyme Sources. PHT, pHPPH, and (±)-5-(4-methylphenyl)-5-phenylhydantion (MPHT) were obtained from Aldrich Chemical Co.

Abbreviations used are: BSA, bovine serum albumin; PHT, phenytoin; OH-TLB, 4-hydroxy tolbutamide; pHPPH, p-hydroxy phenytoin; MPHT, (±)-5-(4-methylphenyl)-5-phenylhydantion; CPPM, chlorpropamide; HLM, human liver microsomes; LC-MS/MS, liquid chromatography/tandem mass spectrometry; \( f_u \), mean unbound fraction of substrate.

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Similarly, triplicate samples of four concentrations of TLB (10, 50, 250, and 100 μM) were prepared in Tris buffer containing 10 mM MgCl2, 1 mM EDTA, and microsomal preparation (0.25 and 0.25 mg of protein/ml) in the presence or absence of BSA. Each sample (0.8 ml) was placed in a Centrifree micropartition system unit (Millipore Corp., Bedford, MA) following incubation (37°C for 30 min) and centrifuged (20 min, 2000g) at 37°C. Control samples without microsomes or BSA were incubated simultaneously. To 50 μl of the filtrate was added 50 μl of MPHT (2.5 μM) followed by 300 μl of an aqueous solution of acetone (30%). Similarly, triplicate samples of four concentrations of TLB (10, 50, 250, and 1000 μM) were incubated in 0.1 M phosphate buffer containing the same components as above and subject to ultrafiltration. To 25 μl of the filtrate was added 50 μl of CPPM (5 μM) followed by 300 μl of 30% acetone aqueous solution. All samples prepared as such were analyzed by LC-MS/MS assay.

Microsomal Incubations. The final incubation conditions were based on preliminary studies to ensure the linearity of product formation. Thus, for the metabolism of phenytoin, the incubation mixture (final volume of 0.5 ml) contained 0.25 mg of microsomal protein/ml, 10 mM MgCl2, 1 mM EDTA, 1 mM NADP+, 10 mM D-glucose 6-phosphate, D-glucose 6-phosphate dehydrogenase (Sigma Type VII, from baker’s yeast, 2 units/ml), and 150 mM Tris-HCl buffer (pH 7.4). The reaction was initiated by the addition of NADPH-generating system and terminated 30 min later by the addition of 0.5 ml of acetonitrile followed by 2 ml of ethyl acetate. The internal standard, MPHT (50 μl of 5 μM), was added to the samples prior to extraction. Following rigorous vortexing on a multiple tube vortexer for 3 min and a brief centrifugation, the organic layer was transferred and evaporated to dryness in Speedvac (Savant Instruments Inc., Holbrook, NY). The residues were constituted in 100 μl of 30% acetonitrile aqueous solution for LC-MS/MS analysis. The recovery of all analytes was greater than 95%.

TLB metabolism was evaluated as described above except that the incubation mixture contained 0.125 mg of microsomal protein/ml in 0.1 M potassium phosphate (final volume of 0.25 ml). The reaction was started by the addition of the NADPH-generating system and terminated 30 min later with acetonitrile (200 μl). The internal standard (CPPM, 50 μl of 5 μM) was added prior to centrifugation. The supernatant was used for LC-MS/MS analysis.

LC-MS/MS Analysis. The separation of PHT, pHPPH and MPHT, and of OH-TLB, TLB and CPPM, was accomplished on a MetaChem Products Inc. (Torrance, CA) ODS-3 column (2.1 × 50 mm, 5 μm). The mobile phase consisting of 0.02% acetic acid (solvent A, pH adjusted to 4.5 with ammonium hydroxide) and acetonitrile (solvent B) was delivered at a flow rate of 0.5 ml/min with a linear increase of solvent B from 25 to 65% over 2 min. Equilibration was allowed for an additional 1.5 min, giving a total chromatographic run time of 3.5 min. The flow was split, such that 2.5 were introduced into the mass spectrometer.

Tandem mass experiments were performed on a Sciex (Concord, Ontario, Canada) model API 3000 triple quadrupole mass spectrometer interfaced to the column eluent via a Sciex turbospray probe (350°C). Operating conditions for PHT, pHPPH, and MPHT were optimized by infusion of a mixture of all analytes (25 μM each) at a flow rate of 5 μl/min, along with the LC flow (200 μl/min, solvent A/B = 50/50), and were determined as follows: nebulizing gas pressure, 14; auxiliary gas flow, 0.7 l/min; curtain gas, 12; ion spray voltage, 5000 V; orifice voltage, 48 V; ring voltage, 195 V; collision gas (nitrogen) flow, 6. Operating conditions for OH-TLB, TLB, and CPPM were identical to those described above except for orifice voltage (30) and ring voltage (100). Multiple reaction monitoring experiments in the negative ionization mode were performed using a dwell time of 200 ms per transition to detect ion pairs at m/z 251/208 (PHT), 267/224 (pHPPH), 265/222 (MPHT), 269/170 (TLB), 285/186 (OH-TLB), and 275/190 (CPPM).

Data Analysis. The apparent enzyme kinetic parameters were determined by fitting the reaction velocities versus substrate concentrations to eq. 1 (GraFit; Erithacus Software Ltd., Staines, UK). Over the range of PHT and TLB concentration tested (1–1000 μM, respectively), a linear Eadie-Hofstee plot suggested a one-site (one Km) Michaelis-Menten kinetics for pHPPH and OH-TLB formation.

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V = \frac{V_{\text{max}} \cdot C}{K_m + C}
\]

The statistical significance in enzyme activities (single substrate concentration) and enzyme kinetic parameter (Km and Vmax) differences at different BSA concentrations were determined using a single factor analysis of variance (p < 0.05 indicating a significant difference). The difference of uncorrected and corrected enzyme kinetic parameters was evaluated by means of the Student’s test, and the statistical difference is denoted by *p < 0.05.

Results

Binding of PHT and TLB to BSA. The unbound fractions of PHT and TLB at different concentrations determined in the presence and absence of BSA are listed in Table 1. Consistent with the report by Ashforth et al. (1995), PHT (1–100 μM) binding to BSA was concentration-independent. The mean unbound fraction (f0) was 0.3 and 0.2 in the presence of 2 and 4% BSA, respectively, and did not significantly change in the presence of microsomes (250 μg/ml, data not shown). On the contrary, TLB binding to BSA was concentration-dependent (Table 1). The f0 increased with TLB concentration (10–1000 μM). Similarly, the presence of microsomes (125 μg/ml) did not change the extent of binding to BSA (data not shown). On the other hand, the f0 of PHT (5 μM) and TLB (50 μM) varied inversely with BSA concentration (Fig. 1). TLB gave rise to a lower f0 than PHT over BSA concentration range tested.

Effect of BSA on the Hydroxylation of PHT and TLB by Human Liver Microsomes. To minimize nonspecific binding of the substrates to microsomal proteins, lower enzyme concentrations were used in the present study (250 and 125 μg/ml for PHT and TLB, respectively), compared with previous reports (Ludden et al., 1997; Carlile et al., 1999). Less than 10% PHT (5 μM) and TLB (50 μM) was bound to microsomal protein, and the value for TLB was negligible when its concentration reached 1000 μM (data not shown). The sensitivity of LC-MS/MS analysis allowed for the determination of small quantity of metabolites generated at low substrate concentrations, especially for PHT metabolism.

The formation of pHPPH in a pooled human liver microsomal sample (HMM-0259, 10 donors) in the absence of BSA was described by monophasic kinetics over a substrate concentration range of 1 to

<table>
<thead>
<tr>
<th>Substrate</th>
<th>2% BSA</th>
<th>4% BSA</th>
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<tbody>
<tr>
<td>PHT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.32 ± 0.03</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>5</td>
<td>0.30 ± 0.01</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>25</td>
<td>0.32 ± 0.01</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>100</td>
<td>0.32 ± 0.01</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>TLB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.022 ± 0.001</td>
<td>0.012 ± 0.001</td>
</tr>
<tr>
<td>50</td>
<td>0.024 ± 0.001</td>
<td>0.012 ± 0.001</td>
</tr>
<tr>
<td>250</td>
<td>0.066 ± 0.002</td>
<td>0.021 ± 0.001</td>
</tr>
<tr>
<td>1000</td>
<td>0.242 ± 0.007</td>
<td>0.098 ± 0.001</td>
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</table>

(251/208 (PHT), 267/224 (pHPPH), 265/222 (MPHT), 269/170 (TLB), 285/186 (OH-TLB), and 275/190 (CPPM).
The rate of OH-TLB formation was also determined under three different lower than the control activity (0.25, 0.5 and 1.0% BSA; and 3) TLB at 20, 10 and 4 microsomal sample was also described by monophasic kinetics over a substrate concentration range of 25 to 1000 μM) was used to facilitate the formation of a sufficient quantity of the metabolite. As outlined in Table 3, the values of \( v_c \) were proportionally lower than those of \( v_a \), but \( v_b \) is always higher than \( v_c \). It is worth noting that the difference between \( v_b \) and \( v_c \) became less significant as the BSA concentration increased along with concomitant decrease in the unbound fraction of the substrate. In contrast to the effect on PHT metabolism, the presence of albumin (>0.5%) brought about a decrease in \( v_b \). However, as with PHT, \( v_b \) was almost doubled in the presence of 0.25% BSA.

The increased rate of pHPPH formation in the presence of BSA (1 and 2%) was consistently observed with microsomes of different organ donors (Table 4). However, the extent of enhancement varied with the particular microsomal preparations and appeared to be associated with the rate of PHT metabolism. Thus, HG30 gave rise to the highest activity among the preparations studied, and the presence of 2% BSA failed to increase the rate of pHPPH formation, in contrast to the rest preparations. It seems that microsomes with higher rate of PHT metabolism tend to be more resistant to the stimulating effect of BSA. Nonetheless, the values of \( v_b \) determined in HG30 were appreciably higher than \( v_c \).

**Kinetic Parameters for the Hydroxylation of PHT and TLB by Human Liver Microsomes in the Presence of BSA.** The apparent kinetic parameters for the hydroxylation of PHT and TLB determined in the presence and absence of BSA are listed in Tables 5 and 6. In both cases, metabolite formation conformed to monophasic Michaelis-Menten kinetics (Figs. 2 and 3). The presence of 0.25% BSA resulted in a significant decrease in apparent \( K_m \) values for both PHT (>3-fold) and TLB (~40%). By comparison, the presence of 4% BSA failed to cause a significant change in the rate of PHT hydroxylation over the substrate concentration range tested (1 to 100 μM), thus resulting in apparent kinetic parameters comparable with those obtained in the absence of BSA (Table 5; Fig. 2). The kinetic parameters for TLB hydroxylation in the presence of 2 and 4% BSA were not determined because of the extensive binding at low substrate concentrations.

When the kinetic parameters were calculated based on unbound substrate concentrations, the unbound \( K_m \) values (\( K_{mu} \)) decreased significantly for both reactions. For PHT hydroxylation (Table 5), the \( K_{mu} \) values in the presence of three different BSA concentrations (0.25, 2.0, and 4%) are comparable (~3 μM), whereas the \( V_{max} \) values remain unchanged. In the case of TLB hydroxylation (Table 6), the \( K_{mu} \) value dropped to 36 μM, and there was a slight decline in \( V_{max} \).

**Discussion**

Based on the free drug hypothesis, in which drugs are subject to nonspecific binding to incubation matrices, the free and available substrate concentration will be less than the added concentration. As a result, the decrease in reaction rates would be expected to be proportional to the extent of protein binding at substrate concentrations below \( K_m \). In addition, the apparent \( K_m \) determined on the basis of the added concentration would then be higher than the "true" \( K_m \), but the \( V_{max} \) should not be affected, as predicted by McLure et al. (2000) and as observed for amitriptyline N-demethylation (Venkatkrishnan et al., 2000). However, Ludden et al. (1997) reported a decreased \( K_{mu} \) whereas \( V_{max} \) remained unchanged for PHT hydroxylation by human liver microsomes when BSA was added to decrease free fraction. Obviously, the decreased unbound \( K_m \) for PHT could only be obtained when the added BSA did not significantly reduce the reaction velocity, or the extent of decline is much less than would be expected on the basis of the free drug hypothesis.

The results presented herein demonstrate that BSA at low concentrations increases hydroxylation of PHT and TLB in human liver microsomes despite a decrease in unbound drug concentration. The enhancement became less significant as the BSA concentration was increased. No appreciable changes in PHT hydroxylation were observed when BSA was high (4%). In contrast, the rate of TLB hydroxylation decreased when BSA was present (~0.5%), although

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**Fig. 1. Unbound fraction of PHT and TLB in varying concentrations of BSA.**

PHT (5 μM) and TLB (50 μM) were incubated in Tris-HCl and phosphate buffer, respectively, with varying concentrations of BSA for 30 min. The data are expressed as mean ± S.D. from three determinations.
the decline was still much less than would be expected from the resulting fall in the unbound TLB concentrations. These results strongly suggest a 2-fold effect of BSA on the metabolism of PHT and TLB: first, facilitation of the reactions and, second, a decrease of free drug fraction.

An increased reaction rate in the presence of BSA suggests that facilitation overcomes the effect of nonspecific binding. The decreased apparent $K_m$ values for both PHT and TLB (calculated using total substrate concentrations in the presence of low BSA levels; Tables 5 and 6) are indicative of increased affinities of the substrates for the enzyme. Albumin is known to be associated with intraluminal region of the smooth endoplasmic reticulum (Peters, 1996), and its presence in the incubation system may effect the tertiary and quaternary structure of the P450 system, resulting in an altered affinity to a substrate. It is also possible that BSA cleans up incubation mixtures of some endogenous inhibitors present in microsomal preparations, such as fatty acids (Yamazaki and Shimada, 1999). This likelihood is supported by the report from Qiu et al. (2000) that in BSA-pretreated and -washed microsomes, the $K_m$ values decreased for both PHT and TLB. To strengthen the observation, future studies will examine whether it occurs with recombinant CYP2C9 and with other CY2C9 substrates.

Another interesting observation is that the response of a reaction to this stimulating effect seems to be inversely related to the intrinsic activity. With a comparable $f_u$ ($\sim 0.4$) and in the same enzyme source, the rate of PHT hydroxylation increased by $\sim 3$-fold, whereas the rate of TLB hydroxylation increased only by $\sim 2$-fold (Tables 2 and 3). For a given reaction (e.g., PHT hydroxylation) catalyzed by different enzyme sources, it was found that the reaction in the microsomal preparation with higher activity donor (HG30) was less responsive (Table 4). This phenomenon may be in part attributable to the susceptibility of high turnover reactions to the effect of nonspecific binding (see below).

Facilitation by albumin has been observed in other processes. Pond et al. (1992) reported that facilitation mechanism was involved in uptake of palmitate by hepatocyte suspensions. They found that the measured unbound clearance of $[^{1}H]$palmitic acid, defined as the initial uptake velocity divided by the unbound $[^{1}H]$palmitic acid concentration in the medium, was enhanced 6.6-fold as the concentration of human serum albumin was increased from $\sim 5$ to 480 $\mu$M. In fact, in many cases, the uptake rate appears to be determined more by the bound than by the unbound ligand concentration (Weisiger et
al., 1981; Forker et al., 1982; Forker and Luxon 1983; Fleischer et al., 1986; Burczynski et al., 1989).

A decreased reaction rate obtained in the presence of BSA (higher concentration) indicates that the effect of nonspecific binding obscures the facilitation. The unbound drug is depleted so substantially by BSA that significantly less enzyme-substrate complex forms and a decrease in velocity is observed. Obviously, the susceptibility to nonspecific binding varies with substrate. The more tightly a drug binds to BSA, the more likely the rate is affected. TLB exhibited higher protein binding and the rate of OH-TLB formation dropped in the presence of BSA (≥0.5%). By comparison, pHPPH formation was increased.

Another important determinant is catalytic capacity ($V_{\text{max}}$). Since binding to albumin is a reversible process, with the conversion of enzyme-substrate complex to product, the release of substrate from the albumin-substrate complex can serve as a constant supply of free substrate. Thus, substrates bound to BSA are not completely futile. It has been suggested that albumin-bound TLB molecules contribute to product formation with an affinity equal to or higher than that for free molecules (Black et al., 1999). For an extremely low turnover reaction, the release may compensate for the drop of free substrate so that the total concentration of enzyme-substrate complex would remain relatively constant, and no appreciable change of velocity would be noticed, even without the involvement of facilitation. On the contrary, a fast turnover reaction would be more sensitive to a drop of free substrate. Rat liver microsomes gave rise to a rate of PHT hydroxy-
lution greater than 30-fold higher than human liver microsomes (data not shown). The presence of low level BSA (≤0.5%) failed to show appreciable enhancement. In addition, the rate started to drop when BSA reached 1% and was significantly inhibited (>35%) in the presence of 4% BSA (data not shown). Again, less enhancement by BSA (1%) was observed in a human liver microsomal preparation (HG30) showing a higher rate of pHPPH formation (Table 4), in which more effect from nonspecific binding (drop in reaction rate) may offset the impact of facilitation. Furthermore, TLB hydroxylation was more sensitive to the effect of nonspecific binding than PHT hydroxylation. In other words, a reaction with high turnover rate and extensive protein binding would be more vulnerable to changes in $f_u$.

For reactions in this category, the predominant effect of nonspecific binding will be revealed as a decrease in velocity when substrate concentration is lower than $K_m$, leading to an increased $K_m$ and unchanged $V_{max}$. Correction for $f_u$ would bring about an unbound $K_m$ comparable with control $K_m$. It appears that free drug hypothesis is applicable to this type of reaction. While exploring the effect of BSA on diclofenac metabolism, we found that the apparent $K_m$ increased with BSA ($V_{max}$ remained largely unchanged). Correction for unbound diclofenac gave rise to a $K_m$ close to $K_m$ (data not shown). By comparison to PHT, diclofenac exhibits high turnover (~150-fold higher) and is extensively protein bound (~300-fold stronger). These characteristics may highlight the significance of $V_{max}$ and protein binding extent in determining the susceptibility of a reaction to the effect of nonspecific binding. Amtriptyline $N$-demethylation is another example (Venkatakrishnan et al., 2000).

Whenever the effects of facilitation and nonspecific binding cancel each other, no significant change in reaction rate would be observed regardless of a considerable fall in unbound fraction. Therefore, there would be no appreciable alteration in apparent $K_m$. However, correction for $f_u$ will lead to a decreased $K_{m}^\text{app}$. This situation can explain what was observed in the present study and by Ludden et al. (1997) with PHT hydroxylation in the presence of 4% BSA.

Based on the results described herein, it is concluded that the multiple dynamic processes present in the incubation systems complicate the effect of BSA on the estimation of in vitro kinetic parameters. The extent of the effect may be descriptively defined by the rate of turnover of enzyme-substrate complex to product, the affinity of substrate to the enzyme and albumin, and the concentration of the albumin. However, more data are required to establish a quantitative way to estimate and correct the effect for the correlation of in vitro and in vivo studies. We demonstrate that using $f_u$ to calculate $K_m$ values may not be applicable to all reactions. Future investigations should compare the effect of protein binding on various enzyme reactions and on different type of substrates.

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