Effect of Albumin on the Estimation, In Vitro, of Phenytoin $V_{\text{max}}$ and $K_m$ Values: Implications for Clinical Correlation

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ABSTRACT

The effect of bovine serum albumin (BSA) on human liver metabolism, in vitro, of $^{14}$C-phenytoin (PHT) was studied. Michaelis Menten parameters were determined for the conversion of PHT to p-hydroxy phenytoin in seven different microsomal preparations with the addition of 0, 2, and 4% BSA. The unbound $K_m$ ($K_{mu}$) values were $30.8 \pm 18.6, 1.57 \pm 0.21$ and $1.50 \pm 0.17 \mu M$ (mean $\pm$ S.D.), respectively; however, there was excellent agreement among the $V_{\text{max}}$ values (29.1, 31.8 and 31.5 pmol/min/mg). With intact tissue slices, BSA (4%) added to incubations of PHT had a minimal effect on the $V_{\text{max}}$ values in two of the four livers studied and resulted in a mean $K_{mu}$ value of $2.20 \pm 0.59 \mu M$, although the $K_{mu}$ in the absence of BSA was $6.64 \pm 3.17$. In scaling-up to the whole body, $V_{\text{max}}$ values were 3.9 and 1.0 mg/kg/day for microsomes and slices, respectively, compared to 5.9 mg/kg/day, in vivo. The $K_{mu}$ values determined in the presence of albumin in both microsomes and slices were similar to those based on in vivo human steady state data ($K_{mu} = 2-3 \mu M$), and the intersubject variation, in vitro, was decreased in the presence of BSA. These findings for phenytoin metabolism suggest that the addition of albumin to incubation media for slices or microsome experiments may yield $K_m$ estimates that are more representative of in vivo values.

Interest continues to grow in the use of metabolic systems, in vitro, for predicting metabolism and drug interactions, in vivo. Thus, it is important to determine the relationship between kinetic parameters obtained in vitro and in vivo.

Intersubject variability in drug clearance is well-documented. However, the underlying source of this variability is largely unexplored. Metabolic drug clearance is determined from the ratio of two independent kinetic parameters: $V_{\text{max}}$ and $K_m$. Although it is relatively straightforward to determine values for $V_{\text{max}}$ and $K_m$ from experiments in vitro, it is much more difficult to separately estimate these parameters from experimental data in vivo.

In particular, $K_m$ is the most important determinant of potential drug-drug interactions, especially for the competitive type. Thus, its accurate estimation is therapeutically important. PHT is one of only a few drugs for which the human $K_m$ value has been estimated in vivo. Based on unbound PHT concentration the mean $K_m$ is about 2 to 3 $\mu M$, in vivo (Grasela et al., 1983; Tozer and Winter, 1992). Therefore, PHT is an ideal model drug for the comparison of $K_m$ values estimated from studies performed in vivo and in vitro.

PHT $K_m$ values, in vivo, have been reported to have an intersubject coefficient of variation of 50% (Grasela et al., 1983). Our studies were initiated to estimate the variability of $K_{mu}$ values, in vitro. The primary metabolite of PHT, pHPH, is generated via cytochrome P450, so initial experiments were conducted with human liver microsomes. It is very convenient to work with subcellular fractions that can be stored indefinitely, but the harsh processing of tissue to generate microsomes, as well as the requirements for exogenous cofactors, may create an altered milieu for metabolism. The conformation of the active site of the enzyme is a key determinant of binding affinity ($K_m$), so we also wanted to investigate metabolism in an intact cellular system in which the enzyme is maintained as close as possible to the environment in situ. Thus, we also studied the kinetic parameters for PHT using fresh human liver slices.

Phenytoin is a highly bound drug (90%) (Tozer and Winter, 1992; Benet et al., 1996) and the unbound fraction of a drug is the portion available for metabolism. Certain disease states are known to alter plasma protein binding, but only preliminary investigation of metabolic effects have been reported (Taburet et al., 1996). Because phenytoin is princi-
pally bound to albumin, we also explored the effect of albumin on the $V_{\text{max}}$ and $K_m$ values obtained, *in vitro*. These studies made it possible to compare $V_{\text{max}}$ and $K_m$ values obtained in the presence and absence of albumin for two model systems, *in vitro*, with the $V_{\text{max}}$ and $K_m$ values observed *in vivo*.

**Materials and Methods**

**Chemicals.** $^{14}$C PHT (specific activity 53.1 mCi/mmol; radiocchemical purity > 99%) was obtained from New England Nuclear (Boston, MA). Ethyl acetate, acetonitrile and tetrahydrofuran were HPLC grade and used as purchased. PHT (sodium salt), bovine serum albumin, fraction V (BSA), Krebs-Henseleit bicarbonate buffer and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

**Protein binding of PHT.** The free fraction of PHT in Tris buffer containing 2 and 4% BSA in and Krebs-Henseleit buffer containing 4% BSA was determined by ultrafiltration. Three replicate samples of two concentrations of $^{14}$C PHT (3 and 15 mM) were prepared in each of the buffers used in the microsome and slice incubations. Approximately 1 ml of each sample was placed in Centrifree Micro- parti tition System units (Amicon Division, W. R. Grace & Co., Beverly, MA) and centrifuged. Duplicate aliquots of each of the resulting ultrafiltrates were counted in a Tri-Carb 2500 TR Liquid Scintillation Analyzer (Packard, Meriden, CT). These counts were compared to counts in duplicate aliquots of the unfiltered solutions to determine the free fraction of PHT in the incubation buffers.

**Procurement and preparation of human liver tissues.** Human liver specimens medically unsuitable for transplantation were acquired under the auspices of the Washington Regional Transplant Consortium (Washington, DC) or the International Institute for the Advancement of Medicine (Exeter, PA). Freshly isolated human liver slices were prepared by *In Vitro* Technologies, Inc. (Catonsville, MD) and yielded slices that were approximately 300-µm thick and 8.0 mm in diameter. Human liver tissue samples were obtained and stored at -80°C until microsomes could be prepared by tissue homogenization and differential centrifugation as described by Lake (1987). The final microsomal pellet was resuspended in 100 mM sodium phosphate, with 5 mM MgCl$_2$ and 1 mM EDTA, (pH 7.4). Protein concentration of the microsomal fractions was measured using the Bio-Rad (Her- cules, CA) method with BSA as the standard. The fractions were washed with 2 and 4% BSA and in Krebs-Henseleit buffer containing 2 and 4% BSA, and centrifuged. Duplicate aliquots of each of the resulting ultrafiltrates were counted in a Tri-Carb 2500 TR Liquid Scintillation Analyzer (Packard, Meriden, CT). These counts were compared to counts in duplicate aliquots of the unfiltered solutions to determine the free fraction of PHT in the incubation buffers.

**Hepatic microsomal incubations.** $^{14}$C PHT was appropriately diluted with unlabeled PHT in 0.01 N NaOH before being added in 10 µl aliquots to a total final volume of 1 ml. Each incubation mixture contained 1.0 mg microsomal protein, PHT (0 and 150 µM), and 10 µl of a NADPH generating system in Tris buffer, pH 7.4. The NADPH generating system consisted of 10 mM glucose-6-phosphate, 1 mM NADP$^+$ and 1 U/ml glucose-6-phosphate dehydrogenase. Duplicate incubations for each concentration were carried out in Tris buffer, pH 7.4, with 0, 2, and 4% BSA. The microsomal mixtures without substrate were preincubated at 37°C for 3 min. After addition of the appropriate concentration of PHT the samples were incubated for 1 hr in a shaking water bath. The samples were removed, the reaction stopped and the protein precipitated by the addition of 3 ml water saturated ethyl acetate. After vortex mixing and centrifugation, 1.5 ml of the organic phase was transferred to clean vials and dried under nitrogen. The dried samples were reconstituted in 1-mm glass beads. All samples were frozen for future analysis.

**Hepatic microsomal incubations.** After incubation with β-glucuronidase the samples were transferred to 15-ml screw cap microfuge tubes containing 1-mm glass beads. All samples were frozen for future analysis.

**Preparation of liver slices for HPLC analysis.** After the slice samples were thawed, Krebs-Henseleit buffer (300 µl) was added to each sample. The microfuge tubes were capped and placed on a Mini Beadbeater (Biospec Products, Bartlesville, OK) and shaken at 50,000 rpm for 10 sec. After homogenization, 300 µl of 0.2 M sodium acetate buffer, pH 5.0, and 10 µl (approximately 1200 U) of β-glucuronidase were added to each sample. The samples were gently mixed and incubated at 37°C for 4 hr. After incubation with β-glucuronidase the samples were transferred to 15-ml screw cap centrifuge tubes. The 2-ml microfuge tubes were washed with 2 x 600 µl of 0.5 M phosphate buffer, pH 7.4, and the washes added to the incubation mixtures in the centrifuge tubes. The pHPPH was extracted by adding 4 ml water saturated ethyl acetate and vortexing for 1 min. After separation of the phases by centrifugation, a 3.5-ml aliquot of the organic phase was transferred into clean vials and dried under nitrogen. The dried samples were reconstituted with 200 µl mobile phase and 25 µl injections were made on the HPLC.

**Preparation of liver slice incubation buffers for HPLC analysis.** The incubation buffer samples were allowed to thaw and 450 µl of 0.2 M sodium acetate buffer, pH 5.0, was added. Ten µl of β-glucuronidase were added, the samples gently mixed and incubated at 37°C for 4 hr. After the glucuronidase incubation, 1.2 µl of 0.5 M phosphate buffer, pH 7.4, was added to each sample. The pHPPH was extracted into ethyl acetate and the samples prepared for HPLC analysis as outlined for the slices.

**HPLC Analysis of PHT and pHPPH.** Samples for HPLC analysis were injected on a Hewlett-Packard 1050 HPLC system. The column effluent was monitored by on-line radioactivity detection using a Radiomatic Flo-One βeta detector (Packard Instrument Co., Meriden, CT). The separation of PHT and pHPPH was accomplished on a Hypersil C$_{18}$ column, (5 µ, 4.6 x 250 mm; Alltech, Deerfield, IL) maintained at 35°C. The mobile phase consisted of 40 mM ammonium acetate:acetonitrile: tetrahydrofuran (58%:32%-10%, v/v) and was pumped at a flow rate of 0.6 ml/min. The retention times for pHPPH and PHT were 7.9 and 11.9 min, respectively as measured at the radioactivity detector.
Data analysis. A response factor for 14C PHT in the radioactive detector was determined by injections of known concentrations of 14C PHT. This response factor was used to estimate concentrations of pHPPh and PHT based on measured peak areas in each sample. V_{\text{max}} and K_{\text{m}} values were estimated using nonlinear regression analysis (SigmaPlot, Jandel Scientific, Version 2.0, San Rafael, CA). All data sets for microsomal incubations were analyzed using both a one and a two enzyme model. The sums of squared residuals for the two models were essentially identical for all data sets. Application of the Akaike Information Criterion (Akaike, 1974) resulted in the choice of a one enzyme model for all cases. V_{\text{max}} values for microsomes and slices are reported per mg of microsomal protein and per gram of liver tissue, respectively. A mean slice weight of 13.9 mg (n = 21, S.D. = 2.4) was used.

The incubation media and the slices from the slice incubations were analyzed separately for total pHPPH and the results added to give the total amount of pHPPH produced. Due to binding of PHT to the slices and high rates of pHPPH formation the concentration of PHT in the media at the end of the incubation in the slice samples was sometimes less than 90% of the concentration added. Therefore, the mean concentration present in the incubation media during the experiment was estimated by averaging the concentration of PHT added at the beginning of the incubation with the concentration measured at the end of the incubation. Unbound PHT concentrations were calculated as the product of the fu, determined by ultrafiltration, and the estimated average PHT concentration. This average unbound concentration is represented in the Michaelis Menten plots and was used in calculating associated parameters.

Recovery of 14C PHT after incubations with the liver slices was evaluated by comparing the combined (buffer and slices), total peak area (PHT + pHPPH) measured in the samples containing slices with the peak area obtained for PHT in the samples incubated without slices. The mean recoveries of radioactivity following glucuronidase hydrolysis for experiments with and without 4% BSA were 97.9 ± 3.09 (mean ± S.D.) and 97.5 ± 1.56, respectively.

For the microsome incubation experiments, unbound phenytoin concentrations were calculated from the fraction unbound to albumin and corrected for partitioning into microsomes.

Results

Unbound fractions of PHT in the buffers containing 2 and 4% BSA were determined to be 0.195 ± 0.003 and 0.126 ± 0.007, respectively. The fu for samples without BSA was assumed to be one.

Partitioning into microsomes was greatest for Tris buffer alone with a 22.4% decrease of the added concentration. The decrease was only 9 to 10% with the 2 and 4% albumin solutions.

Figure 1 shows the curves generated in human liver (HL 23) microsomes in the presence or absence of 4% BSA. V_{\text{max}} and K_{\text{m}} values determined for the microsomal experiments are summarized in table 1. The resulting V_{\text{max}} values with 0, 2 and 4% BSA are 63.8, 1.44 and 1.63 μM, respectively. Although there was excellent agreement among mean V_{\text{max}} values determined with 0, 2 and 4% BSA, there was a 20-fold difference in the mean K_{\text{m}} values determined in the presence and absence of BSA. However, mean K_{\text{m}} values estimated in the presence of 2 and 4% BSA were in excellent agreement. It is also notable that the relative standard deviation expressed as a percent coefficient of variation was 4- to 5-fold greater for incubations performed in the absence of BSA versus with BSA (60 and 12-13%, respectively). The K_{\text{m}} values obtained in the presence of BSA, 1.3 to 1.9 μM, are similar to the mean K_{\text{m}} values of 2 to 3 μM estimated from clinical data (Brown et al., 1985; Grasela et al., 1983; Ludden et al., 1977) assuming a fraction free of 0.1 (Tozer and Winter, 1992).

The results from incubations of PHT with intact slices from HL 23 are shown in figure 2. As was observed with the microsomes, the V_{\text{max}} values for HL 23 are similar between incubations with 0% BSA and 4% BSA (39.4 and 36.0 pmol/min/g, respectively). The HL 23 K_{\text{m}} values for 0 and 4% BSA
based on unbound PHT concentrations are 10.5 and 2.58 μM, respectively.

The \( V_{\text{max}} \) and \( K_m \) values estimated for the studies using human liver slices are presented in table 2. For two of four livers the \( V_{\text{max}} \) values were similar in the presence and absence of BSA. The mean \( K_m \) value in the absence of BSA was 3-fold greater than in the presence of 4% BSA. Thus, the difference in \( K_m \) values between incubations with and without BSA is much less for the slices than for microsomes. As with the results for microsomes, the \( K_m \) values determined in the presence of albumin were similar to those from clinical studies and the coefficient of variation was lower for the 4% BSA results than for the results obtained without BSA (27 vs. 48%, respectively).

**Discussion**

The prediction of human drug metabolism from studies performed in vitro is of great interest to scientists who are attempting to increase the efficiency of the drug development process. These studies may be particularly useful in predicting potential drug-drug interactions (Peck et al., 1993). In vitro studies allow for easy control of experimental variables to obtain data that can be used in scaling-up from in vitro systems to the intact organ and whole body (Pang and Chiba, 1994).

Using the \( V_{\text{max}} \) (31.7 pmol/min/mg) determined in microsomal studies with BSA and assuming 15 mg microsomal protein per gram of human liver tissue (Schmucker et al., 1990), a predicted \( V_{\text{max}} \) \textit{in vivo}, of 3.9 mg/kg/day is obtained. This is somewhat lower than the value of 5.9 mg/kg/day which has been determined \textit{in vivo} (Benet et al., 1996; Grasela et al., 1983). The predicted \( V_{\text{max}} \) \textit{in vivo}, estimated using the slice results is 0.97 mg/kg/day or four times less than the predicted \( V_{\text{max}} \) obtained with microsomes and one-sixth of the \( V_{\text{max}} \) observed \textit{in vivo}. Vickers et al. (1992, 1993) reported that human liver microsomes metabolized cyclosporin A seven times faster than human liver slices, and that, for an ergot derivative, clearance predictions from microsomes were closer to \textit{in vivo} values than were predictions from slices.

In the intact liver slices, enzymes are maintained close to the environment \textit{in situ}; however, it is possible that there is poor penetration of substrate into the interior region of a slice or this region is deficient in metabolic activity. Dogterom (1993) and Worboys et al. (1995) have presented data indicating that the amount of metabolite formed by slices is dependent on the way in which they are incubated, but they report conflicting results on the effect of slice thickness relative to the rate of metabolite formation. Worboys et al. (1995) found that increasing slice thickness produced increased rates of metabolism, although Dogterom (1993) found that increased slice thickness resulted in a decrease in rates when normalized to mg of slice wet weight.

Human \( K_m \) values for PHT determined from \textit{in vivo} studies exhibit high intersubject variability with coefficients of variation being about 50% (Grasela et al., 1983). One explanation for this variability is that PHT is metabolized by two or more pathways, each with different \( K_m \) values. Differing relative amounts of the enzymes could then produce a range of \( K_m \) values. A good correspondence has been found between PHT

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**Table 1**

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<tr>
<th>HL ID</th>
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<th>4% BSA in Tris Buffer</th>
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<td>( K_m^a )</td>
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<td>31.8</td>
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<td>Coefficient of variation, %</td>
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<td>60.4</td>
<td>51.3</td>
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\( ^a V_{\text{max}} \) results expressed as pmol/min/mg microsomal protein. 
\( ^b K_m \) values expressed as μM unbound PHT.

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**Fig. 2.** Concentration dependent total pHPHP formation in human liver slices (HL-23) measured after a 6-hr incubation (A) in the absence of BSA and (B) in the presence of 4% BSA. All samples were incubated with β-glucuronidase to obtain results for total pHPHP. The \( V_{\text{max}} \) values determined by nonlinear regression were similar (39.4 and 36.0 pmol/min/mg), although the unbound \( K_m \) values were 10.5 and 2.58 μM, respectively. Symbols represent mean ± S.D. of three replicate analyses.
characteristics suggestive of significant alterations in PHT binding. Thus, the apparent high variability in human K_m values determined in vivo is unlikely to be explained by unrecognized variation in fu. It can be shown by simulation studies that the apparent intersubject variability in K_m reported from clinical studies is probably due, at least in part, to the use of only a few steady-state concentrations at two or more dosing rates in combination with very small (~5%), unaccounted for, changes in V_max between steady-state observation periods (M.-L. Chen, D. Schuurman, R. Miller, T. M. Ludden and T. N. Tozer, unpublished observations).

The effect of BSA on the K_m values relative to unbound substrate are of particular interest. The mean K_m values relative to unbound PHT obtained in the presence of BSA were very similar to mean values determined in vivo (Grasela et al., 1983) assuming a mean fu of 0.1 (Tozer and Winter, 1992). The K_m values were higher in the absence of BSA (tables 1 and 2). Worboys et al. (1996) suggest that higher K_m estimates in rat slices relative to hepatocytes indicate a delayed access of substrate into the slices. Using parallel reasoning, the effect of BSA on the slice experiments would seem to imply that BSA enhances the rate of penetration of PHT into the cells of the slice.

Another possible explanation for the difference in K_m values is that PHT is not in true solution in the media without albumin. However, the reported aqueous solubility of PHT at pH 7.4 and 37°C is about 120 μM (Schwartz et al., 1977), 60 times the apparent true K_m value relative to free PHT.

The effect of added BSA on the K_m estimate is much greater for micromes than for slices. The results of the same study in liver microsomes suggest that albumin is in some way increasing the apparent affinity between enzyme and substrate. Perhaps albumin is affecting the tertiary and quaternary structure of the P450 system. In microsomes or rat liver microsomes has been reported to be about 0.3 to 0.4 mg/g liver. About 0.5 g of albumin is located in the secretory channels of hepatocytes (Peters, 1966). However, how these values relate to the in vivo concentration of albumin at the enzyme site is unknown. Alternately, because albumin is not a totally purified substance, it is also possible that trace amounts of contaminants might play a role in altering the K_m value.

Future investigations should compare other sources and fractions of albumin. In addition, further studies are needed using additional substrates of CYP2C9 that exhibit a wide range of albumin binding. However, at this time, most known CYP2C9 substrates are substantially bound to albumin. Additional studies are also needed to determine if there is a similar effect of albumin on the activity of other P450 enzymes in vitro.

Our results indicate that the addition of BSA to either human microsomes or liver slices has a marked effect on the apparent PHT K_m values and the variability among different livers. In both cases lower and less variable K_m values are obtained and these values correspond more closely to those observed in vivo. However, there appears to be little or no effect on albumin on the V_max values for PHT hydroxylation, in vitro. However, the V_max values obtained from studies with microsomes were more representative of the V_max.

<p>| TABLE 2 |
| Incubation of 14C PHT with human liver slices |</p>
<table>
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<td>Coefficient of variation, %</td>
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</table>

a V_max results expressed as pmol/min/g liver tissue.

b All K_m values are expressed as μM unbound PHT.
values obtained in vivo than were the V_max values obtained with slices. The cause remains unexplained.

Further investigations of the influence of experimental conditions and the choice of in vitro preparations are needed to increase the predictive potential of quantitative drug metabolism data obtained in vitro.

References


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