PROBENECID-ASSOCIATED ALTERATIONS IN VALPROIC ACID PHARMACOKINETICS IN RATS: CAN IN VIVO DISPOSITION OF VALPROATE GLUCURONIDE BE PREDICTED FROM IN VITRO FORMATION DATA?

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ABSTRACT:

Previous investigations have suggested that probenecid (PRB) alters the in vivo disposition of valproic acid (VPA), perhaps by inhibiting hepatic formation of valproate glucuronide (VG). Because VPA and PRB bind moderately to plasma proteins, protein binding also is a potential locus of interaction. The purpose of this investigation was to determine whether in vitro systems could accurately predict PRB-associated perturbations in the hepatobiliary disposition of VPA and VG in vivo. VPA and PRB were coadministered to rats for 60 min at various infusion rates to examine steady-state VPA disposition. PRB did not alter the binding of VPA in serum or hepatic cytosol. However, PRB decreased the apparent intrinsic clearance of VPA (1.81 ± 0.58 versus 1.23 ± 0.23 ml/min; P = .025) by competitively inhibiting VPA elimination. In a separate study, rat hepatic S9 fractions were incubated with VPA (7.2–721 μg/ml) and PRB (0–2850 μg/ml). VG formation (Vmax = 0.80 ± 0.06 μg/min/mg of protein; Km = 173 ± 28.8 μg/ml) was impaired by PRB in a competitive manner (Ki = 876 ± 559 μg/ml), consistent with the in vivo data. Despite inhibition of phase II metabolism of VPA to VG by PRB, the VG biliary excretion rate at similar unbound VPA concentrations in hepatic cytosol was not lower in PRB-treated rats. These results indicate that VG disposition in the presence of PRB cannot be predicted accurately based solely on in vitro inhibition of glucuronidation and emphasize the complexity of processes associated with the hepatobiliary system.

Hepatic disposition of xenobiotics may be modulated by a number of mechanisms, including alterations in hepatocellular uptake, hepatocellular binding, metabolism, hepatic egress, and biliary excretion. Perturbations in the hepatobiliary disposition of drugs, toxicants, and endogenous substrates may alter pharmacologic and toxic responses. In vitro systems may be useful in predicting xenobiotic interactions at specific sites in the liver. However, the complexity of the in vivo system may require a multiexperimental approach to accurately predict a priori perturbations in the disposition of xenobiotics and metabolites secondary to drug interactions, chemical exposure, or physiologic variations.

The anticonvulsant valproic acid (VPA 1) is a useful model substrate for studying hepatic translocation processes. VPA is relatively lipophilic [octanol:water partition coefficient of ~900 at pH 7.2 (Liu, 1991)] and exists predominantly in the ionized form at physiologic pH (Bellringer et al., 1988). The nonlinear relationship between VPA and its phase II metabolite, valproate glucuronide (VG), have not been investigated.

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1 Abbreviations used are: VPA, valproic acid; AIC, Akaike’s information criterion; MRP, multidrug resistance protein; PRB, probenecid; VG, valproate glucuronide.

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plasma concentrations and dose in rats has been attributed, in part, to saturable hepatic clearance (Dickinson et al., 1979). Although the VPA elimination rate does not increase proportionately with steady-state concentrations of unbound VPA in serum, the relationship between elimination rate and unbound concentration in hepatic cytosol is linear (Brouwer et al., 1993). These data support the hypothesis that the nonlinear elimination of VPA in rats may be due to concentration-dependent distribution of VPA between the hepatocyte and blood. However, studies in freshly isolated rat hepatocytes indicated that hepatocellular uptake of VPA occurred rapidly by passive diffusion (Booth et al., 1996); no evidence for a saturable translocation process was observed. The mechanism(s) underlying the nonlinear hepatic distribution of VPA have yet to be elucidated completely.

VPA is metabolized by β- (in hepatic mitochondria) and ω- (by microsomal enzymes) oxidation, as well as by acyl glucuronidation [mediated by uridine diphosphate glucuronosyltransferase (Schoffen and van der Kleijn, 1982)]. After a 75- to 300-mg/kg dose of VPA, approximately 50% of the administered dose undergoes glucuronidation in male rats (Pollack and Brouwer, 1991) with somewhat more extensive glucuronidation in female animals (Liu and Pollack, 1993). After a similar dose of VPA in female rats, 29% of the dose was excreted in urine, and 53% in bile, as valproate glucuronide (VG) (Liu et al., 1992). The mechanism(s) involved in the formation and biliary excretion of VG, and their contribution to the nonlinear disposition of VPA, have not been investigated.

VPA is bound moderately and in a concentration-dependent fashion to proteins in rat serum and hepatic cytosol (Brouwer et al., 1993). The degree of VPA binding was lower ex vivo than in vitro, and VG...
was bound more highly than VPA ex vivo in serum. These data are consistent with the hypothesis that VG may displace VPA from protein binding sites. The intrahepaticocyte protein binding may alter hepatic translocation of VPA and should be considered as a potential site of interaction/regulation of hepatobiliary VPA disposition.

Previous investigations have suggested that probenecid (PRB) alters the hepatobiliary disposition of VPA, perhaps by inhibiting VG formation (Golden et al., 1993). PRB is an inhibitor of several renal and hepatic transport systems and undergoes extensive phase I and phase II metabolism in the rat (Dayton et al., 1973). PRB ultimately is metabolized to an acyl and two ether glucuronides (Guarino et al., 1969). PRB decreases the clearance of numerous substrates of uridine diphosphateglucuronosyltransferase (Smith et al., 1985) and also directly inhibits glucuronidation of various substrates (Mays et al., 1991). In addition to directly inhibiting glucuronidation, PRB may deplete cofactor supply or inhibit cofactor transport to the enzymatic site (Hauser et al., 1988). PRB binds significantly (~90%) in human serum (Vree et al., 1993), and in vitro studies have shown that PRB glucuronide (a major metabolite of PRB) is ~75% bound in serum (Vree et al., 1992). PRB is transported actively into bile (Guarino and Schanker, 1968) and may impair the biliary excretion of other xenobiotics (Savina and Brouwer, 1992). PRB, a classic inhibitor of organic anion transport, has been shown to reverse multidrug resistance in multidrug resistance-associated protein (MRP) overexpressing cell lines (Gollapudi et al., 1997).

The current investigation characterized the effects of PRB on the in vivo disposition of VPA in rats. To determine whether in vitro systems could accurately predict PRB-associated perturbations in the hepatobiliary disposition of VPA and VG in vivo, the effects of PRB on the ex vivo protein binding and in vitro hepatic glucuronidation of VPA were examined.

Materials and Methods

Chemicals. VPA was obtained from Aldrich Chemical Co. (Milwaukee, WI), and PRB was purchased from Sigma Chemical Co. (St. Louis, MO); all other reagents were purchased from standard commercial sources and were of the highest purity available.

Animals. Male Sprague-Dawley rats (Hilltop Laboratory Animals, Scottdale, PA) were used in all experiments. Animals were allowed to acclimate for at least 1 week before experimentation. Rats were housed individually for at least 1 week before experimentation. Rats were housed individually (Scottdale, PA) were used in all experiments. Animals were allowed to acclimate for at least 1 week before experimentation. Rats were housed individually for at least 1 week before experimentation. Rats were housed individually (Scottdale, PA) were used in all experiments. Animals were allowed to acclimate for at least 1 week before experimentation. Rats were housed individually for at least 1 week before experimentation.
TABLE 1
Serum VPA and VG concentrations, and apparent VPA clearance, after various continuous infusion rates of VPA (n = 1 rat per infusion rate), with and without PRB treatment

<table>
<thead>
<tr>
<th>Continuous VPA Infusion Rate</th>
<th>Serum VPA Concentration at 60 min</th>
<th>Serum VG Concentration at 60 min</th>
<th>Apparent Clearance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/h</td>
<td>µg/ml</td>
<td>µg/ml</td>
<td>ml/min</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.18</td>
<td>7.46</td>
<td>2.65</td>
<td>7.10</td>
</tr>
<tr>
<td>7.36</td>
<td>17.4</td>
<td>18.7</td>
<td>7.05</td>
</tr>
<tr>
<td>14.7</td>
<td>61.1</td>
<td>51.6</td>
<td>4.01</td>
</tr>
<tr>
<td>21.0</td>
<td>132</td>
<td>49.0</td>
<td>2.65</td>
</tr>
<tr>
<td>44.1</td>
<td>301</td>
<td>110</td>
<td>2.44</td>
</tr>
<tr>
<td>72.0</td>
<td>534</td>
<td>272</td>
<td>2.25</td>
</tr>
<tr>
<td>98.0</td>
<td>995</td>
<td>151</td>
<td>1.26</td>
</tr>
<tr>
<td>PRB*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.28</td>
<td>9.01</td>
<td>3.37</td>
<td>4.22</td>
</tr>
<tr>
<td>4.20</td>
<td>36.5</td>
<td>0</td>
<td>1.92</td>
</tr>
<tr>
<td>9.00</td>
<td>57.2</td>
<td>85.7</td>
<td>2.62</td>
</tr>
<tr>
<td>13.2</td>
<td>128</td>
<td>123</td>
<td>1.72</td>
</tr>
<tr>
<td>26.0</td>
<td>401</td>
<td>141</td>
<td>1.08</td>
</tr>
<tr>
<td>49.0</td>
<td>851</td>
<td>90.8</td>
<td>0.960</td>
</tr>
</tbody>
</table>

* Clearance = VPA infusion rate/serum VPA concentration at 60 min.
* Apparent clearance was statistically different across infusion rates, P < .05.
* Apparent clearance was statistically different from control, P < .05.

where \( n \) is the binding capacity, \( K_s \) is the association constant for the specific binding site, and \( m \) is the slope of the nonspecific binding profile. The binding equations each were fit to the data, and the model selection criteria described above were used to determine the optimal model.

In vitro metabolism data also were analyzed by nonlinear least-squares regression to determine \( V_{\text{max}} \) and \( K_m \) for VG formation, the mechanism of inhibition [competitive, noncompetitive, or uncompetitive (Segel, 1976)], and the \( K_i \) for inhibition by PRB. The model selection criteria discussed above were used to identify the optimal inhibition model.

Statistical Analysis. To assess the significance of differences in \( V_{\text{max}} \) and \( K_m \) for VG formation between different PRB treatment groups, one-way ANOVA was used; comparison of PRB treatment groups to controls was performed by a multiple comparison \( t \) test with a Bonferroni correction. Differences between group means in the in vivo and ex vivo studies were assessed with an unpaired Student’s \( t \) test where appropriate. To determine the significance of treatment and infusion rate on the apparent clearance of VPA, a two-way ANOVA was constructed. The influence of treatment and subcellular fraction on hepatic disposition of VPA also was assessed by a two-way ANOVA; a Tukey’s Studentized range test was used to determine which subcellular fraction(s) contributed to the variance in VPA disposition. In all cases, data are expressed as mean ± S.D., symbols represent individual animals (unless otherwise noted), and \( P < .05 \) was predetermined as the criterion of significance.

Results

PRB consistently increased VPA serum concentrations in vivo compared with control rats receiving comparable VPA infusion rates, resulting in a decrease in the apparent clearance of VPA (Table 1). In the presence and absence of PRB, apparent VPA clearance decreased with increasing VPA infusion rate. Two-way ANOVA indicated that both PRB treatment (\( P = .012 \)) and VPA infusion rate (\( P = .028 \)) were significant determinants of VPA clearance.

The model selection criteria indicated that a saturable elimination model provided a better description of the elimination rate versus steady-state serum VPA concentration data (Fig. 1A) than a simple linear model for both the control and PRB-treated data. Estimates for \( V_{\text{max}} \) in control and PRB-treated rats were not significantly different. However, the model-generated estimate of \( K_m \) was nearly 3-fold higher in PRB-treated animals. To increase the accuracy of the model-generated estimate of \( K_m \), a Michaelis-Menten model with a single linear least-squares regression of elimination rate versus cytosolic VPA concentrations provided the best description of the data in both control and PRB-treated rats. The solid line represents the best fit of a Michaelis-Menten elimination rate model to the control data; the dashed line represents model fit to the PRB-treated data.

\[ V_{\text{max}} \text{ and either a control } K_m \text{ or a PRB-treated } K_m \text{ was fit simultaneously to the combined data (serum VPA concentration range of 7.5–1000 µg/ml)} \]

\[ \text{from control and PRB-treated animals. The parameter estimates generated for this model were } V_{\text{max}} = 1914 ± 215 \mu \text{g/min, control } K_m = 442 ± 110 \mu \text{g/ml, and PRB-treated } K_m = 1186 ± 244 \mu \text{g/ml.} \]

In contrast to the relationship between elimination rate and serum VPA concentrations, the rate of VPA elimination at steady-state appeared to increase proportionately with VPA concentrations in hepatic cytosol (Fig. 1B); a linear model provided the optimal fit to the observed data, based on standard model selection criteria (AIC, residual sums of squared error, and condition number of the model). Linear least-squares regression of elimination rate versus cytosolic VPA concentrations provided apparent intrinsic clearance estimates for control (1.81 ± 0.58 ml/min) and PRB-treated (1.23 ± 0.23 ml/min) rats that were significantly different (\( P = .025 \), two-tailed unpaired Student’s \( t \) test).

The effect of PRB on ex vivo VPA protein binding in serum also was evaluated. A model incorporating a single specific binding site provided the best description of the data in both control and PRB-treated rats. The unbound VPA fraction in serum was 0.722 ± 0.129
for control and 0.764 ± 0.093 for PRB-treated animals. Because PRB did not alter the relationship between bound and unbound VPA in serum, estimates of binding parameters were obtained by combining data from both control and PRB-treated rats to increase the accuracy of the estimation (Fig. 2A; data from both control and PRB-treated rats to increase the accuracy of the estimation). The unbound VPA fractions in cytosol were 0.275 ± 0.082 for control and 0.345 ± 0.092 for PRB-treated animals. Because PRB treatment did not affect this relationship, the data for control and PRB-treated animals were combined as in Fig. 2A (slope = 2.13). VG was not detected in hepatic cytosol.

The nonlinear relationship between the VPA elimination rate and serum VPA concentrations shown in Fig. 1A appeared to be associated with nonlinear distribution of VPA between serum and hepatic cytosol. The cytosol-to-serum partition coefficient decreased from 559 (173 ± 28.8 versus
584 ± 278 μg/ml) and no change in $V_{max}$ of 0.80 ± 0.06 versus 0.76 ± 0.37 μg/min/mg of protein) at PRB concentrations of 0 versus 0.2850 μg/ml (Table 3).

### Discussion

In the in vivo disposition studies performed in this investigation, PRB decreased the apparent clearance of VPA due to a significant decrease in intrinsic clearance. These findings are consistent with the in vitro study. PRB significantly increased the $K_m$ but not the $V_{max}$ for VPA glucuronidation in vitro. These data support the hypothesis that PRB competitively inhibits the elimination of VPA and are consistent with previously published observations in which PRB treatment increased the $K_m$ for VPA elimination in rats (Golden et al., 1993).

PRB treatment did not alter VPA protein binding in serum or hepatic cytosol or the binding of VG in serum. VPA binding parameters in serum were similar to previously published ex vivo results [{$nP_{app} = 0.229 ± 0.079$ mg/ml; $K_p = 3.25 ± 1.86$ ml/mg} (Brouwer et al., 1993)]. VPA, PRB, and the associated glucuronide conjugates are moderately to highly protein-bound in serum (Vree et al., 1992; Brouwer et al., 1993). The apparent lack of any binding interactions in the present study suggests that these compounds are bound to different sites.

The relationship between unbound VPA concentrations in serum and hepatic cytosol appeared to be nonlinear. These results are in agreement with previously published in vivo data (Brouwer et al., 1993) suggesting that a regulatory process is involved in the uptake of VPA into hepatocytes. Although the mechanism underlying this nonlinear distribution is unclear, PRB did not alter the distribution of VPA between cytosol and serum.

In general, the VG biliary excretion rate increased as unbound VPA concentrations in serum increased ($P < .0194$, one-way ANOVA). Bile flow and VG excretion determined during each 15-min bile collection interval in control animals (○) and PRB-treated animals (●) (baseline bile flow, i.e., intercept, = 9.92 μl/min; slope = 29.0 μl/μmol by orthogonal linear least-squares regression).

### Table 3

<table>
<thead>
<tr>
<th>Subcellular Fraction</th>
<th>Vehicle (n = 7)</th>
<th>Probenecid (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear</td>
<td>0.523 ± 0.110</td>
<td>0.621 ± 0.137</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>0.212 ± 0.052</td>
<td>0.175 ± 0.032</td>
</tr>
<tr>
<td>Lysosomal</td>
<td>0.211 ± 0.092</td>
<td>0.210 ± 0.042</td>
</tr>
<tr>
<td>Microsomal</td>
<td>0.248 ± 0.178</td>
<td>0.194 ± 0.070</td>
</tr>
<tr>
<td>Cytosolic</td>
<td>2.82 ± 0.90</td>
<td>3.37 ± 0.77</td>
</tr>
</tbody>
</table>

$a$ Data are expressed as mean ± S.D.; concentrations of VPA in each fraction were normalized for the respective concentration in whole liver homogenate.

$b$ Significantly different across fractions; $P < .001$.

$c$ Significantly different from all other subcellular fractions (Tukey’s Studentized range test); $P < .05$. 

### Table 2

<table>
<thead>
<tr>
<th>Concentration-dependent PRB inhibition of VPA glucuronidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>VPA concentration (μg/ml)</td>
</tr>
<tr>
<td>---------------------------</td>
</tr>
<tr>
<td>7.14</td>
</tr>
<tr>
<td>14.3</td>
</tr>
<tr>
<td>143</td>
</tr>
<tr>
<td>714</td>
</tr>
<tr>
<td>2850</td>
</tr>
</tbody>
</table>

$a$ Data are expressed as mean ± S.D. (n = 3) based on five VPA concentrations (7.2–721 μg/ml) per preparation.

$b$ Significantly different across PRB treatment; $P = .0194$, one-way ANOVA.
conjugate, the VG formation rate could not be calculated in the present study.

PRB is a potent inhibitor of the MRP efflux pump (Laupeze et al., 1999). In previous studies, PRB significantly enhanced fluorescein accumulation in primary cultured bovine microvessels endothelial cells (Hua-Yun et al., 1998), indicating that inhibition occurs at the MRP transport site. Additionally, PRB was reported to increase MRP2-ATPase activity and to inhibit ATPase activity of MRP1 (Bakos et al., 2000). Because the mechanism(s) of VG canaliculal transport have not been characterized, a clear hypothesis regarding the effects of PRB on the biliary excretion of VG could not be formulated. In the present study, PRB decreased the elimination rate of VPA due to inhibition of metabolism. Because PRB decreased the phase II metabolism of VPA to VG, and because PRB is a known inhibitor of organic anion transport, it was anticipated that the rate of VG appearance in bile would also decrease in PRB-treated rats. However, this effect was not observed (Fig. 4). Two explanations for these results are possible. PRB may inhibit oxidative VPA metabolism in addition to inhibiting VG formation. This could result in increased amounts of VPA available for glucuronidation and, therefore, no apparent change in cytosolic VG concentrations (Ward et al., 2001). Previous work has indicated that PRB inhibits the oxidative metabolism of other compounds (Damm and Erttmann, 1975). It also is possible that PRB inhibits VG translocation from the hepatocyte into blood [via MRPs (Konig et al., 1999) or some other transporter located on the basolateral hepatocyte membrane (Muller et al., 1996)], thereby increasing the driving force for VG biliary excretion. The mechanism(s) responsible for maintenance of VG biliary excretion rates at similar unbound cytosolic VPA concentrations despite inhibition of VG formation by PRB require further investigation.

Canaliculal bile formation is attributed to the mechanisms generating bulk movement of water into bile canaliculi; possible mechanisms include filtration, vesicular transport, and active transport of certain solutes leading to passive water flow (Klaassen and Watkins, 1984). According to Fig. 5, there is a linear relationship between bile volume and the amount of VG transported into bile. The intercept of the line (or the baseline bile formation) was ~10 μL/min (similar to published reports; Shaw and Heath, 1975; Watkins and Klaassen, 1982; Erlinger, 1988) and represents both bile acid-dependent and -independent flow. VG clearly was choleretic; this effect has been noted previously for VG and a number of bile acids, including taurocholic acid (Klaassen, 1974; Watkins and Klaassen, 1981; Liu et al., 1992).

Because VPA binds significantly to proteins and is metabolized by both mitochondrial and microsomal enzyme systems, a differential distribution of the drug within the hepatocyte was anticipated. In general, VPA appeared to localize preferentially in cytosol, in agreement with previously reported data (Brouwer et al., 1993). PRB did not alter the subcellular distribution of VPA. Lack of measurable VG concentrations in cytosol was not surprising because VG is excreted readily into bile and translocated rapidly into blood.

In vitro metabolism studies often are proposed as a surrogate for, or at least a supplement to, more costly and time-consuming in vivo disposition studies. The results of this study demonstrate an inherent difficulty in predicting in vivo interactions from in vitro data. Typically, a particular metabolic pathway is of interest, and in vitro conditions are adjusted to optimize formation of the resulting metabolite. This approach allows assessment of metabolic interactions only for the optimized pathway. In the case of competing metabolic reactions, such as the competition between VPA oxidation and glucuronidation in the present study, in vitro data may not predict the scope of metabolic interactions in vivo. Although perturbations in the hepatoiliary disposition of VPA by PRB may be attributed, at least in part, to competitive inhibition of VPA glucuronidation, other PRB-associated alterations in VPA metabolism or VG hepatobiliary translocation must be operative in the intact organism. Accurate predictions of alterations in vivo disposition based upon in vitro data require a complete understanding of potential sites of interaction in the hepatobiliary system.

References


