EFFECT OF ZONAL TRANSPORT AND METABOLISM ON HEPATIC REMOVAL: ENALAPRIL HYDROLYSIS IN ZONAL, ISOLATED RAT HEPATOCYTES IN VITRO AND CORRELATION WITH PERFUSION DATA

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ABSTRACT:

Previous studies showed that the transport of enalapril occurred homogeneously among zonal rat hepatocytes. However, the metabolism of hepatic arterially delivered enalapril, swept into the rat liver by the portal or hepatic venous flow (HAPV and HAHV perfusion), was more abundant in the perivenous (PV) than the periportal (PP) region. Hence, metabolic activities toward enalapril in 9000g supernatant (S9) fractions of enriched rat PP and PV hepatocytes were examined. Although Michaelis-Menten kinetics were invariably observed, the metabolic activity toward enalapril (intrinsically clearance or \( V_{\text{max}}^{\text{met}}/K_m^{\text{met}} \) of 0.008 ml/min/mg of S9 protein, \( V_{\text{max}}^{\text{met}} \) of 21 ± 6 nmol/min/mg of S9 protein, and \( K_m^{\text{met}} \) of 2612 ± 236 \( \mu \)M) was greater in PV than in PP (\( V_{\text{max}}^{\text{met}} \) of 5.5 ± 3.1 nmol/min/mg of S9 protein and \( K_m^{\text{met}} \) of 1049 ± 335 \( \mu \)M; intrinsic clearance of 0.0052 ml/min/mg of S9 protein) hepatocytes. These metabolic intrinsic clearances were much lower than the sinusoidal influx clearances observed from previous transport studies, revealing metabolism as the rate-limiting step. Substitution of the scaled-up transport and metabolic intrinsic clearances into the “well stirred”, “parallel-tube”, and “dispersion” models predicted higher steady-state extraction ratios for HAHV perfusion. By contrast, integration of the scaled-up in vitro parameters on zonal metabolism and homogeneous transport into a “zonal-compartment” model of three zonal subcompartments (1, 2, and 3) provided an improved description of the extraction ratios during HAPV and HAHV. Zonal factors are important for the scale-up of data in vitro to the whole organ.

Enalapril is a prodrug that is hydrolyzed to yield the active diacid metabolite, enalaprilat. Three members of the type-B carboxylesterase family that are thought to be involved in hepatic xenobiotic metabolism, namely, hydrolases A (Robbi et al., 1990), B (Yan et al., 1994), and C (Yan et al., 1995) have been cloned from rat liver. The expressions of both hydrolases A and B are greater in the perivenous (PV) than the periportal (PP) region of rat liver. Additionally, a 59-kDa carboxylesterase that exhibits a PV zonation has been purified from rat liver (Satoh et al., 1985; Pohl et al., 1991). Hydrolase B is expressed in rat kidney as well as liver (Yan et al., 1994) and is able to hydrolyze aspirin (Mentlein and Heymann, 1984) and the angiotensin converting enzyme inhibitor ester-prodrugs benazepril, delapril, and temocapril (Luan et al., 1997). Hydrolase A is involved in the hydrolysis of clofibrate and procaine (Mentlein and Heymann, 1984). Hydrolases A and B are both paraoxon-inhibitable, and are distinguished from each other by their affinity in catalyzing the hydrolysis of p-nitrophenol acetate (Morgan et al., 1994). The substrate specificity and role played by hydrolase C are, however, less well characterized (Yan et al., 1995).

The highest carboxylesterase activity toward enalapril exists in the liver, where metabolism is characterized by a \( K_m \) value of 710 \( \mu \)M (Tocco et al., 1982; Tabata et al., 1990). Rat liver perfusion studies showed that enalapril was highly removed with a steady-state extraction ratio (\( E_{\text{u,HAPV}} \)) of 0.77 when arterially delivered enalapril was swept into the liver by the flow of blank perfusate through the portal vein in the antegrade direction (HAPV perfusion), whereas a much reduced \( E_{\text{u,HAHV}} \) of 0.12 existed with HAHV perfusion when arterially delivered drug was confined to the PP end of the acinus with retrograde (hepatic venous) perfusion with blank perfusate. The observation inferred that hydrolysis of enalapril occurred predominantly in the PV region (Pang et al., 1991). However, the mechanism, whether transport or metabolism is responsible for the zonation, has not been identified.

The interplay of transport and metabolism and the rate-determining step are important considerations in the description of hepatic processing of drugs. Recently, uptake of enalapril was shown to be mediated by Oatp1, the organic anion transporting polypeptide 1 cloned from rat liver (Pang et al., 1998), and transport activities were found to be identical among zonal hepatocytes (\( K_m^{\text{uptake}} = 344 \mu M, V_{\text{uptake}}^{\text{max}} = 11 \text{nmol/min/10}^6 \text{cells} \) (Abu-Zahra et al., 2000). The definitive answer to the question of whether transport or metabolism is responsible for the zonated hydrolysis in perfused liver required the direct comparison of metabolic and uptake activities. In this

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nication, metabolic activities in the 9000g supernatant (S9) fractions of PV and PP rat hepatocytes were studied to test the hypotheses that metabolism and not transport is rate-determining in the overall hydrolysis of enalapril and that metabolism is greater in the PV region. A correlation was also sought between the in vitro and perfusion data with existing models of hepatic drug clearances.

Theoretical

In vitro parameters obtained from studies in cellular and subcellular fractions may be scaled up to represent parameters for physiological variables such as transport and intrinsic clearances for the whole organ. The correlation between in vitro and whole organ in vivo data is of interest in pharmacokinetics for the understanding of the physiological processes underlying the disappearance of a substrate and the formation of metabolites by the liver. Models of hepatic drug clearances have been proposed to interrelate the physiological variables. These models vary with respect to the description of how blood flow is channeled through the sinusoid as well as how a substrate is dispersed across the acinus, resulting in a concentration gradient in the acinus. Historically, the “well stirred” (or venous equilibration) model (Rowland et al., 1973), which assumes the liver as a single well mixed compartment receiving bulk flow, and the “parallel-tube” (or sinusoidal perfusion) model (Winkler et al., 1973), which describes enzymes being distributed evenly in single sheets of hepatocytes lining the flow path (plug flow), have been used widely. The models represent boundary conditions of perfectly mixed and nonmixed systems, respectively. According to the well stirred model, the concentration of drug in liver is constant and is in equilibrium with that in venous blood, whereas according to the parallel-tube model, drug concentration declines exponentially in the direction of flow. Other models exist and render predictions that are intermediate to those of the well stirred and parallel-tube models: the “dispersion” model (Roberts and Rowland, 1986); the “series-compartment” model (Gray and Tam, 1987); and the “zonal-compartment” model (Meijer and Groothuis, 1991; Tirona and Pang, 1996). The dispersion model describes nonideal blood flow through sinusoids of different lengths, whereas the series-compartment model describes well stirred subcompartments connected in series. Other combinations have also surfaced to depict the “enzyme-distributed” occurrence of enzymatic activities (Pang and Stillwell, 1983).

Models of Hepatic Drug Clearances

The models of drug clearances (Fig. 1) are simplified when viewed under first order conditions. Under these instances, the permeability surface area products (or transport clearances) into and out of the cell (PS$_{in}$ and PS$_{out}$) as well as the metabolic (CL$_{int,liver}$) and biliary (CL$_{bile}$) intrinsic clearances are independent of concentration; protein binding is also linear with concentration. The $E_{ss}$ value for enalapril was predicted using the well stirred, parallel-tube, dispersion, and zonal-compartment models according to eqs. 1 to 6.

**Well Stirred Model.** For the well stirred model, the value of $E_{ss}$ for the whole liver is (Gillette and Pang, 1977):

$$E_{ss} = \frac{f_u \cdot CL_{t,h,liver} \cdot PS_{in}}{PS_{out} + \left(f_u \cdot CL_{t,h,liver} \cdot PS_{in} + Q \cdot CL_{int,liver}\right)}$$

where $Q$ is the plasma flow rate through the liver and enalapril is confined to plasma; $f_u$ is the unbound fraction of drug in the plasma; and $CL_{int,liver}$ is the total intrinsic (metabolic plus biliary) clearance.

**Parallel-Tube Model.** For the parallel-tube model, $E_{ss}$ is given by (Pang and Chiba, 1994):

$$E_{ss} = 1 - e^{\frac{-f_u \cdot CL_{t,h,liver} \cdot PS_{in}}{Q \cdot (PS_{in} + CL_{int,liver})}}$$

**Dispersion Model.** For the dispersion model, $E_{ss}$ is given by (Roberts and Rowland, 1986):

$$E_{ss} = 1 - e^{\frac{1 - \sqrt{1 + 4DN/N}}{(2N)}}$$

where $D_N$ is the dispersion number relating to the degree of dispersion of the system and varied between 0.15 to 0.24 (Tirona et al., 1998). $R_N$, the efficiency number, is a composite parameter of the transport clearances, the intrinsic clearances, flow, and unbound fraction.

$$R_N = \frac{f_u \cdot CL_{t,h,liver} \cdot PS_{in}}{Q \cdot (PS_{in} + CL_{int,liver})}$$

**Zonal-Compartment Model.** The zonal-compartment model, a version of the series-compartment model (Gray and Tam, 1987), described single cells being aligned in series along the sinusoidal flow path (Fig. 2). Three is the smallest number of units to denote the zonal regions, 1, 2, and 3, and both transport and metabolic activities are allowed to vary (Tirona and Pang, 1996). The mass balance rate equations describing the sinusoidal and cellular enalapril concentrations in each ($i^{th}$) of three compartments in series are:

For the change of sinusoidal enalapril concentration in the $i^{th}$ compartment:

$$\frac{d[E]_i}{dt} = \left(Q[E]_i^{-1} - \frac{PS_{in} - f_u \cdot PS_{out} \cdot [E]_i - Q\cdot [E]_i}{N \cdot (PS_{in} + CL_{int,liver})} \right)/V_p$$

and the equation describing the change of cellular enalapril concentration in the $i^{th}$ compartment is:

$$\frac{d[E]_i}{dt} = \left[\frac{PS_{out} \cdot [E]_i}{N \cdot (PS_{in} + CL_{int,liver})} - \frac{PS_{in} \cdot [E]_i - CI_{int,liver} \cdot [E]_i}{N \cdot (PS_{in} + CL_{int,liver})} \right]/V_c$$

where $[E]$ is the concentration of enalapril in either the sinusoid or cell (subscripts $p$ and $c$, respectively) of the $i^{th}$ compartment of $N$ total...
compartments (three in this case). The metabolic intrinsic clearance $CL_{int}^{met}$, for each compartment, or the total metabolic intrinsic clearance ($CL_{int}^{met, liver}$) divided by $N$, may vary. But this is not the case for the biliary intrinsic clearance $CL_{int}^{bile}$, for each compartment [the total biliary intrinsic clearance ($CL_{int}^{bile, liver}$) divided by $N$], which was reported to be constant for all regions of the liver (Pang et al., 1991).

The unbound fractions of enalapril in the vasculature and in tissue are denoted as $f_u$ and $f_t$, respectively. $V_p$ is the volume of the sinusoidal plasma space and $V_s$ is the volume of cell water. For the first compartment ($i = 1$), $[E]_p^{-1} = C_{in}$.

Experimental Procedures

Materials. $[^3H]$Enalapril, synthesized as described previously (Pang et al., 1998), was 98.5% pure, as judged by thin-layer chromatography (TLC) [1-propanol/1 M acetic acid/water (10:1:1, v/v/v), with silica gel GF TLC plates]. Enalaprilat and unlabeled enalapril were obtained from Merck Sharp and Dohme Research Laboratories (West Point, PA). Collagenase A (clostridium histolyticum) was purchased from Boehringer Mannheim (Darmstadt, Germany). All other reagents and solvents were of HPLC grade.

Isolation of Rat Hepatocytes. The isolation of rat hepatocytes from all zones and from zones 1 (PP) and 3 (PV) of the rat liver and from the whole liver was identical with that previously described (Abu-Zahra et al., 2000). The enrichment of PP or PV hepatocytes was routinely verified by marker enzymes (Tang et al., 1999). The activity of the PP marker enzyme, alanine aminotransferase, was 2.20 ± 0.09 times higher (ANOVA $P < .05$) in PP (461 ± 150 nmol/min/mg of protein) than in PV (210 ± 49 nmol/min/mg of protein) hepatocytes, whereas glutamine synthetase, an enzyme expressed exclusively in the distal PV hepatocytes, exhibited significantly lower activity in PP (0.52 ± 0.96 nmol/min/mg of protein) than in PV preparations (27.7 ± 11.6 nmol/min/mg of protein) (ANOVA $P < .05$); the PP/PV ratio was 0.018 ± 0.03.

Preparation of S9 Fraction from Homogeneous and Zonal Hepatocytes. Hepatocyte suspensions of >90% viability were centrifuged for 5 min at 850g. The incubation buffer supernatant was aspirated, and ice-cold Krebs-Henseleit bicarbonate buffer (containing 120 mM NaCl, 25 mM NaHCO$_3$, 4.75 mM KCl, 2.54 mM CaCl$_2$, 1.19 mM MgSO$_4$7H$_2$O, and 1.19 mM KH$_2$PO$_4$) was added in an amount three times the volume of the hepatocyte pellet. The mixture was shaken by vortex, then homogenized for 1 to 2 min (Ultra Turrax T25 homogenizer; Janke and Kunkel, IKA-Labortechnik, Staufen i.m. Briesgau, Germany). Homogenates were centrifuged (Beckman J2-21 M centrifuge; Beckman Canada, Mississauga, ON) at 9000g at 4°C for 20 min. The resulting supernatant (S9 fraction) was either used immediately or stored at −70°C until use.

Metabolic Studies. Preliminary studies had shown that identical rates of enalapril hydrolysis were obtained with freshly prepared and thawed (frozen at −70°C) S9 preparations. Thawed S9 preparations were diluted to a concentration of approximately 2 mg of protein/ml then preincubated in a rotating water bath for 10 min at 37°C. Aliquots (100 µl) of S9 were added to 100 µl of enalapril (and tracer $[^3H]$enalapril, about 2000000 dpm/ml) in Krebs-Henseleit bicarbonate buffer to result in concentrations of 50 to 4000 µM in 0.2 ml of incubation mixture. Samples (100-µl) were retrieved after 15 min, a predetermined time in which metabolic formation rates were found to be linear with time. The samples were placed into 1.5-ml microfuge tubes containing 300 µl of ice-cold CH$_3$CN to halt the reaction. The tubes were centrifuged, and the supernatants were stored at −20°C for later analysis by TLC. Fifty microliters of the remaining incubation mixture was removed for liquid scintillation counting (model 5801; Beckman Coulter, Mississauga, ON, Canada).

Protein was measured by the method of Lowry et al. (1951).

Assays of Enalapril and Enalaprilat. $[^3H]$Enalapril and $[^3H]$enalaprilat were separated by TLC (Pang et al., 1991). Silica gel GF TLC plates (Analtech, Newark, DE) were initially prepared by placement of approximately 150 µl of a solution containing authentic standards of enalapril and enalaprilat in CH$_3$CN and H$_2$O. Two hundred microliters of the deproteinized sample in CH$_3$CN was spotted at the origin of the TLC plate, which was preloaded with the authentic standards. The plate was developed in a solvent system of 1-propanol/1 M acetic acid/water (10:1:1, v/v/v). After resolution, the standards, which had comigrated with their radiolabeled counterparts, were visualized under a UV lamp, and the bands were scraped off into 20-µl glass vials containing 0.5 ml of water and 10 ml of scintillation fluid (Ready Protein; Beckman Instruments). After mixing, the vials were stored in darkness for a minimum of 48 h before liquid scintillation counting. Additionally, 50 µl of sample was sampled for determination of on-plate recovery. The relative amounts of radioactive enalapril and enalaprilat present on the TLC plates and the specific activity of the incubation mixture were used to calculate the amounts of metabolite formed per 15 min. The information provided the rate of metabolism, $v$ (nanomoles of enalapril formed per min per milligram of protein), for each incubation mixture.

Fitting. Fitting of the in vitro metabolic data was performed by an iterative, nonlinear least-squares procedure (Scientist, v.2; Micromath Scientific Software, Salt Lake City, UT). A weighting scheme of 1/(observation)$^2$ was used because this provided the best optimization of the data. The best fit was described by the simple Michaelis-Menten equation:

$$v = \frac{v_{max}^{met}[S]}{K_m^{met} + [S]}$$

where $K_m^{met}$ represents the Michaelis-Menten constant and the $v_{max}^{met}$ denotes the maximal velocity.

In Vitro Data Scale-Up for Use in Models of Hepatic Clearances. Values for plasma flow $[12(1 - \text{hematocrit})\ \text{ml/min}]$, the input enalapril concentration ($C_{in}$), liver weight, and unbound fraction of drug in plasma ($f_u$) were taken from the literature (Pang et al., 1991). Other parameters were derived from in vitro data. The maximal velocity for uptake, $v_{max}^{uptake}$, determined in vitro from isolated hepatocytes in the presence of sodium (Abu-Zahra et al., 2000) was scaled up by multiplication with the scaling factor or $1.25 \times 10^3$ cells/g of liver (Lin et al., 1980), and the ratio $v_{max}^{uptake}/K_m^{uptake}$ provided the sinusoidal influx clearance, $PS_{in}$. Analogously, the maximal velocities for metabolism determined from $S9$ of PP, homogeneous, and PV hepatocytes (Table 1) were scaled up to per gram of tissue by multiplication with the scaling factor, 100 mg of $S9$ protein/g of liver (Mahler and Cordes, 1966). Because HAHV perfusion revealed perfusion volumes of about one-third those for whole liver (Pang et al., 1991), the volume and weight, and the associated intrinsic clearances for transport and metabolism for the PP region were taken as one-third the whole liver; a similar assumption was made for the midzonal (MZ) and PV regions. The metabolic intrinsic clearances $CL_{int,PP}^{met}$, $CL_{int,MZ}^{met}$, and $CL_{int, PV}^{met}$:

$$CL_{int,liver}^{met} = CL_{int,PP}^{met} + CL_{int,MZ}^{met} + CL_{int, PV}^{met}$$

FIG. 2. Schematics of the zonal compartment model consisting of three $i^{th}$ subcompartments (1, 2, and 3); an expansion of any $i^{th}$ compartment is shown.

See text for definition of the terms.
TABLE 1

Comparison of Scaled-Up Transport and Metabolic Intrinsic Clearances. Although no difference was observed for transport among zonal cells (Abu-Zahra et al., 2000), differences were obtained for metabolic intrinsic clearance within zonal hepatocytes CL_{int,liver}. The metabolic intrinsic clearance of the PV region was 1.5 times that of the PP region (Table 2). The scaled-up data revealed a high PS_{out} (4 ml/min/g of liver) in relation to the metabolic intrinsic clearances (0.52–0.8 ml/min/g) among the zonal regions (Table 2). The PS_{out} was 5 to 7.7 times the metabolic intrinsic clearances. The data suggest that metabolism and not transport is the rate-limiting step in the removal of enalapril.

Predictions of PS_{out} and E_{ss,HAHV} with the Well Stirred, Parallel-Tube, and Dispersion Models. Values of PS_{out} based on the scaled-up PS_{in} and intrinsic clearances and the observed E_{ss,HAPV} value of 0.77 (Pang et al., 1991) were different among the well stirred, dispersion, and parallel-tube models (Table 3). Varying from a total mixing model to a nonmixing model, the estimated PS_{out} values decreased with increased mixing (1.93 < 9.2 < 15.5 ml/min/g), giving rise to predicted E_{ss,HAHV} values of the reverse rank order (0.53 > 0.44 > 0.39) (Table 3). The lower PS_{out} rendered higher extraction ratios due to entrapment of drug in the cell. The predicted values of E_{ss,HAHV} were 3.3 to 4.5 times the observed value, as were the ratios E_{ss,HAHV}/E_{ss,HAPV}.

Predictions of the Series-Compartment Model of Three Subcompartments. Because three different PS_{out} values resulted with the well stirred, parallel-tube, and dispersion models, simulations were performed with each of the PS_{out} values for each of the enzyme distributions (sets 1–3; zonal-compartment models of even-enzyme distribution, zonal-enzyme distribution based on observed data, and modified, zonal-enzyme distribution incorporating variability; Table 4), yielding a total of nine different simulations of enalapril removal with the zonal-compartment model (Table 5). Among the simulations based on similar intrinsic clearances, values for E_{ss,HAHV} were generally similar, regardless of the enzyme distribution due to the high transport clearance of drug, rendering enzymic distribution unimportant for whole organ removal of the unienzyme substrate (Pang and Stillwell, 1983). Again, the lower PS_{out} rendered higher extraction ratios due to entrapment of drug in the cell. E_{ss,HAHV} and E_{ss,HAHV} were greatest with the lowest PS_{out} of 1.93 ml/min/liver, and both parameters were progressively diminished with increasing PS_{out} (9.2 and 15.5 ml/min/liver). Although the E_{ss,HAHV} values were generally similar for all three scenarios of the zonal-compartment model (Table 5), values of E_{ss,HAHV} began to diverge due to differences in enzyme content with increasing PS_{out}. The best correspondence existed for the PS_{out} of 9.2 or 15.5 ml/min/liver with the modified zonal-enzyme system (sets 2 and 3, Table 5).

Results

Enalapril Metabolism in S9 Fraction of Homogeneous and Zonal Hepatocytes. In the S9 fractions of homogeneous, PP, or PV hepatocytes, metabolism of enalapril was linear within 30 min (data not shown). Other preliminary studies failed to show loss of activity in thawed versus fresh preparations (data not shown). Metabolism was concentration-dependent among all preparations (Fig. 3), and the fit was best with eq. 7, which described simple Michaelis-Menten kinetics with a single saturable component. The fitted V_{max,PP} was 5.5 ± 3.1 nmol/min/mg of S9 protein, and the corresponding K_{m,PP} was 1049 ± 335 μM for PP hepatocytes. These values were not statistically different from those obtained from the S9 of homogeneous hepatocytes: V_{max,HO} of 8.0 ± 2.99 nmol/min/mg of S9 protein and K_{m,HO} of 1308 ± 419 μM (Table 1; P > .05, ANOVA), and the K_{m,met} values were generally similar to the value of 710 μM reported by Tabata et al. (1990). However, the fitted V_{max,HAPV} (21.0 ± 6.0 nmol/min/mg of S9 protein) and K_{m,HAPV} (2612 ± 236 μM) for the S9 of PV hepatocytes were both significantly higher than those observed for the S9 of either homogeneous or PP hepatocytes (Table 1; P < .05, ANOVA).

The weighting scheme of 1/observation^2 was optimal. Significant (P < .05) compared with S9 of homogeneous hepatocytes. Significant (P < .05) compared with S9 of PP hepatocytes.

**TABLE 1**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>V_{max, S9} b</th>
<th>K_{m, S9} b</th>
</tr>
</thead>
<tbody>
<tr>
<td>S9 from homogeneous hepatocytes</td>
<td>1308 ± 419</td>
<td>8.0 ± 3.0</td>
</tr>
<tr>
<td>(n = 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S9 from PP hepatocytes (n = 4)</td>
<td>1049 ± 335</td>
<td>5.5 ± 3.1</td>
</tr>
<tr>
<td>S9 from PV hepatocytes (n = 4)</td>
<td>2612 ± 236</td>
<td>21.0 ± 6.0</td>
</tr>
</tbody>
</table>

The biliary intrinsic clearance (CL_{int,liver}) for the whole liver was estimated according to the ratio of the rates of excretion to metabolism (0.032/0.734) in previous perfusion studies (Pang et al., 1991) and total metabolic intrinsic clearance of the liver.

The biliary clearance (CL_{int,liver}) for the entire liver was approximated as:

\[
\text{CL}_{\text{int,liver}} = \frac{\text{V}_{\text{ss,HAHV}}}{\text{E}_{\text{ss,HAHV}}} \approx \text{E}_{\text{ss,HAHV}} = \frac{\text{V}_{\text{ss,HAHV}}}{\text{E}_{\text{ss,HAHV}}} = \frac{\text{V}_{\text{ss,HAHV}}}{\text{E}_{\text{ss,HAHV}}} = 0.049 \quad (9)
\]

The total intrinsic clearance (CL_{total, liver}) was the sum of biliary and metabolic intrinsic clearances.

\[
\text{CL}_{\text{total, liver}} = \text{CL}_{\text{int, liver}} + \text{CL}_{\text{int, liver}} = \frac{\text{V}_{\text{max, PP}}}{\text{K}_{\text{m, PP}}} + \frac{\text{V}_{\text{max, HO}}}{\text{K}_{\text{m, HO}}} = \frac{\text{V}_{\text{max, PP}}}{\text{K}_{\text{m, PP}}} + \frac{\text{V}_{\text{max, HO}}}{\text{K}_{\text{m, HO}}} = 0.049 \quad (9)
\]

Because no data on the sinusoidal efflux of enalapril was available, values of the sinusoidal efflux clearance (PS_{in}) were calculated by setting E_{ss,HAPV} (the steady-state extraction through whole liver) equal to 0.77 and solving for PS_{in} with the equations for well stirred, parallel-tube, and dispersion models (eqs. 1–3). These PS_{in} values were in turn used to predict the E_{ss,HAHV} (steady-state extraction ratio for HAHV perfusion) for the well stirred, parallel-tube, and dispersion models, correspondingly, after considering that values of the PS_{out}, PS_{in}, and the intrinsic clearances pertaining to the PP region were one-third the values of whole liver (Table 3).

The Alternative, Zonal-Compartment Model. A model of three zonal compartments in series (repeated sets of eqs. 5 and 6; Tirona and Pang, 1996) was used to describe the segregated, metabolic activities in different zones of the liver (Fig. 2). Within each zone, the metabolic intrinsic clearance, CL_{int, zone}, was varied and ascribed various values according to the observations (Table 2), whereas the transport clearances were kept constant (Table 4). Three possible scenarios were presented: in the first instance, the total metabolic and biliary intrinsic clearances were divided evenly into three equal portions for zones 1, 2, and 3 (set 1); in the second case (set 2), the metabolic intrinsic clearances were calculated from the scaled-up mean (V_{max, PP} / K_{m, PP}) in vitro parameters of each zone (see Table 2); and in the third case, a modified, zonal distribution (set 3) was adopted to encompass variability of the data. For the PP zone, the value V_{max, PP} was given by the value of (mean − S.D.) whereas K_{m, PP} was given the value (mean + S.D.). For the PV zone, V_{max, PP} and K_{m, PP} were modified to yield the highest V_{max, PP} (mean + S.D.) and lowest K_{m, PP} (mean − S.D.), whereas parameters for the MZ region were not modified. This manipulation provided a much steeper activity gradient of metabolic activities.

Simulations. Simulations for the zonal-compartment model (three scenarios) were performed on Scientific C.2 (Micromath Scientific Software) with the differential equations presented previously (eqs. 5 and 6) and values shown in Table 4.

Statistics. The data were presented as mean ± S.D. The means were compared by use of ANOVA or the paired r-statistic accordingly, with P = .05 denoting the level of significance.
Drug Removal within Zonal Regions of the Zonal Compart-ment. Because the zonal-compartment model with the modified zonal-al enzyme distribution best described the data, simulation was performed to determine the effects of the metabolic intrinsic clearance \( CL_{\text{int,PP}} \), at the given influx transport clearance (\( PS_{\text{in}} \)) on the removal within each zone (\( F_i \)), whereas all other parameters were held constant. The overall availability of the liver (\( F_{\text{liver}} \)) was given by the product of the individual availability of each zone (\( F_i \)), as expected of the relation for compartments in series (Table 6).

### Table 2

Parameters obtained from the literature (Pang et al., 1991) or scaled up from data of in vitro studies

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C_{\text{in}} ) (( \mu \text{M} ))</td>
<td>Initial input concentration of enalapril</td>
<td>2.5a</td>
</tr>
<tr>
<td>( f_{\text{unb}} )</td>
<td>Unbound fraction in sinusoid or tissue</td>
<td>0.82a</td>
</tr>
<tr>
<td>( Q ) (ml/min)</td>
<td>Plasma flow rate ( = [\text{blood flow rate} \times (1 - \text{hematocrit})] )</td>
<td>10.3b</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>Weight of whole liver</td>
<td>12.8c</td>
</tr>
<tr>
<td>( V_{\text{up}} ) (max) (ml/min/g liver)</td>
<td>Maximal velocity for uptake in all zones</td>
<td>1377 \pm 186c</td>
</tr>
<tr>
<td>( K_{\text{in}} ) (( \mu \text{M} ))</td>
<td>Michaelis-Menten constant for uptake in all zones</td>
<td>344 \pm 52b</td>
</tr>
<tr>
<td>( F_{\text{SS,HAHV}} )</td>
<td>Influx permeability surface area product</td>
<td>4.0</td>
</tr>
<tr>
<td>( V_{\text{max,PP}} ) (ml/min/g liver)</td>
<td>Maximal velocity of metabolism in periporal zone</td>
<td>547 \pm 310b</td>
</tr>
<tr>
<td>( K_{\text{m,PP}} ) (( \mu \text{M} ))</td>
<td>Michaelis-Menten constant for metabolism in periporal zone</td>
<td>1049 \pm 335a</td>
</tr>
<tr>
<td>( V_{\text{max,MZ}} ) (ml/min/g liver)</td>
<td>Maximal velocity of metabolism in midzonal region</td>
<td>805 \pm 300b</td>
</tr>
<tr>
<td>( K_{\text{m,MZ}} ) (( \mu \text{M} ))</td>
<td>Michaelis-Menten constant for metabolism in midzonal region</td>
<td>1308 \pm 419b</td>
</tr>
<tr>
<td>( V_{\text{max,FV}} ) (ml/min/g liver)</td>
<td>Maximal velocity of metabolism in perivenous zone</td>
<td>2096 \pm 600b</td>
</tr>
<tr>
<td>( K_{\text{m,FV}} ) (( \mu \text{M} ))</td>
<td>Michaelis-Menten constant for metabolism in perivenous zone</td>
<td>2612 \pm 236b</td>
</tr>
<tr>
<td>( PS_{\text{in}} ) (ml/min/g liver)</td>
<td>Metabolic intrinsic clearance in periportal zone</td>
<td>0.52 \pm 0.34</td>
</tr>
<tr>
<td>( CL_{\text{INT,PP}} ) (( \mu \text{M} ))</td>
<td>Metabolic intrinsic clearance in midzonal region</td>
<td>0.62 \pm 0.30</td>
</tr>
<tr>
<td>( CL_{\text{INT,MZ}} ) (( \mu \text{M} ))</td>
<td>Metabolic intrinsic clearance in perivenous region</td>
<td>0.80 \pm 0.24</td>
</tr>
<tr>
<td>( CL_{\text{INT,FV}} ) (( \mu \text{M} ))</td>
<td>Metabolic intrinsic clearance for whole liver</td>
<td>8.27</td>
</tr>
<tr>
<td>( K_{\text{m,PP}} ) (( \mu \text{M} ))</td>
<td>Biliary intrinsic clearance for whole liver</td>
<td>0.408</td>
</tr>
<tr>
<td>( K_{\text{m,MZ}} ) (( \mu \text{M} ))</td>
<td>Total intrinsic clearance for whole liver</td>
<td>8.68</td>
</tr>
</tbody>
</table>

### Table 3

Calculated clearances for the well-stirred, parallel tube, and dispersion models

<table>
<thead>
<tr>
<th>Clearance Model</th>
<th>( PS_{\text{in}} )(^b)</th>
<th>( E_{\text{HAHV}} )(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well-stirred model</td>
<td>1.93</td>
<td>0.527</td>
</tr>
<tr>
<td>Dispersion model</td>
<td>9.19</td>
<td>0.441</td>
</tr>
<tr>
<td>Parallel-tube model</td>
<td>15.5</td>
<td>0.387</td>
</tr>
</tbody>
</table>

\( a\) Calculated from Table 1.
\( b\) Calculated from Table 2.

### Table 4

Parameters used to describe transport and metabolism in the zonal-compartment model

<table>
<thead>
<tr>
<th>Zonal Intrinsic Clearances</th>
<th>Even Distribution(^a) (Set 1)</th>
<th>Zonal Distribution (Set 2)</th>
<th>Modified Zonal Distribution (Set 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( PS_{\text{in}} ) (ml/min)</td>
<td>17.1a</td>
<td>17.1a</td>
<td>17.1a</td>
</tr>
<tr>
<td>( CL_{\text{INT,PP}} ) (ml/min)</td>
<td>2.76</td>
<td>547 • (12.8/3)b = 2.22</td>
<td>(547 - 310)(12.8/3)c ÷ (1049 + 335) = 0.74</td>
</tr>
<tr>
<td>( CL_{\text{INT,MZ}} ) (ml/min)</td>
<td>2.76</td>
<td>805 • (12.8/3)b = 2.62</td>
<td>805 • (12.8/3)b ÷ 1308 = 2.62</td>
</tr>
<tr>
<td>( CL_{\text{INT,FV}} ) (ml/min)</td>
<td>2.76b</td>
<td>2096 • (12.8/3)b ÷ 2612 = 3.43</td>
<td>(2096 + 600) • (12.8/3)c ÷ (2612 - 236) = 4.85</td>
</tr>
</tbody>
</table>

\( a\) Value is one-third those for whole liver (\( PS_{\text{in}} \) and \( CL_{\text{INT,PP}} \) in Table 2); \( f \) denotes PP, MZ, or PV.
\( b\) Values of \( CL_{\text{INT,PP}}, CL_{\text{INT,MZ}}, \) and \( CL_{\text{INT,FV}} \) were calculated from the ratios of the corresponding \( V_{\text{max}} \) and \( K_m \) shown in Table 2, after adjustment for liver weight.

### Discussion

The correlation of in vitro data to whole organ/in vivo is a widely investigated topic in drug metabolism. Varying successes have been encountered (Lin et al., 1980; Houston, 1994; Pang and Chiba, 1994; Iwatsubo et al., 1997; Ito et al., 1998). Poor correlations have been blamed on neglect of a transport barrier at the sinusoidal membrane and lack of consideration of metabolic zonation (Pang and Chiba, 1994). It was shown that the membrane barrier poses a major source of discrepancy when transport and not metabolism/excretion is the rate-limiting step (de Lannoy and Pang, 1987; Geng et al., 1995; Tirona and Pang, 1999). In such an instance, the overall removal rate mimics the transport rate. Moreover, zonal transport and zonal metabolism are important for whole organ removal (Meijer and Groothuis, 1991; Kwon and Morris, 1997). The in vitro and in vivo correlation should be stratified when such zonal data exist.

In vitro data now exist for enalapril, whose uptake by rat hepatocytes was homogeneously distributed (Abu-Zahra et al., 2000). The S9 fraction from PV hepatocytes, however, exhibited a significantly higher \( V_{\text{max}} \) and \( K_m \) than those for homogeneous or PP hepatocytes (Table 1). If there were a greater expression of a single enzyme,
and PV, the intrinsic metabolic clearance under first-order conditions discern individual systems of similar component. The condition exists if there is a lack of resolution to individual enzyme despite the good fit of data to a single saturable values of pooled activities of multiple enzymes rather than those of an multiple and not a single carboxylesterase.

The greater maximal velocity in S9 of PV is due to greater expression substrate specificities and varying affinities, it is highly probable that the carboxylesterases are in greater abundance in the PV region (Pohl et al., 1991; Yan et al., 1995) and that these enzymes have overlapping et al., 1991; Yan et al., 1995) and that these enzymes have overlapping

Due to differences observed for both $V_{\text{max}}^\text{met}$ and $K_{m}^\text{met}$ in the S9 of PP and PV, the intrinsic metabolic clearance under first-order conditions ($C_{\text{out}}^\text{met} / K_{m}^\text{met}$) was highest for PV hepatocytes, followed by that for the homogeneous, then PP hepatocytes (Table 2). These values were much lower than the $P_{\text{in}}$ for transport, suggesting that metabolism and not transport is rate-limiting in the overall removal of enalapril in the intact organ. Hence, the higher metabolic intrinsic clearance in the PV region dictates the higher removal in the intact organ, an inference that is consistent with the findings of Pang et al. (1991). Through comparison of the in vitro data, it could be concluded that zonal metabolism and not transport constitutes the observations of Pang et al. (1991).

The test remains, however, whether the in vitro transport and metabolic activities, when scaled up, would adequately reflect activities of the whole organ. Cellular uptake by the rat liver has been found to correlate closely with that in isolated rat hepatocytes (Yamada et al., 1997; Tirona and Pang, 1999), and there is demonstrable success in correlating metabolic drug clearance with in vivo (Lin et al., 1980; Houston, 1994; Ito et al., 1998). The simplistic models—well stirred, parallel-tube, and dispersion models that do not account for transport or metabolic heterogeneity—are, however, inept in predicting the associated $E_{\text{ss}}$ values (Table 3). All of the $E_{\text{ss}}$ values predicted by these models were much higher than the observed $E_{\text{ss}}$, suggesting that these idealized and simplified models of hepatic drug clearances were unable to predict the hepatic removal of enalapril in the absence of zonation (Table 3). Not unexpectedly, the parallel-tube and well stirred models, representing boundary conditions for the degree of mixing for drug removal in the liver, were found associated with extreme values of the $PS_{\text{out}}$ (1.93 and 15.5 ml/min), with an intermediate value (9.2 ml/min) for the dispersion model.

Because zonation was detected for metabolic activities among zonal rat hepatocytes, the data could be viewed with respect to a zonal-compartment model that describes the liver as three subcompartments or zonal regions consisting of different transport or metabolic activities or both (Tirona and Pang, 1996). Improved correlation was attained between data in vitro and in vivo with the zonal-compartment model. The low $PS_{\text{out}}$ value associated with the well stirred model (1.93 ml/min) was inconsistent with the observed $E_{\text{ss,HAPV}}$ and $E_{\text{ss,HAHV}}$ values in the perfused liver preparation (Pang et al., 1991). Absence of metabolic zonation (even enzyme case) was also incongruent with the observations. By contrast, the $PS_{\text{out}}$ value predicted by both the dispersion and parallel-tube models, together with zonal metabolic heterogeneity (parameter set 2, PV/PP = 1.5 from observations; and parameter set 3, PV/PP activity of 6.6 for the steeper distribution of esterase activity-gradient) better correlated with the observed data (Table 5). The closeness between the in vitro zonation patterns with perfusion data validates the model of HAPV-HAHV perfusion in determining the preponderance of zonal activity of the liver (Pang et al., 1991).

However, the predicted values of $E_{\text{ss}}$ for HAHV remained higher than observed. This is partially attributed to a lesser volume reached by HAHV perfusion (< one-third liver) as inferred by the intracellular water space accessed (Pang et al., 1991). Also, the gradient in metabolic activity, increasing from the PP to the PV zone, is underestimated by the present extrapolation with in vitro data. The same artifact was observed for the gradient of glutathione S-transferase activity for ethacrynic acid within zonally enriched hepatocytes although a steeper activity gradient

\[ \text{TABLE 5} \]

Estimations of $E_{\text{ss}}$ for HAPV and HAHV with the various $PS_{\text{out}}$ for the zonal-compartment model\(^a\)

<table>
<thead>
<tr>
<th>$PS_{\text{out}}$</th>
<th>$E_{\text{ss,HAPV}}$</th>
<th>$E_{\text{ss,HAHV}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.93 ml/min</td>
<td>0.894</td>
<td>0.526</td>
</tr>
<tr>
<td>Even (Set 1)</td>
<td>0.894</td>
<td>0.517</td>
</tr>
<tr>
<td>Zonal (Set 2)</td>
<td>0.880</td>
<td>0.440</td>
</tr>
<tr>
<td>Modified zonal (Set 3)</td>
<td>0.781</td>
<td>0.389</td>
</tr>
<tr>
<td>9.2 ml/min(^b)</td>
<td>0.781</td>
<td>0.373</td>
</tr>
<tr>
<td>Zonal (Set 2)</td>
<td>0.747</td>
<td>0.233</td>
</tr>
<tr>
<td>Modified zonal (Set 3)</td>
<td>0.695</td>
<td>0.328</td>
</tr>
<tr>
<td>15.5 ml/min(^b)</td>
<td>0.695</td>
<td>0.300</td>
</tr>
<tr>
<td>Even (Set 1)</td>
<td>0.666</td>
<td>0.165</td>
</tr>
<tr>
<td>Zonal (Set 2)</td>
<td>0.661</td>
<td>0.165</td>
</tr>
<tr>
<td>Modified zonal (Set 3)</td>
<td>0.77</td>
<td>0.12</td>
</tr>
</tbody>
</table>

\(^a\) See Table 4 for enzymatic distributions.
\(^b\) Equal to the simulated extraction ratio for the first zone of the liver.

\[ \text{TABLE 6} \]

Zonal extraction ratios and the overall extraction ratio of the liver predicted for set 3

<table>
<thead>
<tr>
<th>$PS_{\text{out}}$</th>
<th>$E_{\text{ss,PV}}$</th>
<th>$E_{\text{ss,MZ}}$</th>
<th>$E_{\text{ss,PV}}$</th>
<th>$E_{\text{ss,liver}}$(^a)</th>
<th>$E_{\text{ss,liver}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.5</td>
<td>0.165</td>
<td>0.322</td>
<td>0.401</td>
<td>0.338</td>
<td>0.661</td>
</tr>
<tr>
<td>9.2</td>
<td>0.233</td>
<td>0.393</td>
<td>0.458</td>
<td>0.252</td>
<td>0.748</td>
</tr>
</tbody>
</table>

\(^a\) Equals the product of $(1 - E_{\text{ss,PP}})(1 - E_{\text{ss,MZ}})(1 - E_{\text{ss,PV}})$. 

\[ F I G . 3 . \] Metabolism of enalapril by S9 obtained from PP, whole liver (HO), and PV hepatocytes (mean ± S.D., n = 4).
was obtained with lysates from the farthest cells of the PP and PV zones prepared by dual-pulsing of digitonin (Tirrona et al., 1999). Unfortunately, accurate patterns of metabolic zonation could not be determined from the present zonal hepatocyte studies (Kera et al., 1987), and the preparation of lysates is not applicable to the study of enalapril metabolism because membrane-associated proteins, such as the microsomal esterases thought to be involved in enalapril metabolism, are not present in a functional form in lysates (Witters et al., 1993; Fang et al., 1998). It is likely that the shape of the actual gradient is continuous as would be expected for gradient-type distribution of an enzyme (Ononen and Lindros, 1998), rather than stepwise as modeled presently. Gradient distributions of cytochrome P450 isoforms have been estimated from mRNA distributions in lysates that exist evenly (CYP2C12) across the acinus or increased from PP to PV regions (CYP3A1), to exclusively PV expression (CYP2C12) (Ononen and Lindros, 1998). It is also surmised that the correlation may improve with optimization of a better definition of the enzymic distribution and PSpout. A simulation with a zonal-compartment model of six subcompartments (two equal subcompartments within each zonal region) was also performed (data not shown). However, the results were less compliant with the observations.

Notwithstanding the above comments, it appears that the parameters for metabolism and transport determined in various zones in vitro reflect the total intrinsic metabolic and transport activities in the whole organ, albeit the gradient is less shallow than in reality. The metabolic intrinsic clearance of the liver could be approximated by the sum of the metabolic intrinsic clearances of the zonal regions. Moreover, drug extraction among zonal regions occurs successively, and the product of the zonal bioavailabilities determines the overall bioavailability of the liver (Table 6). By modeling the liver as a series of subcompartments with different zonal transport/metabolic intrinsic clearances, a more complete description of the hepatic removal is attained, as shown in this first exercise on the removal of the enalapril in the perfused liver. Metabolism and not transport was found to be rate-determining for hydrolysis. The zonation observed in perfused liver is attributed to a greater PV metabolism, as verified by the in vitro data. Modeling along similar strategies should improve future efforts in in vitro and in vivo correlation.

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References


