Evaluation of Route of Input on the Hepatic Disposition of Diazepam

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ABSTRACT

Diazepam, a drug of high intrinsic clearance, was studied in the in situ rat liver dually perfused with Krebs-bicarbonate buffer containing human serum albumin (HSA; 0–1%) and unlabeled diazepam (1 mg/l) under constant hepatic arterial (3 ml/min) and portal venous (PV; 12 ml/min) flow rates. Events after a unit impulse (using [14C]diazepam) and at steady state (using unlabeled diazepam) were evaluated. In the absence of HSA the fractional effluent recovery (F) after hepatic arterial infusion (0.046 ± 0.013) was about twice that after PV infusion (0.019 ± 0.006). With HSA present, regardless of input route, F increased as unbound diazepam fraction in perfusate decreased (e.g., for PV, F = 0.58 ± 0.05 and 0.69 ± 0.02 for unbound diazepam fraction values of 0.18 ± 0.01 and 0.037 ± 0.01 at 0.25% and 1% HSA). The effluent [14C]diazepam profile was also dependent upon HSA. On decreasing HSA from 1 to 0.25% the early sharp peak (at 12–20 s) was replaced by a flatter unimodal profile with a later peak (at 60–80 s). Comparison of estimated effective permeability-surface area product to perfusate flow ratios (4.4 for 1% HSA and 21 for 0.25% HSA) indicated a shift from a perfusion rate-limited uptake with 0.25% HSA to one intermediate between permeability and perfusion at 1% HSA. Recognizing that orally absorbed drug enters the liver only via PV and i.v. drug via both vascular routes, this study emphasizes the difference in hepatic extraction of compounds depending on route of input, and the role that alteration in perfusate binding has on hepatic drug disposition.

There is growing interest in the use of the dual perfused liver preparation to explore the relative contributions of the hepatic artery (HA) and portal vein (PV) to events occurring within the liver, particularly given that intestinally absorbed compound enters the liver only via the PV, whereas systemically administered compound perfuses the liver via both vascular routes. One aspect of this dual input is the anatomical and functional relationships between these two vessels. Observations obtained from such efforts favor the idea that there are both common and separate channels within the liver. Although the majority of the sinusoids are perfused by the mixed blood, a small fraction of the vascular bed remains separate (about 10%; Field and Andrews, 1968; Ahmad et al., 1984; Sahin and Rowland, 1998a) and receives 17% of the mixed blood, a small fraction of the vascular bed remains separate (about 10%; Field and Andrews, 1968; Ahmad et al., 1984; Sahin and Rowland, 1998a) and receives 17% of the hepatic extraction of compounds depending on route of input. Second, the greater sensitivity of cellular uptake to protein binding than cellular permeability (Diaz-Garcia et al., 1992) also

ABBREVIATIONS: HA, hepatic artery; PV, portal vein; HSA, human serum albumin; fu, unbound fraction of [14C]diazepam; AUC, area under the outflow concentration-time profile; MTT, mean transit time; F, fractional effluent recovery; CL, clearance; PS, permeability surface area product; CYP, cytochrome P450.

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1 S.S. thanks the Turkish Government for a studentship.
allows an assessment on the influence of altered binding on the uptake kinetics for the two routes of input.

**Materials and Methods**

**Chemicals**

Iodine-125-labeled albumin ([125I]-human serum albumin (HSA), 1.02 mCi/ml), was purchased from ICN Biomedicals (Costa Mesa, CA) and [2-14C]diazepam (57 mCi/mmol) from Amersham International (Horsham, UK). Diazepam was obtained from Sigma Chemical (St. Louis, MO). HSA was a gift from Pharmacia Upjohn AB (Uppsala, Sweden). All other chemicals were of analytical grade and were obtained from commercial sources.

**Perfusion Procedure**

All animals were handled in compliance with the UK Home Office guidelines. They were fed on a normal laboratory diet with free access to drinking water and kept under a 12-h light/dark cycle in a temperature-controlled environment.

The single-pass dual perfused in situ rat liver system, with male Sprague-Dawley rats (330–380 g; wet liver weight, 11–15 g) was essentially the same as that described previously (Sahin and Rowland, 1998b). Krebs-bicarbonate buffer containing HSA and/or drug (concentrations specified below) was used as the perfusion medium. Under anesthesia, the bile duct was cannulated, and loose ligatures were placed around the PV ensuring exclusion of the HA. The abdominal contents were displaced to the animal’s right, and branches of the celiac artery (i.e., left gastric and lienal arteries) were tied very close to their junctions to the celiac artery. The gastroduodenal artery (branch of the common hepatic artery) was also ligated. After all these steps only the HA was left patent. The PV was cannulated with a 16GA catheter (Argyle Medicut, o.d. 1.7 mm × 45 mm), and the perfusion was started immediately at a flow rate of 12 ml/min. Exsanguination of the liver was facilitated by inserting a tubing into the vena cava via the right atrium. The HA was cannulated with an 18GA (Argyle Medicut, o.d. 1.3 mm × 45 mm) or 20GA (Argyle Medicut, o.d. 1.1 mm × 45 mm) catheter, and the second perfusion started immediately at a flow rate of 3 ml/min. Liver viability was routinely assessed by monitoring bile flow, perfusate recovery, and arterial perfusion pressure, and by macroscopic appearance.

**Injection Preparation**

One drug ([14C]diazepam) and one extracellular marker ([125I]-HSA) were injected together as a bolus [50 μl in saline containing HSA (at a concentration equal to that in the perfusate) and unlabeled drug (1 mg/ml diazepam)]. Doses used were as follows: 0.18 to 0.25% HSA. In the absence of protein, diazepam infusion was altered between the PV and HA inputs; diazepam was delivered into the liver initially via the PV for 20 min and then via the HA for 25 min. During diazepam infusion via one of the inputs (e.g., PV) the alternate vessel (e.g., HA) was perfused with drug-free perfusate. Subsequently, the perfusion medium was changed, and the liver was perfused with diazepam in the presence of 0.25% HSA. The infusion period and order of diazepam delivery to the liver were the same as in the absence of protein; first PV and then HA. During constant perfusion with unlabeled diazepam in 0.25% HSA, a bolus (50 μl) containing [14C]diazepam and [125I]-albumin was injected into the liver via the vessel in which unlabeled diazepam was infused. Immediately after an injection, the total effluent from the liver was automatically collected every 2 s for 2 min, and thereafter (into siliconized test tubes) at increasing time intervals up to 6 min. Perfusion samples were also collected before and after the bolus injection to determine the extraction of unlabeled diazepam at steady state. In group B (n = 6), the design of the experiment was the same as with group A except a different protein concentration (i.e., 1% HSA) was used.

**Radiochemical Analysis**

The activities of [14C]diazepam and [125I]-albumin in effluent samples were determined by radiochemical analysis after the addition of 4 ml of liquid scintillation fluid, with the results expressed as dpm.

**HPLC Assay of Diazepam**

Diazepam was analyzed by HPLC using a modification of the method of Raisys et al. (1980). The HPLC system consisted of a Kontron model 420 HPLC pump (Zurich, Switzerland), which delivered mobile phase (acetoniitrite:water with 1% triethylamine adjusted to pH 3.0 with 85% orthophosphoric acid; 50:50, v/v) at a flow rate of 1 ml/min; an autosampler (Kontron model 360); and a Jasco model 100-IV variable wavelength UV detector (Tokyo, Japan). A Newguard column (Brownlee Labs, UK) with a disposable cartridge (Newguard RP-18, 15 × 3.2 mm, 7 μm) was also used to protect the analytical column. Separation of diazepam was performed at ambient temperature on a Spheri-5, RP18 cartridge column (25 cm × 4.6 mm; 5 μm), with detection at 254 nm. Quantitation of diazepam was determined by peak area ratio of drug to internal standard (prazepam) with reference to an appropriate calibration curve. The retention times for diazepam and prazepam were 9 and 17 min, respectively.

When the concentration of diazepam in the outflow samples was high (i.e., in the presence of HSA in the perfusate), samples were measured directly after precipitation of HSA with acetonitrile. Briefly, 75 μl of internal standard solution (prazepam; 1/10 dilution of 2 mM stock solution) and 500 μl of acetonitrile were added to 500 μl of outflow sample. After vortex mixing (1 min) and centrifugation at 3500 rpm for 20 min, the supernatant was injected into the HPLC system. The calibration curves were constructed over the range of 100 to 1500 ng/ml. The determination coefficient (r²) for the calibration curve was always greater than 0.95.

Hepatic outflow samples obtained using protein-free perfusate contained concentrations of diazepam too low to be measured accurately using the method described above. Therefore, it was necessary to perform a simple extraction procedure before HPLC analysis. An aliquot (6 ml for PV and 8 ml for HA samples) of the outflow sample was transferred into a siliconized test tube, and 50 μl of internal standard solution (prazepam; 1/10 dilution of 2 mM stock solution) and 6 ml of hexanecetethyl acetate (8:2, v/v) were then added. The samples were mixed using a horizontal mixer for 45 to 60 min and then centrifuged (3500 rpm; 20 min). The lower aqueous layer was frozen by immersion in liquid nitrogen, and the upper organic layer was then transferred to another siliconized tube and evaporated to dryness under a smooth nitrogen stream (35°C). The residue was reconstituted in mobile phase and vortexed immediately before injection into the HPLC system. Calibration curves were constructed over the range of 5 to 150 ng/ml. The determination coefficient (r²) for the calibration curve was always greater than 0.95.
To avoid nonspecific adsorption (binding) of diazepam, all glassware was silanized by immersion in 4% dichlorodimethylsilane in chloroform for 24 h followed by rinsing with methanol. Furthermore, protein (HSA) used throughout the study was defatted and freeze-dried.

**Purification of HSA.** The method for removal of fatty acids from serum albumin was a modification of that of Chen (1967). Two liters of HSA solution (20% w/v) was diluted with the twice the volume of distilled water and then placed in an ice bath. After mixing in activated charcoal powder (200 g; British Drug House, Poole, UK) and lowering the pH to 3.0 by addition of 1 N HCl, the solution was magnetically stirred for 1 h, at a constant temperature of 4°C. The charcoal suspension was then further diluted with distilled water to obtain a concentration of 4% HSA (w/v) and centrifuged (3000 rpm) at 4°C for 20 min to remove the majority of the charcoal. The remaining finely suspended charcoal was removed by filtration through a bed of Kieselguhr (British Drug House), followed by filtration through 0.8-, 0.45-, and 0.22-μm filters (Millipore, Watford, UK), respectively. The solution was then brought up to pH 7.4 by the addition of 1 M NaOH and stored at 4°C. Because the albumin solution was prepared once as a large batch, to ensure stability, its water content was removed by freeze-drying technique. The defatted solution was prepared once as a large batch, to ensure stability, its addition of 1 M NaOH and stored at 4°C. Because the albumin protein (HSA) used throughout the study was defatted and freeze-dried and albumin powder was stored at 4°C in tightly closed containers until used.

**Equilibrium Dialysis.** Equilibrium dialysis was performed using two-chambered Teflon dialysis cells (Dianorm, Switzerland), each of 1-ml capacity, separated by a membrane (Spectropor-2; Spectraceutical Medical Industries Inc., CA). To assess the unbound fraction of [14C]diazepam (fu), 1 ml of drug solution [unlabeled, 1 mg/l, and [14C]diazepam, 0.045 μCi/ml, in HSA-buffer solution; HSA concentrations (g/dl): 0, 0.1, 0.25, 0.5, and 1] was dialysed against 1 ml of protein-free perusate at 37°C in a thermostatically controlled water bath, with the cell system continuously rotated at a fixed rate of 20 rpm. At the end of the equilibration time (4 h), the solution from each chamber was expelled by pushing air through the cells, and collected into test tubes. After the addition of 4 ml of liquid scintillation fluid (Optiphase “Hisafe” II; Wallac, Gaithersburg, MD), 14C activity was determined in duplicate aliquots (200 μl) from each compartment by radiochemical analysis. The unbound fraction at equilibrium was calculated as follows:

\[ fu = \frac{\text{dpm}_{\text{buffer}}}{\text{dpm}_{\text{protein}}} \]

where \( \text{dpm}_{\text{buffer}} \) and \( \text{dpm}_{\text{protein}} \) are the radioactivity (dpm) in the buffer and protein compartments.

**Data Analysis**

**Bolus Experiments.** After bolus administration the outflow data were transformed to frequency data \( f(t) \), \( 1/f \) using the following equation:

\[ f(t) = \frac{C(t) \cdot Q}{\text{Dose}} \]

where \( C(t) \) is the concentration of radioactivity associated with the unchanged compound, and \( Q \) is the total perfusate flow (ml/s). Time was taken as the difference between the midtime of the sampling interval and the lag time, corresponding to the delay in the nonhepatic region of the experimental system (catheter and tubing: PV, 2.5 s and HA, 2.7 s). The maximum frequency output \( (f_{\text{max}}) \) and the time reached \( (t_{\text{max}}) \) were determined experimentally.

Moments of the frequency outflow versus time profiles and the parameters related to these moments (e.g., CV2) were estimated using the following equations:

\[ \text{AUC} = \int_0^\infty C(t) \cdot dt \]

\[ \text{MTT} = \frac{\int_0^\infty t \cdot C(t) \cdot dt}{\text{AUC}} \]

\[ \text{VTT} = \frac{\int_0^\infty t^2 \cdot C(t) \cdot dt}{\text{AUC}} - \left( \frac{\text{MTT}}{1.83} \right)^2 \]

where AUC, MTT, and VTT are area under the outflow concentration-time profile, mean transit time, and the variance of the transit times, respectively.

The normalized variance (CV2) is given by the following equation:

\[ \text{CV}^2 = \frac{\text{VTT}}{\text{MTT}^2} \]

CV2 is a dimensionless parameter and has been used as a measure of relative dispersion of drug within the liver (Roberts et al., 1988). These moment equations apply equally both to eliminated (diazepam) and noneliminated (albumin) compounds because they are only dealing with characterization of the material associated with transit through the liver. Obviously, such moments can only be used to calculate the volume of distribution of a fully recovered compound such as albumin.

For the calculation of the volume of distribution, \( V_H \), of albumin, the liver was taken to consist of two spaces: a specific arterial space receiving 17% of the arterial flow and a common space receiving all the portal flow and the remaining fraction (83%) of the arterial flow. The volumes of distribution associated with each input were calculated as follows (Sahin and Rowland, 1998a, 1999).

After the venous injection:

\[ V_{PV} = Q_{PV} \cdot 0.83 \cdot \frac{Q_{HA}}{Q_{PV}} \cdot \text{MTT}_{PV} \]

After the arterial injection:

\[ V_{HA} = Q_{HA} \cdot \frac{[\text{MTT}_{HA} - 0.83 \cdot \text{MTT}_{PV}]}{Q_{PV}} + V_{PV} \]

where \( Q_{PV} \) and \( Q_{HA} \) are the PV and HA flow rates, respectively, and \( \text{MTT}_{PV} \) and \( \text{MTT}_{HA} \) are the corresponding mean transit times. Equations 7 and 8 assume that albumin is not eliminated in the liver.

Fractional hepatic recovery (F) of diazepam in the hepatic effluent is defined as follows:

\[ F = \frac{\text{AUC} \cdot Q}{\text{Dose}} \]

where AUC is area under the outflow concentration-time profile obtained after bolus administration of the labeled diazepam.

**Steady-State Experiments.** The fractional hepatic recovery at steady state, F, is calculated from the following relationship (Ahmad et al., 1984):

\[ F = \frac{\text{Rate of exit}}{\text{Rate of presentation}} = \frac{C_{\text{out}} \cdot Q_{\text{out}}}{C_{\text{in}} \cdot Q_{\text{in}}} \]

where \( C_{\text{in}} \) and \( C_{\text{out}} \) are the perfusate drug concentrations entering and leaving the liver, and \( Q_{\text{in}} \) and \( Q_{\text{out}} \) are the influent (3 ml/min for HA and 12 ml/min for PV) and effluent (15 ml/min) flow rates, respectively. With diazepam infused into either the HA or PV, the rate of presentation is \( Q_{HA}C_{in} \) or \( Q_{PV}C_{in} \) as appropriate.

Regardless of the mode of administration (bolus or infusion) the hepatic clearance (CLH) of a drug across the liver may be defined as
the rate of drug elimination normalized to the influent drug concentration; thus \( \text{CL}_{\text{H}} \) is given by the following equation:

\[
\text{CL}_{\text{H}} = Q_{\text{in}} \cdot E
\]

where \( E = 1 - F \) is the extraction ratio.

All tabulated results were expressed as mean \( \pm \) S.E. The results were compared by means of Student’s \( t \) test (paired or unpaired) and one-way analysis of variance. A \( P \) value less than .05 was taken as significant.

**Results**

**Protein Binding.** The fractions of diazepam unbound in fresh perfusate at different defatted HSA concentrations are summarized in Table 1. These results are in a close agreement with those of Diaz-Garcia et al. (1992). It has been shown previously that equilibrium is reached within 4 h, and that diazepam does not bind to the membrane and dialysis apparatus. Furthermore, the albumin-to-buffer volume ratio for diazepam 4 h after dialysis with different albumin concentrations was about 1, indicating that the aqueous volume shift was negligible.

**Outflow Profiles.** Figures 1 and 2 show representative frequency outflow versus time profiles for \(^{14}\text{C}\)diazepam obtained in the presence of various HSA concentrations (0.25 and 1%), after bolus administration into the PV and HA, respectively. In all cases the shape of the outflow profile was dependent upon the albumin concentration used. In the presence of 1% HSA, the hepatic output profiles of \(^{14}\text{C}\)diazepam appeared as a sharp peak (\( f_{\text{max}} = 0.0147 \pm 0.0035 \) l/s at 12.2 \( \pm \) 4.7 s for PV administration, and 0.0043 \( \pm \) 0.0003 l/s at 19.7 \( \pm \) 3.2 s for HA administration), which eluted over the first 25-s interval, followed by a slowly eluting tail. With a decrease in the perfusate protein concentration from 1 to 0.25%, the earlier first peak disappeared and the profiles displayed a unimodal characteristic with a later and much flatter peak (\( f_{\text{max}} = 0.0029 \pm 0.0006 \) l/s at 60.1 \( \pm \) 4.9 s for PV administration; 0.0012 \( \pm \) 0.0001 l/s at 76.8 \( \pm \) 15.5 s for HA administration). In contrast, \(^{125}\text{I}\)-albumin displayed a unimodal outflow profile irrespective of both HSA concentration and route of input (Fig. 3). Regardless of both the HSA concentration (0.25 and 1%) and compound (\(^{125}\text{I}\)-albumin and \(^{14}\text{C}\)diazepam), the outflow profiles after arterial input were flatter than those after venous input (e.g., for diazepam Fig. 1 versus Fig. 2, and for \(^{125}\text{I}\)-albumin \( f_{\text{max}} \), l/s: 0.045 \( \pm \) 0.001 versus 0.098 \( \pm \) 0.011 for 1% HSA; Fig. 3).

**Albumin.** Moment analysis results for \(^{125}\text{I}\)-albumin are summarized in Table 2. Regardless of both route of input (HA and PV) and perfusate albumin concentration (0.25 and 1%), MTT after arterial administration was longer than that after venous administration (\( P < .05 \) and \( P < .001 \) for the corre-}

**TABLE 1**

<table>
<thead>
<tr>
<th>Albumin Concentration</th>
<th>( f_{\text{u}} )a</th>
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<tbody>
<tr>
<td>%</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.016 ( \pm ) 0.005</td>
</tr>
<tr>
<td>0.1</td>
<td>0.402 ( \pm ) 0.012</td>
</tr>
<tr>
<td>0.25</td>
<td>0.175 ( \pm ) 0.012</td>
</tr>
<tr>
<td>0.5</td>
<td>0.077 ( \pm ) 0.002</td>
</tr>
<tr>
<td>1</td>
<td>0.037 ( \pm ) 0.001</td>
</tr>
</tbody>
</table>

a Protein binding experiments were performed using diazepam solution containing 1 mg/l unlabeled and 0.045 \( \mu \text{g} \) labeled compound.

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**Albumin.** Moment analysis results for \(^{125}\text{I}\)-albumin are summarized in Table 2. Regardless of both route of input (HA and PV) and perfusate albumin concentration (0.25 and 1%), MTT after arterial administration was longer than that after venous administration (\( P < .05 \) and \( P < .001 \) for the corre-
experiments protein concentration, MTT after arterial administration was diazepam are shown in Table 2. Regardless of the perfusate TABLE 3 Moment analysis (mean

Fig. 3. Fractional rate of efflux of albumin obtained after bolus injection into the portal vein (■) and hepatic artery (□). Profiles are obtained in the presence of 1% perfusate albumin concentration.

Diazepam. Results of the moment analysis for labeled diazepam are shown in Table 2. Regardless of the perfusate protein concentration, MTT after arterial administration was longer than that after venous administration (P < .05 for 0.25% HSA and P < .01 for 1% HSA). On the other hand, the transit times decreased with an increase in the albumin concentration from 0.25 to 1% (P < .001; Table 2). When administered via the HA, spreading of labeled diazepam within the liver was not influenced by the changes in the perfusate albumin concentration. In contrast when administered via the PV, there was a small but significant increase in the CV² values with an increase in the HSA concentrations in the perfusate (P < .05). However, for a given perfusate protein concentration, no significant difference was obtained between HA and PV inputs with regard to CV² estimates.

Disposition parameters (e.g., F and CL₄₅) for diazepam estimated from the bolus and steady-state experiments are listed in Table 3. In the absence of binding protein, the F value after arterial perfusion (0.046 ± 0.013) was approximately twice that after venous perfusion (0.019 ± 0.006; P < .01), irrespective of the mode of input; with an increase in the protein concentration fractional hepatic recovery of diazepam was increased. In the case of 0.25% albumin, the fractional hepatic recovery of labeled diazepam, estimated from the area under the frequency outflow versus midtime point profile was 0.65 ± 0.02 for PV and 0.54 ± 0.03 for HA injections (P < .01). For the same protein concentration, the fractional hepatic recovery of diazepam, estimated as the ratio of rate of exit/rate of presentation (e.g., Eq. 10) for unlabeled material was 0.58 ± 0.05 and 0.54 ± 0.06 for the corresponding routes, with no significant difference between them. When the per-

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HSA (%)</th>
<th>Portal Vein</th>
<th>Hepatic Artery</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTT (s)</td>
<td>0.25</td>
<td>9.72 ± 0.29</td>
<td>18.43 ± 2.13</td>
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<td>1.0</td>
<td>13.03 ± 1.12</td>
<td>26.61 ± 1.47</td>
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<tr>
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<td>P &lt; .001</td>
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<tr>
<td>V₄₅ (ml/g)</td>
<td>0.25</td>
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<td>0.21 ± 0.01</td>
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<td>1.0</td>
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<td>0.31 ± 0.02</td>
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<tr>
<td></td>
<td>P &lt; .05</td>
<td>NS**</td>
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<tr>
<td>CV²</td>
<td>0.25</td>
<td>0.55 ± 0.11</td>
<td>0.60 ± 0.03</td>
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<td>0.50 ± 0.05</td>
<td>0.73 ± 0.08</td>
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<tr>
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<td>NS**</td>
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</tbody>
</table>

<table>
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<th>Parameter</th>
<th>HSA (%)</th>
<th>Portal Vein</th>
<th>Hepatic Artery</th>
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<tr>
<td>MTT (s)</td>
<td>0.25</td>
<td>224.17 ± 24.54</td>
<td>467.28 ± 53.36</td>
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<tr>
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<td>1.0</td>
<td>92.12 ± 8.29</td>
<td>173.00 ± 11.35</td>
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<tr>
<td></td>
<td>P &lt; .001</td>
<td>P &lt; .01</td>
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<tr>
<td>CV²</td>
<td>0.25</td>
<td>0.93 ± 0.10</td>
<td>0.93 ± 0.01</td>
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<td>1.0</td>
<td>1.29 ± 0.11</td>
<td>0.97 ± 0.04</td>
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<tr>
<td></td>
<td>P &lt; .05</td>
<td>NS**</td>
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*Portal vein versus hepatic artery; ** 0.25 versus 1.0% HSA.

<table>
<thead>
<tr>
<th>Mode of Administration</th>
<th>HSA %</th>
<th>F</th>
<th>CL₄₅ (ml/min)</th>
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<td></td>
<td></td>
<td>Portal Vein</td>
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<td>Bolus</td>
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<td>0.68 ± 0.01</td>
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<td>0.046 ± 0.013</td>
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<td>0.25</td>
<td>0.58 ± 0.05</td>
<td>0.54 ± 0.06</td>
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<tr>
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<td>1.0</td>
<td>0.69 ± 0.02</td>
<td>0.56 ± 0.04</td>
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* Overall mean of groups A and B, obtained in the absence of albumin in the perfusate; ** portal vein versus hepatic artery; *** 0.25 versus 1% albumin for bolus administrations; **** comparison of all conditions (0, 0.25, and 1% albumin) for infusion.
Diazepam was chosen as the model compound because in the absence of protein, it is not only highly cleared by the liver but also a substantial body of data for this drug already exists in the single PV-perfused rat liver. In addition, based on the good agreement between specific (HPLC) and nonspecific (radiochemical) methods, it has been shown previously that in the presence of 1% HSA, metabolites of [14C]diazepam do not contribute significantly to the total radioactivity in hepatic outflow over the period of assessment (Diaz-Garcia et al., 1992). Similar observations were also made in the presence of 0.25% HSA (e.g., F = 0.54 ± 0.06 versus 0.54 ± 0.03 for HA infusion and bolus administration of [14C]diazepam; Table 3). Furthermore, in the rat, especially at early times, metabolites are in significantly lower concentrations compared with those of diazepam (Igari et al., 1982), and are predominantly excreted in bile (Andrews and Griffiths, 1984). These findings suggest that the hepatic effluent radioactivity after an impulse dose in the single-pass system comprises predominantly parent diazepam, with perhaps a contribution of metabolite in the tail region.

**Discussion**

**Choice of Compound.** The liver is a heterogeneous organ in terms of its flow distribution: 83% of the arterial flow mixes with the venous flow in a common space, with the remainder perfusing a specific space (Sahin and Rowland, 1998a). Despite such heterogeneity, currently it is not known how these two spaces differ with respect to enzyme content. Unlike PV administration, hepatic extraction after HA input will be governed by events in both the common and specific spaces. Any difference in fractional hepatic recovery as a function of route of input could therefore be attributed to the specific space and its enzyme content because the extraction of compound from the common space will be the same whether administered via the HA or PV. Nevertheless, demonstration of any differences in fractional hepatic recovery requires the use of highly cleared compounds (e.g., ones with an extraction ratio in excess 0.90–0.95; Ahmad et al., 1984) because the specific space receives only 17% of the arterial flow and hence the arterial dose. At the extreme, if no enzyme is present in this space 17% of arterial dose will escape elimination. However, in the case of slowly extracted compounds, problems may arise in demonstrating a difference in hepatic extraction.

In the present study, diazepam was chosen as the model compound because in the absence of protein, it is not only highly cleared by the liver but also a substantial body of data for this drug already exists in the single PV-perfused rat liver. In addition, based on the good agreement between specific (HPLC) and nonspecific (radiochemical) methods, it has been shown previously that in the presence of 1% HSA, metabolites of [14C]diazepam do not contribute significantly to the total radioactivity in hepatic outflow over the period of assessment (Diaz-Garcia et al., 1992). Similar observations were also made in the presence of 0.25% HSA (e.g., F = 0.54 ± 0.06 versus 0.54 ± 0.03 for HA infusion and bolus administration of [14C]diazepam; Table 3). Furthermore, in the rat, especially at early times, metabolites are in significantly lower concentrations compared with those of diazepam (Igari et al., 1982), and are predominantly excreted in bile (Andrews and Griffiths, 1984). These findings suggest that the hepatic effluent radioactivity after an impulse dose in the single-pass system comprises predominantly parent diazepam, with perhaps a contribution of metabolite in the tail region.

**Albumin.** Comparison of the \( V_H \) values indicates that labeled albumin has access to larger space after arterial than venous administration, confirming our previous observation made with labeled albumin and other reference markers, including erythrocytes, sucrose, urea, and water (Sahin and Rowland, 2000). This excess space was about 15 to 20% of the total extracellular space. Furthermore, longer MTT values and also slightly higher CV^2 values obtained after arterial administration could be attributed to the presence of more tortuous pathways (i.e., peribiliary capillary plexus) taken by the arterial blood.

**Outflow Profiles.** Regardless of route of input, changes in albumin concentration had a clear effect on the hepatic outflow profiles of labeled diazepam. When 1% HSA was used, [14C]diazepam outflow profiles were characterized by a sharp early peak (throughput component) followed by a slowly eluting tail, produced by the material returning from the cellular space having escaped metabolism and biliary excretion (returning component). This is in accordance with previously observations with diazepam in the single PV perfused rat liver and is indicative of a compound whose fractional distribution from the vascular space within this organ is not instantaneous (Diaz-Garcia et al., 1992). When the perfusate HSA concentration was decreased to 0.25%, the outflow profiles displayed monophasic characteristics. Such discrepancies in outflow profiles as a function of protein concentration can be attributed to the hepatic uptake processes involved.

It is well recognized that hepatic uptake is dependent on binding of drug within blood, perfusate (blood) flow, and cellular permeability. Inherently, a permeability problem seems extremely unlikely for diazepam because its permeability surface area product (PS) is 137 ml/min/g of liver, which is more than 100 times greater than the blood (perfusate) flow (Diaz-Garcia et al., 1992). Therefore, protein binding rather than cellular permeability would be expected to be an important factor in determining the hepatic uptake kinetics of diazepam. A quantitative analysis (Chou et al., 1995) revealed that the uptake is rate limited by permeability when the effective permeability surface area product to flow rate ratio (i.e., \( f_{ub} \cdot PS/Q \); where \( f_{ub} \) is unbound fraction in perfusate) is less than 0.07 and is perfusion rate limited when \( f_{ub} \cdot PS/Q \) is greater than 5.7. When \( f_{ub} \cdot PS/Q \) lies between these two limits, both permeability and flow influence the hepatic uptake. Furthermore, an attempt to correlate physicochemical properties (e.g., logD) with cellular permeability led to the conclusion that for compounds with logD values greater than zero, cellular uptake is flow rate limited in the absence of protein or negligible binding in perfusate, but may shift toward a permeability rate limitation in the presence of extensive protein binding (Chou et al., 1995).
Diazepam, with a logD value of 2.8, fulfills these requirements.

Based on this information, given a perfusate flow of 1.15 ml/min/g of liver, a PS of 137 ml/min/g of liver, and fu values (Table 1), comparison of estimated effective surface area to blood flow ratios ($f_{u,PS}/Q = 121$, 21, 9.2, and 4.4 for the HSA concentrations of 0, 0.25, 0.5, and 1%, respectively) clearly indicates that two different uptake patterns operate for diazepam-intermediate with 0.25% HSA and perfusate flow-limited uptake with 0.25% HSA (or even 0.5%), which is reflected in the difference in shape of the outflow profiles.

**Effect of Route of Administration on the Hepatic Recovery.** A survey of available data on the hepatic extraction or clearance of various compounds in relation to the route of presentation to the liver is currently ambiguous. Hepatic clearances of both bromosulphthalein and CrPO$_4$ colloid (Brauer et al., 1959) and of $^{32}$Kr (Hollenberg and Dougherty, 1966) in dog liver were not found to be influenced by route of hepatic input. When equal doses of bile salts were infused into the hepatic artery or portal vein, taurocholic acid produced equal effects on bile flow regardless of the infusion site (Lautt and Daniels, 1983). Hepatic extraction of indocyanine green (Lautt et al., 1984), and extraction ratio of vitamin D$_3$ (Gascon-Barre et al., 1988) were unchanged whether administered into the HA or PV. Ikeda et al. (1993) also observed no difference in the hepatic extraction of arterially or portally delivered insulin, glucagon, and epinephrine. Furthermore, no significant difference was reported between regional kinetics (i.e., hepatic extraction and hepatic clearance) and systemic exposure of 5-fluorouracil and 5-bromo-2’-deoxyuridine after hepatic arterial versus portal venous infusions of drugs (Kuan et al., 1996). In contrast, more epinephrine (Meyerholz et al., 1991) and noradrenaline (Gardemann et al., 1991; Zimmermann et al., 1992) were extracted when administered via the HA. Furthermore, hepatic recovery was 18 and 3 times greater for lidocaine and meperidine, respectively, when infused via the HA compared with the PV (Ahmad et al., 1984). Recently, small but significant decreases were also observed in the extraction ratios of [14C]phenceto and tritiated acetaminophen delivered simultaneously into the HA and PV, with increment of HA flow, within the same liver preparation (Pang et al., 1994). Additionally, two chemotherapeutic agents, Adriamycin (Shiotani et al., 1995) and doxorubicin (Iwasaki et al., 1998), were more effectively extracted by the liver when administered via the HA than PV, leading to the conclusion that intra-arterial administration of these drugs are superior to intraportal administration in terms of reduction of systemic drug exposure and systemic toxicity.

Our findings with regard to fractional hepatic recovery of diazepam may be discussed under two headings: absence and presence of albumin. In the absence of protein, fractional hepatic recovery is low (indicating extensive and rapid metabolism) with that for the arterially administered diazepam being approximately twice that of portally administered drug. One possible explanation is that the enzymes responsible for hepatic extraction of diazepam (e.g., CYP3A2, CYP2C11, CYP2D1; Cheryn et al., 1987; Reilly et al., 1990; Neville et al., 1993) are diminished in the specific compared with the common space. When HSA is present in the perfusate, irrespective of the route of input, fractional hepatic recovery of diazepam was increased with an increase in the perfusate albumin concentration. The fractional hepatic recovery ratio (PV-to-HA) after constant infusion in the presence of HSA was close to unity, indicating that the difference in recovery of diazepam, as a function of route of input, was now small. When diazepam was administered as a bolus, the fractional recoveries were slightly higher than those after constant infusion (with 1% HSA, 0.85 versus 0.69 for PV and 0.68 versus 0.56 for HA). This discrepancy could be an artifact due to the errors associated with extrapolation in the tail region after bolus dose administration, a problem that does not arise with the steady-state approach.

The results of this study highlight several major points. First, the protein concentration dependence of the hepatic efflux profiles clearly demonstrates that different uptake patterns prevail for diazepam, perfusate flow-limited uptake with 0.25% HSA and intermediate between permeability and flow limitations with 1% HSA. Second, in the absence of albumin, diazepam is highly extracted independent of route of input. However, an elevated fractional hepatic recovery after HA than PV input suggests that the activity of the enzymes responsible for diazepam metabolism is less within the specific arterial than common space. These findings have potential implication concerning the influence of route of administration on the hepatic extraction of compounds. After oral administration, compounds are delivered to the liver via the PV only and so will perfuse the common space with its higher enzymatic activity and extraction ratio. Although both the HA and PV convey the molecules to the liver after i.v. or parenteral administration, some will perfuse the specific arterial space, yielding a somewhat lower overall extraction ratio. The extreme situation arises with HA administration, which is occasionally used for the treatment of hepatic tumors, where a much higher fraction of the dose may escape into the systemic circulation than experienced with oral administration.

**References**


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