Determining Intestinal Metabolism and Permeability for Several Compounds in Rats. Implications on Regional Bioavailability in Humans

Patrick J. Sinko[1,2] and Peidi Hu

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Purpose. To investigate the regional differences in small intestinal (SI) metabolism and permeability for several compounds and to ascertain the potential significance of these differences on the reported reductions in regional bioavailability in humans.

Methods. The regional SI metabolism and permeability of captopril, didanosine (ddI), mannitol, ofloxacin and zidovudine (ZDV) were investigated in rats using a Single Pass Intestinal Perfusion (SPIP) procedure or intestinal homogenates.

Results. ddI was metabolized to a greater extent in the upper SI whereas captopril was metabolized to a greater extent in the lower SI. Relatively low homogenate concentrations resulted in significant degradation of captopril in the upper and lower SI. All other compounds were stable and changes in the buffer system or the initial concentration did not affect the results. The SI permeabilities of all compounds, with the exception of mannitol, decreased linearly with respect to SI location and the slopes of the corresponding normalized regression lines were not significantly different.

Conclusions. It has been reported that captopril and ddI demonstrate regional intestinal bioavailability in several species including humans. The current results suggest that the reported reduction in the lower SI bioavailability of captopril may be a result of a reduction in permeability and an increase in intestinal metabolism whereas for ddI, the reduction in the lower SI bioavailability appears to be attributable to a reduction in intestinal permeability. Other factors such as luminal metabolism may also significantly effect regional differences in the intestinal bioavailability of ddI or captopril. Based on these results, a strong possibility exists that ofloxacin and ZDV may also demonstrate regional differences in intestinal bioavailability.

KEY WORDS: Bioavailability; captopril; didanosine; intestinal permeability; mannitol; metabolism; ofloxacin; regional oral absorption; site-specific; zidovudine.

INTRODUCTION

As early as 1904, differences in the regional intestinal absorption of ions and fluids were described in isolated segments of rabbit small intestine (SI) by Nobécourt and Vitry (1). Recently, regional differences in intestinal permeability and oral bioavailability have been reported for a variety of compounds in rats (2–4), dogs (5–6) and humans (4,7–9). Seta et al. (6) demonstrated that when two solutions of captopril were injected into isolated loops of the upper and lower SI in anesthetized dogs, captopril was significantly less bioavailable from the lower SI. Similar reductions in captopril bioavailability were reported in humans when captopril was delivered to the lower SI and colon by intubation (7) and by pulsatile dosage forms (9). Using scintigraphy, Wilding et al. (9) demonstrated that the reduction in bioavailability in the terminal ileum and colon of humans was not due to a dosage form effect. Several published reports suggest that the oral bioavailability of ddI may also be regionally dependent. Bramer et al. (3) reported that the time to peak ddI plasma levels was greater after ileal infusion than after duodenal infusion in rats suggesting that the absorption rate was slower in the lower SI. They also found that ddI metabolism in upper and lower SI rat homogenates was approximately equal and less than 2%, further suggesting that the slower rate of absorption was probably due to changes in permeability and/or absorptive surface area. Sinko and coworkers (4) recently demonstrated that the SI permeability of ddI in rats decreased 35% and 65% in the middle and lower SI, respectively. Evidence of site-specific SI absorption of ddI has also been reported in dogs (5) and humans (8) after the administration of various dosage forms. Although dosage form effects may contribute to the reduction in bioavailability of ddI, Sinko et al. (7), using a correlation, demonstrated that the reduction in SI permeability is a potentially significant contributing factor. In this report, the regional SI metabolism and permeability are investigated for several compounds. The results suggest that regional differences in metabolism and/or permeability may significantly effect the regional intestinal bioavailability of the compounds studied.

MATERIALS AND METHODS

Materials

Ketaset and Rompun were obtained from A.J. Buck, Owings Mills, Maryland, ddI was supplied by Bristol-Myers Squibb Company, ofloxacin was obtained from the R.W. Johnson Pharmaceutical Research Institute. Captopril and captopril disulfide were obtained from Sharmatek, Inc. and ZDV was obtained from Burroughs-Wellcome Company. ^{3}H-mannitol and ^{14}C-PEG 3350 were obtained from Du Pont NEN, Boston, Massachusetts. All other materials were obtained from Fisher Scientific, Fair Lawn, New Jersey or Sigma Chemical Co., St. Louis, Missouri and were used as received.

All animal studies were performed under approved protocols of the Institutional Animal Care and Use Committee at Rutgers University.

Acute Single Pass Intestinal Perfusion Method

The acute single pass intestinal perfusion procedure (10) was used for the intestinal permeability studies. Experimental parameters: flow rate = 0.191 mL/min and lengths ranged from 7 cm to 15 cm. All other experimental conditions are found in the caption of Figure 1. All compounds were found to be stable in perfused blank buffer (pH 6.5, 37°C) during the period of sample collection (10–15 min).

Preparation of Intestinal Homogenates

The intestinal homogenates were prepared by using es-

[1] Department of Pharmaceutics, College of Pharmacy, Rutgers University, P.O. Box 789, Frelinghuysen Road, Piscataway, New Jersey 08855.

[2] To whom correspondence should be addressed.

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tablished methods (3) with modifications. The excised intestine of viral-free, male Sprague-Dawley rats weighing 280–420 g were used. The rats were fasted for a period of 12–18 h prior to the study with water freely available. Two animals were used in each experiment and all experiments were performed in triplicate. Anesthesia was induced as previously reported (4) and 30 cm segments of the intestine were removed and immediately flushed with ice-cold iso-osmotic Ringer's buffer (pH 7.4, 290 mOsm/kg). The rats were euthanized using previously reported methods (4). The tissue was blotted dry and, using a spatula, the intestinal mucosa was removed from the segment by gentle scraping and was weighed. Ringer's buffer was then added to the intestinal mucosal tissue (9:1, V/W) and homogenized using a Yamato LSC Low Shear Tissue Homogenizer for 50 strokes at 1100 RPM in an ice bath. The protein content of the mucosal homogenate preparation was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Virginia). The homogenate was either used immediately or stored at frozen at −70°C.

Presence and Activity of Intestinal Cellular and Subcellular Enzymes

The presence and activity of brush border and subcellular enzyme markers were confirmed in upper and lower SI tissue homogenate preparations using established methods (11–14). The activities in upper and lower SI homogenates were 0.080 and 0.023, 0.0039 and 0.018, 0.00011 and 0.00014, and 0.017 and 0.026 μmol/m/mg protein for alkaline phosphatase (brush border), lactate dehydrogenase (cytosol), NADPH cytochrome C reductase (microsome) and succinate cytochrome C reductase (mitochondria), respectively, confirming enzymatic activity in the mucosal homogenate preparation.

Metabolism Studies

For ZDV and Mannitol. A pilot study was performed to determine the stability of ZDV and mannitol in (0.5% and 4%, w/v) intestinal mucosal homogenates. After the addition of the homogenate suspension to the drug solution (1:19, in Ringer's buffer, pH 7.4), the mixture was incubated in shaking water bath at 37°C. Final concentrations of 1 mM, 10 mM and 0.001 mM, 0.01 mM were achieved for mannitol and ZDV, respectively. Triplicate samples were taken at 0 m, 30 m and 60 m. One-tenth ml of 0.25 mM NaCN was added to each sample to quench the reaction. All post-hoc procedures were performed on ice or under refrigeration. Samples were then mixed and centrifuged at 34,000 ×g for 10 m. The supernatant was filtered using a 0.45 μm pipette tip filter. ZDV and mannitol were also found to be stable when the initial concentration and pH were altered.

For Captopril, ddI and Ofloxacin. Pilot studies were performed using similar conditions as for ZDV and mannitol. 2 ml of homogenate suspension was added to 3 ml of drug solution to achieve the desired concentration. Samples were taken at 0 m, 30 m and 60 m. Ofloxacin (0.01 mM) was stable under these conditions. Captopril and ddI were significantly unstable so full studies were performed. For captopril, the homogenate concentration used in the studies was 0.08% (w/v). Initial captopril concentrations were 0.1 mM, 0.5 mM, 1.0 mM and 5.0 mM. For ddI, three initial concentrations were studied 0.001 mM, 0.01 mM and 0.1 mM using 4% (w/v) homogenates. Four-tenths ml samples were taken at 0, 5, 10, 15, 20, 30, 40, 50 and 60 m. 0.4 ml of 0.25 mM NaCN or 1 N HCl were immediately added to quench reactions for ddI and captopril, respectively. Samples were mixed and centrifuged at 34,000 ×g for 10 min at 4°C. The supernatant was then filtered using a 0.45 μm pipette tip filter. Processed samples were found to be stable when stored on ice until analyses could be performed. Controls (drug with no homogenates and drug with boiled homogenates) and blanks (homogenate and buffer) were also included in the studies.

Analytical Methods

HPLC analyses were performed using a Waters solvent delivery module (Model 600E), a UV detector (Model 590E) and a WISP (Model 717) (Waters, Milford, Massachusetts). Separations were performed on a Supelcosil LC-18S column (25 cm × 4.6 mm) protected by a guard column Supelguard LC-18S (2 cm) (Supelco, Inc. Bellefonte, Pennsylvania). For ZDV, a Waters Nova-Pak C18, 15 cm × 3.9 mm column was used. For mannitol, a Supelco Gel C610-H column was used. For ddI: The flow rate was 1.0 mL/m and the detection wavelength was 254 nm. For perfusion studies, the mobile phase consisted of 0.1 M potassium phosphate containing 20% (v/v) methanol whereas for the metabolism studies the mobile phase consisted of 0.033 M potassium phosphate containing 6.7% (v/v) methanol. For captopril: the flow rate was 1.0 mL/m, the detection wavelength was 210 nm and the mobile phase consisted of 44.98% water, 54.97% methanol and 0.05% phosphoric acid (85%). For ofloxacin: the flow rate was 1.8 mL/m, the detection wavelength was 280 nm and the mobile phase consisted of water: tetrabutylammonium hydroxide (0.4 M) buffer adjusted to pH 2.85: acetonitrile in the ratio of 86.5:3.5:10. For ZDV: the flow rate was 1.0 mL/m and the detection wavelength was 254 nm and the mobile phase consisted of 0.1 M potassium phosphate containing 25% (v/v) methanol. The mobile phase was filtered through 0.45 μm HPLC certified membrane filters (Gelman Sciences, Ann Arbor, Michigan). ^{14}C-PEG 3350 and ^{3}H-mannitol were measured using liquid scintillation counting (Beckman Instruments, LS 5000TD, Fullerton, CA) by adding 0.5 mL of the sample to a liquid cocktail ScintiVerse BD. Initial and final ^{3}H-mannitol concentrations were validated using an HPLC method by examining the coincidence of eluting peaks. The flow rate was 0.5 mL/m, the detection wavelength was 190 nm and the mobile phase consisted of 0.1% phosphoric acid in water. All analytical procedures were validated and all standard curves were linear and reproducible at relevant concentrations. The inter- and intra-day assay variability ranged from 0.5% to 3.47% and 0.5% to 3.0% in the relevant concentration ranges, respectively.

Data Analysis

Calculation of Mean Regional Intestinal Permeability. The perfusion data were analyzed using the Modified Boundary Layer (MBL) analysis. A detailed description of the MBL analysis was presented elsewhere (10).

Analysis of Intestinal Metabolism Data. The initial rate of drug disappearance was determined from the linear portions of the log concentration versus time curves. This period ranged from 10 to 25 m. Linear regression analysis was performed on the data and the initial drug concentration was taken as the intercept of the log concentration axis. The metabolism rate constant was taken from the slope of the regression line. The degradation rate was the product of the initial rate constant and the initial concentration of drug. V_{max} (maximal velocity of enzymatic reaction) and K_m (Michaelis constant) were estimated using weighted nonlinear regression (The Scientist for Windows, MicroMath, Salt Lake City, Utah) and the Michaelis-Menten equation: $V = V_{max}*C/(K_m + C)$. The fit was weighted by $1/SD^2$.

Statistical Analyses. Results are expressed as mean ± SEM (standard error of the mean). All statistical calculations were performed using BMDP/Dynamic statistical software (BMDP Statistical Software, Inc., Los Angeles, California). A p-value of 0.05 was used as the significance level for all tests. One-way ANOVA and two-group t-tests were performed to test for differences in the means between groups. Pooled (classical) or separate t-tests were used based on the results of Levene's test for equality of variance between the groups.

RESULTS AND DISCUSSION

Regional Intestinal Uptake

The regional dimensionless intestinal permeabilities (P^*_w) of captopril, mannitol, ofloxacin and ZDV are plotted in Figure 1. The P^*_w of ddI was reported by our group (4); however, regional metabolism was not studied. Nonabsorptive loss of all compounds studied (i.e., due to lumenal degradation, etc.) was found to be insignificant during the SPIP experiment and the results for ddI were previously reported (4). The lower SI P^*_w of captopril, ddI, ofloxacin and ZDV demonstrated significant ($p < 0.05$) decreases of 49.6%, 67.1%, 53.0% and 61.3%, respectively, when compared to upper SI P^*_w. Mannitol permeability did not vary with SI location. Net fluid absorption occurred for all drugs in all regions but the regional differences were not significant for a given drug. This is meaningful since the enhanced intestinal permeability of small, hydrophilic molecules has been observed with increased net fluid absorption or "solvent drag" (15–16). Therefore, the observed reduction in regional P^*_w is probably due to regional morphological differences rather than due to an artifact in the methods. The trend in the permeability reduction was further analyzed by normalizing P^*_w by its corresponding upper SI permeability, $P^{*,L^*}_w / P^{*,upper}_w$. Linear regression analysis was performed and the slopes of the lines were -0.62 ± 0.04, -0.81 ± 0.07, -0.65 ± 0.15 and -0.76 ± 0.06 for captopril, ddI, ofloxacin and ZDV, respectively. The slopes were not significantly different from each other suggesting that a common physiological factor or group of factors are responsible for the observed reductions.

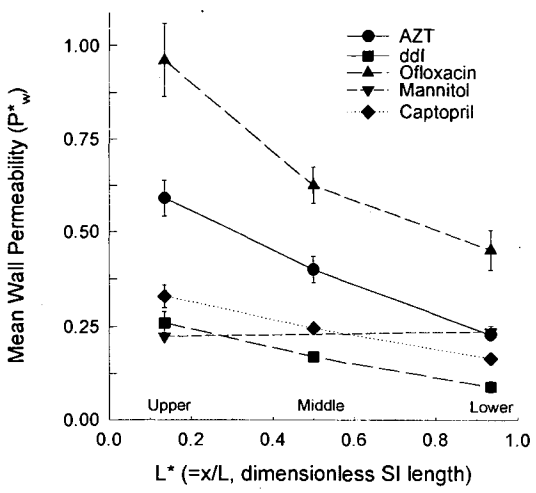

Fig. 1. Plot of the mean P^*_w (±SEM, n = 4–7) of captopril (1 mM), ddI (0.01 mM), ofloxacin (0.1 mM), ZDV (0.01 mM) and mannitol (1 mM) versus dimensionless intestinal length (L*). P^*_w were determined at pH 6.5. Perfusion buffers were maintained at 37°C and were iso-osmotic. The intestinal length (x) at the midpoint of the perfused segment is normalized by the total small intestinal length in rats (L ≈100 cm). Therefore, $x^* = 0.5$ represents the midpoint of the small intestine.

Captopril

Captopril is metabolized to several forms including a disulfide dimer and other mixed disulfides with endogenous thiol compounds such as cysteine (17–19), therefore, post-hoc sample processing methods were established to minimize disulfide formation. Captopril was stable in perfused blank buffer (37°C) during the sample collection period (≈10 min). Since the average intestinal residence time during perfusion is less than 4 min (flow rate of 0.19 ml/min and an average length of 10 cm) and the samples were stabilized immediately after collection, lumenal metabolism was not considered a significant contributing factor in the data analysis. Therefore, perfusate samples did not require correction (17). The absorption of the captopril disulfide could lead to an underestimate of the amount of captopril oxidized during the perfusion experiment and could affect the permeability results. However, since the P^*_w of the disulfide was reported to be only 20% that of captopril (17), the loss of captopril disulfide to absorption during the experiment would be minor.[3] The upper SI P^*_w of captopril determined in this study is substantially lower than that reported by Hu and Amidon (17) for a 1 mM perfusion; however, the variability in the reported P^*_w and the experimental conditions (anesthetics used, flow conditions, composition of the perfusion buffers, etc.) probably accounts for these differences. More important than the absolute value of the reported differences is that captopril P^*_w decreased significantly from the upper to the lower SI in this study in a manner similar to the other water-soluble compounds studied.

[3] The maximum permeability of captopril was determined to be 0.329 in the upper small intestine. According to Hu and Amidon (17), the intestinal permeability of captopril disulfide approximately 20% of the permeability of captopril or 0.066. Using $F = 1 - e^{-2(1.27*0.066)} *100$ (ref 20), only 15% of the disulfide present would be absorbed or <0.015% in our studies.

Mannitol

Fordtran and coworkers (23) reported that the effective pore radii of unperturbed human SI epithelium is about 7–9 Å in the jejunum and 3–4 Å in the ileum. Therefore, it is reasonable to expect that molecular size should not limit mannitol's absorption since it has a Stokes radius of 3.5 Å (22). Although the merits of "aqueous pore" theory may be argued, it has been applied to a variety of pharmaceutical applications including a recent application by Higuchi and coworkers (24). In 1962, Hindle and Code (21) demonstrated that mannitol was absorbed from the upper and lower SI in dogs at equal rates. Since the initial mannitol concentrations in those studies were equal and, using a simple model of absorption rate constant ($k = 1/r\ P_e$, where k is the absorption rate constant, r is the intestinal radius and P_e is the effective permeability), it can be argued that the permeability was approximately equal in the two regions. The geometric differences between the regions are probably not significant since the increase in the intestinal radius in the ileum is probably offset by the decrease in ileal surface area. Other studies in rat ileal and colonic tissue (25) are consistent with the current results whereas, in a study using rabbit tissue (26), an increase in mannitol permeability was reported. The differences may be species-related since all other studies were performed in rats.

Zidovudine

The decrease in ZDV SI permeability (61.3%) is comparable to that reported by Park and Mitra (2) who found that the SI permeability of ZDV decreased 63% in the mid to lower SI using a recirculating perfusion technique in rats.

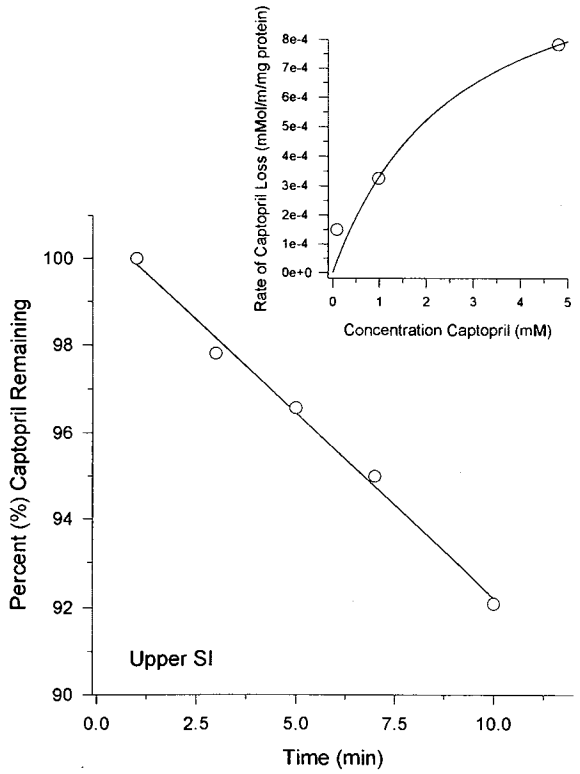

Fig. 2. Metabolism of captopril by 0.08% (w/v) upper small intestinal mucosal homogenates at 37°C was linear for approximately 10 min. Initial captopril concentrations were 0.1 mM, 1.0 mM and 5.0 mM. Initial rates were used to calculate the metabolism parameters, V_{max} and K_m. Inset: The rate of captopril loss demonstrated saturable kinetics between 0.1 mM and 5.0 mM.

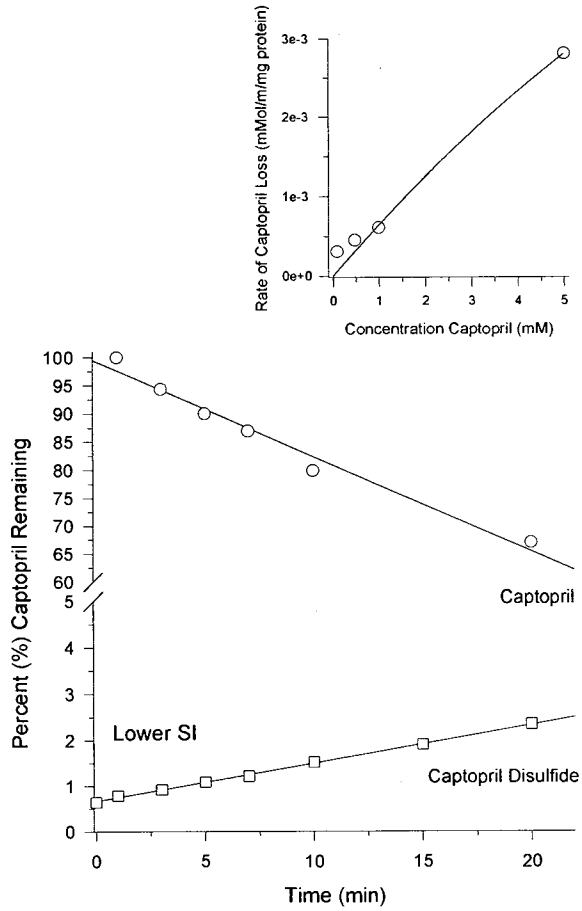

Fig. 3. Metabolism of captopril by 0.08% (w/v) lower small intestinal mucosal homogenates at 37°C was linear for approximately 20 min. Initial captopril concentrations were 0.1 mM, 0.5 mM, 1.0 mM and 5.0 mM. Initial rates were used to calculate the metabolism parameters, V_{max} and K_m. A representative plot of the appearance of captopril disulfide. Captopril disulfide formation in the upper SI was similar (not shown). Inset: The rate of captopril loss demonstrated saturable kinetics between 0.1 mM and 5.0 mM.

Regional Intestinal Metabolism

Typical plots for the loss of captopril (0.1 mM) and ddI (0.001 mM) in upper and lower SI homogenates are shown in Figures 2–5, respectively. Similar profiles were obtained for the other concentrations that were studied. The relationship between degradation rate and initial drug concentration is shown in the inset plots of Figures 2–5 and is nonlinear in all cases. For captopril, V_{max} and K_m were $9.8 \times 10^{-4} \pm 0.39 \times 10^{-4}$ μmol/m/mg protein, 2.65 ± 0.71 mM and $24.49 \times 10^{-4} \pm 22.50 \times 10^{-4}$, 0.017 ± 0.012 for the upper and lower SI, respectively. For ddI, V_{max} and K_m were $9.8 \times 10^{-4} \pm 0.39 \times 10^{-4}$ μmol/m/mg protein, 0.092 ± 0.007 mM and 0.0011 ± 0.0002, 0.157 ± 0.039 for the upper and lower SI, respectively. The ratio of V_{max} to K_m is commonly referred to as intrinsic metabolic clearance. The ratio of V_{max}/K_m for the upper, lower SI are 3.7×10^{-4}, 6.9×10^{-4} and 0.011,

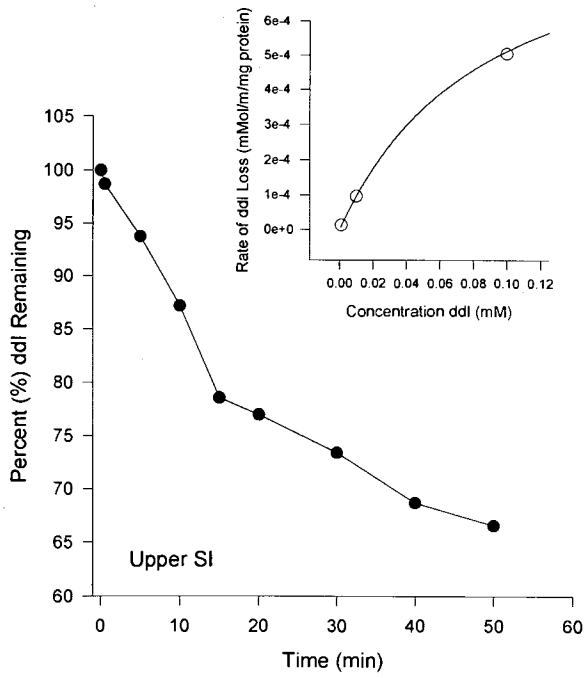

Fig. 4. Metabolism of ddI by 4% (w/v) upper small intestinal mucosal homogenates at 37°C was linear for approximately 15 min. Initial ddI concentrations were 0.001 mM, 0.01 mM and 0.1 mM. Initial rates were used to calculate the metabolism parameters, V_{max} and K_m. Inset: The rate of ddI loss demonstrated saturable kinetics between 0.001 mM and 0.1 mM.

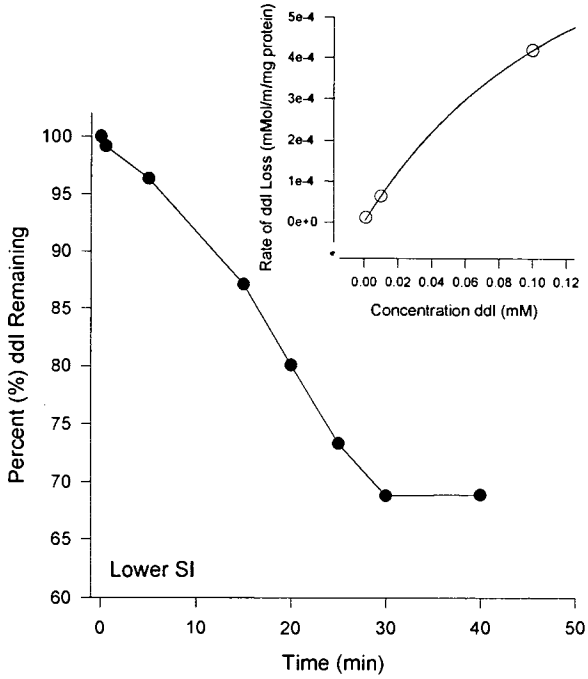

Fig. 5. Metabolism of ddI by 4% (w/v) lower small intestinal mucosal homogenates at 37°C was linear for approximately 30 min. Initial ddI concentrations were 0.001 mM, 0.01 mM and 0.1 mM. Initial rates were used to calculate the metabolism parameters, V_{max} and K_m. Inset: The rate of ddI loss demonstrated saturable kinetics between 0.001 mM and 0.1 mM.

0.007 ml/m/mg protein for captopril and ddI, respectively. A representative plot of the appearance of captopril disulfide in the lower SI homogenate is shown in Figure 3. The appearance of captopril disulfide in upper SI homogenates was similar (not shown). The ratio of upper to lower SI intrinsic clearance for captopril is 0.54:1 and 1.56:1 for ddI. These results suggest that the regional intestinal bioavailability reduction of captopril (6–7,9) may be due to an increase in intestinal metabolism and a decrease in intestinal permeability. The trend in intrinsic metabolic clearance of ddI is similar to that reported by Bramer et al. (3). Neither our current results or those of Bramer et al. demonstrate that ddI is metabolized to a greater extent in the lower SI as compared to the upper SI; however, the colonic-lumenal metabolism reported by their group combined with our finding of a dramatic permeability reduction in the lower SI and colon offer a reasonable explanation for the distal intestinal bioavailability reduction reported for ddI (3,5,8).

CONCLUSIONS

For captopril and ddI, it appears that permeability reductions and regional differences in intestinal metabolism may play a role in the regional intestinal bioavailability of these compounds. In addition to brush border and cellular metabolism, lumenal metabolism may also be significant for captopril and/or ddI. Even though lumenal metabolism was not studied in the present work, it has been suggested as a potential route for the presystemic elimination of ddI by flora (3) and for captopril (17). Even though ofloxacin and ZDV were stable in both regions of the SI, the reduction in intestinal permeability suggests that these compounds may also demonstrate regional intestinal bioavailability. Although the correlation between P^*_w in rats and extent of absorption in humans is well established, the differences in intestinal metabolism may be species dependent and, therefore, extrapolation to bioavailability in humans is difficult without further characterization of metabolism in humans. In spite of these limitations and based on the current results, it is reasonable to expect that permeability and metabolism may have a significant role in regional differences in intestinal bioavailability of these compounds.

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