Desolvation Energy: A Major Determinant of Absorption, But Not Clearance, of Peptides in Rats

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The oral delivery of peptidic drugs is problematic because of their degradation in the gastrointestinal tract and low absorption through the intestinal mucosa. Earlier *in vitro* studies with two series of digestion-resistant, radiolabeled peptides that varied in physical properties (molecular weight, lipophilicity, and hydrogen bonding sites) had suggested that intestinal transport of these peptides was most influenced by the number of hydrogen bonding sites, the major determinant of desolvation energy. To determine whether this correlation could be confirmed *in vivo*, intestinal absorption was determined by comparing the biliary and urinary recovery of these radiolabeled peptides in rats given intravenous or intraduodenal doses. Absorption was inversely correlated to the number of calculated hydrogen bonding sites for the model peptides, similar to what had been found *in vitro*. Clearance by liver and kidneys appeared to be unaffected by desolvation energy but was well correlated with lipophilicity.

KEY WORDS: peptides; intestinal absorption; clearance; hydrogen bonding; lipophilicity.

INTRODUCTION

Advances in biotechnology have made peptides and peptidic hormones available in quantities suitable for pharmaceutical development. Most peptides and proteins, however, even when protected from digestion, are poorly absorbed from the gastrointestinal tract. Since the oral route is preferred for most chronic therapy, many of these proteinaceous molecules are excluded from development.

Earlier studies suggested that membrane transport of many solutes was dependent upon the total number of hydrogen-bonding sites on the molecule, suggesting that the energy of desolvation of the compound greatly influenced transport (1,2). Recently, Conradi *et al.* (3; Burton *et al.*, unpublished) found that this relationship could account for the permeability coefficients of model peptides determined in human intestinal Caco-2 cell monolayers. These peptides had systematic modifications which enabled the contribution of molecular weight and hydrogen bonding potential to be separated. Lipophilicity, which had been classically used to predict absorption with nonpeptidic solutes (4,5), was not positively correlated with Caco-2 transport of these model peptides.

To extend these findings and confirm them *in vivo*, intestinal absorption of the same model peptides was determined in anesthetized rats. Biliary and urinary clearances were also examined. The relationships of lipophilicity, molecular weight, and desolvation energy appear to be substantially different for clearance and gut absorption of these peptides.

MATERIALS AND METHODS

Animals

Sprague Dawley, Upjohn strain, male rats having a mean weight of 340 g were fasted overnight without water for approximately 15 hr.

Peptides

Both peptide series (Fig. 1) were uncharged and presumably enzymatically stable, due to the unnatural "D" configuration of the phenylalanine residue. The Phe1–3 series systematically increased in size, molecular weight, lipophilicity, and hydrogen bond number through the successive additions of phenylalanine (3). For this series, all of these parameters were interrelated. The second series (Me1–4) decreased in hydrogen bond number, while size, molecular weight, and lipophilicity were essentially constant (Burton *et al.*, unpublished). Thus, the second series separated the hydrogen bonding potential from the other two parameters. Hydrogen bond number for each peptide was calculated according to Stein (1), where each amide N–H and carbonyl are assigned a value of 1, and each terminal NH_2 a value of 2.

The peptides were radiolabeled with ^{14}C in the acetamide [C1] carbon position and had a specific activity of approximately 110 mCi/mmol (3; Burton *et al.*, unpublished). They were all stored in methanol at −20°C.

Formulation and Dosage

All intraduodenal (id) injections were aliquots of the peptide methanol stock solutions diluted into 1 ml of 0.1 *M* citric acid vehicle, while all intravenous injections (iv) were diluted from the stock into 1 ml of 0.003 *M* citric acid vehicle.

Dosage amounts varied with available stock of the peptides. Rats receiving $AcPheNH_2$ (Phe1) were given either 2.8×10^{-3} μmol intraduodenally (id) or 2.5×10^{-3} μmol intravenously (iv). Rats administered $AcPhe_2NH_2$ (Phe2) were also given 2.8×10^{-3} μmol id but received 1.0×10^{-3} μmol iv. Rats dosed with the other peptides all received either 8.8×10^{-3} μmol iv or 1.2×10^{-2} μmol id.

In all cases, peptides were repurified immediately prior to preparation of the stock solutions by previously described procedures (3; Burton *et al.*, unpublished).

Anesthetic

The anesthetic was prepared by placing 40 g urethane, 40 g ethylurea, and 10 g 5,5-diallyl barbituric acid into a 250-ml graduated cylinder. A solution of 50 mg disodium calcium salt (EDTA) dissolved in 10 ml sterile water was added to the mixture and hot tap water was used to heat the cylinder until the mixture dissolved. The solution was then

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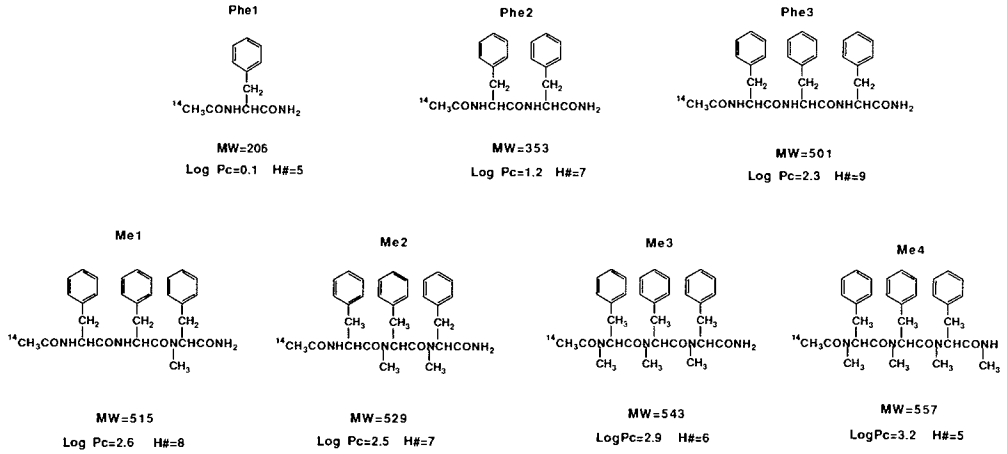

Fig. 1. Structure and properties of model peptides. Octanol–water partition coefficients from references 3 and Burton *et al.*, unpublished. H is the hydrogen bond number according to Stein (1).

allowed to cool to room temperature and the volume was brought up to 100 ml with sterile water.

Animal Procedure I: Bile and Urine Collection

Prior to surgery, all rats were anesthetized with 0.65 to 0.75 ml/kg of a solution of diallybarbital-urethane (dial-urethane) administered intraperitoneally (ip). A midline incision was then made to expose the duodenum. Next the bile duct, found within the mesentery of the duodenum, was cannulated with polyethylene tubing (I.D., 0.28 mm; O.D., 0.61 mm). The urinary bladder was then voided by massage, and the urethral opening was sealed with a drop of cyanoacrylate adhesive. When all rats to be used in a particular study had been prepared as described above, seven rats were injected with each peptide id and five rats were injected with each peptide iv. The rats were then sutured with autoclips and placed under a towel to maintain core temperature.

The bile and urine were collected for 3 hr. The anesthetized rats were then sacrificed by KCl injection and the volume of bile (collected in a graduated test tube) and urine (aspirated into a syringe from the urinary bladder) was measured and recorded. Since bile should be produced at the rate of approximately 1 ml/hr, samples from rats where less than 1.5 ml of bile was collected were discarded. Duplicate studies were performed if animals were lost due to exclusion or death, and data from both studies combined.

Animal Procedure II: Blood and Luminal Fluid Collection

Cannulations and peptide preparation were performed as follows: mesenteric vein cannulation and ligation of the small intestine were performed as described by a previously reported procedure (6), and superior vena cava cannulation was performed by a previously reported procedure (7). Peptides, prepared in 1 ml of a 0.1 *M* citric acid vehicle, were injected id into heparinized rats. Rats were infused continually with donor blood via the superior vena cava cannulae as the blood from the mesenteric vein cannulae was collected in 5-min fractionations until the blood flow ceased (approximately 35 min). The blood was separated by centrifugation. The serum was collected, analyzed by scintillation counting, diluted with water when necessary to obtain 3000 dpm/100 μl, and analyzed by HPLC.

Analytical Methods

Each radioactive peptide was quantitated in a Beckman LS 3801 Scintillation counter. This was accomplished by counting a 0.1-ml aliquot of urine, a 0.5-ml aliquot of bile, or a 0.1-ml aliquot of serum in 12 ml of Beckman Ready Safe scintillation cocktail.

Bile, urine, and serum samples were analyzed by HPLC using procedures previously described (3; Burton *et al.*, unpublished). Briefly, the system consisted of a Beckman Model 110-A pump, a HP-3380-A integrator, and a Flo-One HS detector from Radiomatic fitted with a 2500-μl flow cell. The column was a Brownlee RP-18 Spheri-5 and measured 4.6 × 10 mm. The ratio of scintillant (Flo-Scint II) volume to column effluent volume was 4:1; 100-μl samples of the biological fluids were injected neat.

Statistical Analysis

Absorption was calculated by the following formula:

$$\% \text{ absorption} = \frac{\text{total \% ID recovery}}{\text{total \% IV recovery}} \times 100 \qquad (1)$$

Since the iv recovery is reflective of what would be expected after 100% absorption, the total percentage id recovery is normalized to iv recovery.

The standard error of the mean was calculated for the percentage recoveries after iv and after id dosing found for that particular day. Then, the following equation (8) was used to find the standard error (SE) in the absorption:

$$\text{SE of absorption} = \sqrt{\left(\frac{\text{\% id Rec}}{\text{\% iv Rec}}\right)^2 \left(\left(\frac{\text{\% SE of id Rec}}{\text{\% id Rec}}\right)^2 + \left(\frac{\text{SE of iv Rec}}{\text{\% iv Rec}}\right)^2\right)} \times 100 \qquad (2)$$

where Rec = recovery.

Table I. Biliary and Urinary Recovery of Model Peptides (% Recovery)[a]

Peptide	iv administration			id administration				
	Bile	Urine	Total	Bile	Urine	Total	% Absorption	N^b
Phe1	2.1	30.7	32.8	2.07	22.3	24.4	76.6 ± 4.2	13/11
Phe2	60.2	15.6	75.8	38.6	5.0	43.5	58.1 ± 3.0	13/9
Phe3	54.5	6.9	61.4	7.6	0.30	7.9	12.9 ± 1.0	20/14
Me1	66.4	10.1	76.5	16.1	0.7	16.8	22.0 ± 1.3	13/9
Me2	77.0	3.0	80.0	22.9	1.4	24.3	30.2 ± 2.3	13/8
Me3	78.3	0.7	79.0	23.5	0.6	24.1	30.5 ± 1.4	7/5
Me4	63.8	0.8	64.6	26.8	1.6	28.4	44.0 ± 2.2	7/5

[a] Mean ± standard error of the mean.
[b] Number of rats id/number of rats iv.

Linear regressions were performed in order to determine the equation of the line and estimate the probabilities.

RESULTS

Following an iv or id dose of the peptides (Animal Procedure I), a percentage of the radiolabel was recovered in the bile and urine (Table I). There was some evidence of metabolism as exemplified by HPLC analysis, which showed multiple radioactive peaks for both bile and urine (data not shown). If this transformation occurred after absorption, the radioactivity found in the bile and urine can still be used to quantitate absorption. To determine whether transformation occurred before absorption, samples of mesenteric blood and luminal contents from rats receiving id doses (Animal Procedure II) of the peptides were analyzed by HPLC. Only peaks with retention times corresponding to the standard were found (data not shown), suggesting that transformation occurred after absorption. Since the peptides appear to be absorbed intact, the percentage absorption can be estimated by comparing recovery of the radioactive label in rats given iv and id doses of peptides.

After iv injection, the recovery in the bile of the Phe1–3 series peptides ranged from 2.1 to 60.2%, while the recovery in the urine was inversely related to biliary recovery and ranged from 30.7 to 6.9% (Table I). The total percentage recovery (bile + urine) had a narrow range of 61–80% for six of seven of the peptides. Only 33% of the Phe1 was recovered after iv dosing, which suggests that the elimination and/or disposition of this peptide was different from the other six peptides.

The percentage recovery in bile after intravenous administration displayed a highly significant positive linear correlation ($P < 0.01$) with the lipophilicity (Fig. 2A). Conversely, the percentage recovery in urine exhibited a highly significant negative linear correlation with this parameter (Fig. 2B). Since molecular weight changes parallel alterations in lipophilicity, similar correlations were seen with this parameter (data not shown). The bile and urine recovery did not correlate with hydrogen bonding number (Fig. 3).

Intestinal absorption, however, was strongly inversely correlated with hydrogen bond potential for both series (Fig. 4). Absorption decreased as lipophilicity increased (Fig. 5).

Permeability coefficients calculated from transport across Caco-2 cells (3, Burton *et al.*, unpublished) correlated strongly with gut absorption for the two series (Fig. 6).

DISCUSSION

Validity of the absorption findings in this study rests on the assumption that the peptides are not degraded before

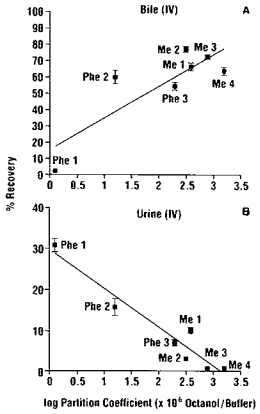

Fig. 2. Relationship between percentage recovery of the model peptides in the bile (A) and urine (B) and the log octanol/water partition coefficient after iv dosing. (A) bile: $y = 13 + 20.68 \cdot 10^6 x$; $R^2 = 0.6968$; $P < 0.01$. Urine: $y = 31.13 - 10.62 \cdot 10^6 x$; $R^2 = 0.9112$; $P < 0.01$.

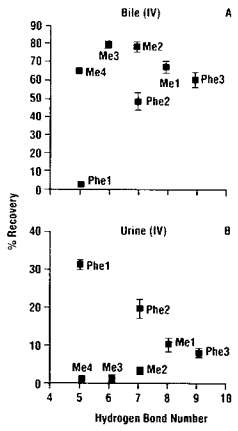

Fig. 3. Relationship between the hydrogen bond number and the percentage recovery after iv dosing for the model peptides in the bile (A) and the urine (B).

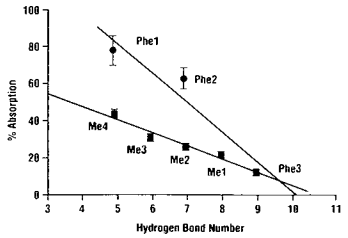

Fig. 4. Relationship between the percentage intestinal absorption and hydrogen bonding potential for both series of peptides. Phe1–Phe3 series, $y = 157.4 - 15.6\ x$; $R^2 = 0.8228$; $P < 0.01$. Methyl series, $y = 74.9 - 6.8\ x$; $R^2 = 0.6956$; $P < 0.01$.

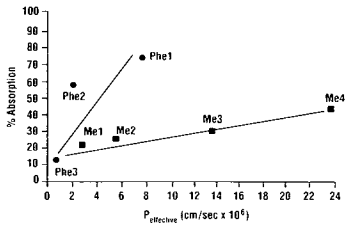

Fig. 6. Relationship between the percentage absorption of the model peptides and the effective permeability coefficient in a Caco-2 cell. Phe1–Phe3 series, $y = 25.264 + 7.13 \cdot 10^6\ x$; $R^2 = 0.659$ and $P = 0.397$. Methyl series, $y = 16.203 + 1.166 \cdot 10^6\ x$; $R^2 = 0.927$ and $P < 0.01$.

absorption occurs. Since the peptides were prepared from the unnatural D-amino acids (3; Burton *et al.*, unpublished), enzymatic hydrolysis was not expected. Evidence of some transformation was found, however, when urine and bile samples were analyzed by HPLC after both iv and id administration. While the identity of the metabolites was not ascertained, it is conceivable that oxidation of the amino acid side chains occurred in the liver, similar to what has been seen for the metabolism of cyclosporin. Cyclosporin is thought to cross the intestine intact but is extensively oxidized after absorption, with little or no evidence of peptide bond hydrolysis taking place (9). In any event, the lack of metabolite present in the portal vein serum after id administration strongly argues that the peptides are absorbed intact and metabolized once in the systemic circulation and/or during first pass through the liver.

Lipophilicity of the peptides in the Phe1–3 series significantly increased as the two phenylalanine residues were added to Phe1. However, the overall absorption decreased from 76.6% for Phe1 to 12.8% for Phe 3. If lipophilicity alone had been the major determinant of absorption, as has been found with other classes of compounds (5), the opposite pattern should have been observed. However, in this series molecular weight and hydrogen bond number also increase, along with lipophilicity. Thus, the results argue that either or both of these two parameters dominate over lipophilicity.

In the Me1–4 series, on the other hand, the molecular weight and lipophilicity change only slightly with each subsequent methylation, while the hydrogen bond number decreases dramatically. The overall absorption increase of from 12.8% for Phe 3 to 44.0% with the addition of the four methyl groups argues that the prevalent factor influencing absorption of these peptides is hydrogen bond potential. These results are all qualitatively identical with what had been found previously for the transport of these peptides

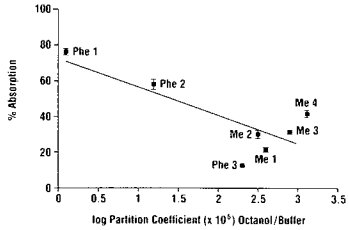

Fig. 5. Relationship between the percentage absorption of the model peptides and the log octanol/water partition coefficient after id dosing. $y = 81.29 - 20.07 \cdot 10^6\ x$; $R^2 = 0.8552$; $P < 0.01$.

across confluent Caco-2 cell monolayers (3; Burton *et al.*, unpublished).

There is a significant difference between these results and those from the Caco-2 cell studies. For pairs of peptides with equivalent numbers of hydrogen bonds, Phe1 and Me4 or Phe2 and Me2, for example, a greater difference is seen for absorption of the smaller peptide than was found in the cell culture model. This suggests that there may be a more significant molecular size component to peptide absorption in the *in vivo* rat model. However, it must be pointed out that, while the exercise of counting hydrogen bonds is useful for demonstrating the concept, it naively assumes that all hydrogen bonds are energetically equivalent. This is clearly not true. Hydrogen bond energies are dependent upon a number of factors including proximity effects (10). Thus, the members of these two pairs of peptides may not be quite equivalent in terms of hydrogen bonding potential.

Also, as has been pointed out previously, the influence of desolvation energy applies only to a transcellular diffusion pathway (3). An organized epithelium, such as the intestinal mucosa, possesses an alternate pathway. This is the paracellular route, involving diffusion of the solute between adjacent intestinal cells, restricted by the tight junctions (11). Since the solute never leaves the aqueous environment in this mechanism, desolvation is not required. The tight junction acts as a sieve, however, so there is a dependence on overall size of the solute (11). The results found in this study may suggest that the Caco-2 cell system has a more restricted "pore" pathway than the rat. In fact, the higher transepithelial electrical resistance found in Caco-2 cell monolayers relative to that found in normal intestinal mucosa is consistent with this possibility (12). Distinguishing among these several possibilities will require further experimentation.

Application of these findings to the development of proteinaceous drugs would be a useful tool in improving absorption. Developing drugs with an inherently lower hydrogen bond number would probably be of more use than emphasizing high lipophilicity for this compound class. For peptides that require hydrogen bonding functionality for activity, the prodrug approach might be useful. A potential drug could be chemically modified to lower its hydrogen bond number and, after absorption, converted back to its original active form.

Even with such potentially improved absorption, however, peptides might have little therapeutic utility if the same modifications which promote absorption also augment clear-

ance by the liver and kidneys. With the model peptides, this did not appear to be the case. While increased lipophilicity (and the interrelated parameter molecular weight) did not correlate well with increased absorption, it does show a fairly good correlation with biliary elimination. Thus it may be possible to design orally absorbable peptides with reasonable circulating half-lives.

In summary, these studies demonstrate that hydrogen bonding potential is a primary determinant of *in vivo* absorption. In this regard, the Caco-2 cell model accurately predicts the *in vivo* findings. In contrast to absorption, liver clearance is less influenced by hydrogen bonding considerations but more by lipophilicity and/or molecular weight.

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