

Intestinal Absorption of Peptides and Peptide Analogues: Implications of Fasting Pancreatic Serine Protease Levels and pH on the Extent of Oral Absorption in Dogs and Humans

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In order to describe and predict the impact of intestinal metabolism on peptide absorption, intestinal chymotrypsin activity, flow rate, and pH were characterized in fasted, duodenally fistulated dogs as a function of gastrointestinal (GI) motility phase. GI motility was classified as either active or quiescent. Cumulative volume, $F(t)$, and volumetric flow rate, $Q(t)$, curves were constructed and the data were sorted according to motility phase. The mean \pm SE active phase pH was 6.4 ± 0.3 , whereas the quiescent phase pH was 7.3 ± 0.3 . The difference between the mean active and the mean quiescent phase pH values was significant. The active and quiescent phase flow rates (ml/min) were also significantly different, at values of 1.2 ± 0.2 and 0.28 ± 0.07 , respectively. The active phase flow rates were consistent among the dogs studied; however, the quiescent phase flow rates were highly variable among the dogs. The variability of the quiescent phase flow rates was expected since phase II of the GI motility cycle is characterized by intermediate, irregular spike activity. The mean active and quiescent phase chymotrypsin activities were $1.87 \times 10^{-5} \pm 0.53 \times 10^{-5}$ and $1.56 \times 10^{-5} \pm 0.65 \times 10^{-5}$ M, respectively. The active phase values were not statistically different among dogs, however, the quiescent phase values were found to be highly variable among dogs. The difference between the active and the quiescent phase chymotrypsin mean levels, however, was not statistically significant. The chymotrypsin levels determined in dogs were found to be approximately 10 times greater than those reported in humans. The significance of fasted-state chymotrypsin levels is discussed with respect to the impact of GI metabolism on peptide and peptide-like drug absorption in dogs. Further, given the intestinal metabolic differences between dogs and humans, the suitability of using the dog model for predicting the oral absorption of peptides in humans is discussed.

KEY WORDS: chymotrypsin; dog; human; intestinal metabolism; oral absorption; pancreatic serine protease; peptide; peptide-like drugs.

INTRODUCTION

The therapeutic use of orally delivered peptides and peptide analogues is advantageous since these compounds are usually potent, safe, and rapidly eliminated from the body. Moreover, the oral route is generally less expensive

and more convenient for the patient than other routes of administration. The oral delivery of peptides, however, is compromised by chemical and proteolytic instability as well as by intestinal membrane transport limitations. Small peptides (3 amino acid residues or smaller) are absorbed primarily by a carrier-mediated absorption mechanism, whereas larger peptides (4 to 10 amino acid residues) may be absorbed by paracellular and endocytotic mechanisms (1). Even though the intestinal transport of large peptides (>10 amino acid residues) and proteins may be possible, presystemic metabolism by intestinal proteolytic enzymes presents a significant obstacle for their oral delivery. In fact, the oral bioavailability of larger peptides is usually less than 10% (1).

The preclinical absorption screening of orally delivered, peptidic drug candidates is commonly performed in dogs, however, metabolic and physiological differences between dogs and humans limit the usefulness of the dog model unless these differences are well characterized. While extensive comparisons have been made between dogs and humans with regard to intestinal pH (2-4), the presystemic metabolism of peptides by intestinal proteolytic enzymes has not been well characterized and could differ significantly from humans. In this report, the results of studies characterizing the upper GI pH, volumetric flow rate, and activity of chymotrypsin in mongrel fistulated dogs as a function of fasted GI motility phase are presented. The significance of fasted-state chymotrypsin levels is discussed with respect to the impact of GI metabolism on peptide and peptide-like drug absorption in dogs. Furthermore, the suitability of using the dog model for predicting intestinal peptide absorption in humans, given the intestinal metabolic differences between dogs and humans, is also discussed.

MATERIALS AND METHODS

Chemicals and Reagents

BTEE (benzoyl-L-tyrosine ethyl ester), calcium chloride, and hydrochloric acid were obtained from Sigma Chemical Company (St. Louis, MO). HPLC-grade methanol was obtained from J. T. Baker Chemical Company (Phillipsburg, NJ).

Experimental

Five healthy female, mongrel dogs weighing 18 to 23 kg were implanted with a chronic duodenal Thomas fistula. This procedure has been described by Meyer *et al.* (5) and Sirois *et al.* (6). The fistula was placed 12-19 cm distal to the pylorus (proximal to mid-duodenum). A recovery period of 2 weeks was allowed after surgery. The dogs were fasted for 12 to 18 hr prior to each experiment. Water was given ad libitum except for 2 to 3 hr prior to the experiment. During the experiments the dogs were fully conscious and retained in slings (Alice King Chatman Medical Arts, Los Angeles, CA). A small volume of water (50 ml) was given during phase I by a natural swallowing technique. Phase I was ascertained by observing the discharge from the cannula as described by Gupta and Robinson (7). Mucus and bile discharges were taken as phase II activity, and 20 min after cessation of any

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discharge from the cannula, the motility was assumed to be phase I. The intestinal discharge was collected from the duodenal fistula at 10 min intervals. If the motility phase was considered active, the sampling time would be shortened to 2 to 5 min. Sampling continued for 210 min. Effluent sample color, enzyme activity, pH at 37°C (Beckman P31 pH meter, Beckman Instruments, Inc., Fullerton, CA), and volume were recorded for each sample. Enzyme determinations were performed as described below. Two to four experiments were completed in each dog.

Chymotrypsin Assay

Chymotrypsin activity was determined using the method of Hummel (8). Briefly, the rate of hydrolysis of the substrate BTEE (benzoyl-L-tyrosine ethyl ester) is determined from the change in absorbance at 256 nm. One unit is equivalent to 1 μ mol of substrate hydrolyzed per min at pH 7.8 and 25°C. The concentration of protein is determined by multiplying the absorbance at 280 nm by 0.49 (the molar absorptivity of the protein). Finally, the activity per milligram of protein was calculated from the following equation:

$$\text{Enzyme activity per mg} = \frac{\text{rate of change absorbance (per min at 256 nm)} * 3000}{964 * \text{amount enzyme used (mg)}}$$

where 964 is the molar absorptivity of *N*-benzoyl-DL-tyrosine.

Data Analysis: Intestinal Motility Patterns and Determination of Active and Quiescent Phases

The active and quiescent GI motility phase definitions of Chen (9) are used to classify the discharge samples. Since gastric and pancreaticobiliary secretions occur sequentially before the sweeping action of the gastric phase III contractions and since the mucous discharge is known to occur at nearly the same time (late phase II/phase III), the experimental method of determining the motility phase is to observe the effluent collected from the fistula. Therefore, the quiescent phase occurs when there is little or no fistula effluent (<0.2 ml/min), whereas the active phase discharge rate is greater than 0.2 ml/min and is accompanied by bile and mucous secretions.

A post hoc method for determining the motility phase of the discharge sample was also used. In this method, both the cumulative volume curve, $F(t)$, and the volumetric flow rate curve, dV/dt or $Q(t)$, of the fistula effluent was plotted with time. The cumulative data were fit (BMDPAR-Derivative Free Nonlinear Regression, BMDP Statistical Software Inc., Los Angeles, CA) to the equation

$$F(t) = \sum_{i=1}^n \frac{P_{3i}}{2} \left(1 - \text{erf} \left\{ \frac{P_{3i-1}}{2} \left[\frac{1 - (t/P_{3i-2})}{\sqrt{t/P_{3i-2}}} \right] \right\} \right) \quad (1)$$

where i is the number of MMCs (gastric motility cycles) observed in the study. Kerlin *et al.* (10) reported that during the fasted state, volumetric flow rate in the small intestine is related to the motility cycle. Therefore, the volumetric flow

rate $Q(t)$ curve for one MMC was constructed using the following equation and the fitted parameters from the previous equation:

$$Q(t) = \sum_{i=1}^n \frac{P_{3i}}{2 \sqrt{\pi P_{3i-1} (t/P_{3i-2})}} e^{-\left[\frac{\left(1 - \frac{t}{P_{3i-2}}\right)^2}{\left(\frac{4}{P_{3i-1} P_{3i-2}}\right)} \right]} \quad (2)$$

GI motility cycles were identified based on the fitted volumetric flow rate curves. The length of one MMC was defined as beginning with the first peak of the $Q(t)$ curve and continuing to the next peak. For example, four representative studies are shown in Figs. 1a through d. Cumulative volume (ml) is plotted versus time (min) and volumetric flow rate (Q ; ml/min). Figure 1a demonstrates two active phases with a MMC length of approximately 120 min. The quiescent phase occurs when the volumetric flow rate is approximately 0 [i.e., when the $Q(t)$ curve is horizontal]. Two error functions were used ($n = 2$) in determining the regression model for data in Fig. 1a. In Fig. 1b, only one active phase was detected ($n = 1$), so the length of the MMC could not be determined. In Fig. 1c, two active phases were determined with an MMC length of 70 min. Finally, in Fig. 1d, two active phases were also determined.

Data Analysis: Statistical Methods

The statistical analysis was performed using SYSTAT: The System for Statistics, SYSTAT, Inc. Evanston, IL (version 4). The Kolmogorov-Smirnov-Lilliefors (KSL) test (11) was used to examine the probability distributions for all three parameters (chymotrypsin activity, flow rate, and pH). The KSL tests for normality without assuming a particular mean or standard deviation for the distribution. Moreover, the KSL test standardizes the data in such a way so that it is concerned only with the shape of the distribution and not the absolute scale. This enabled the testing for a log normal distribution by simply taking the log transformation of the data and testing using KSL. Interdog parameter statistics were examined using one-way ANOVA and a Tukey-Kramer analysis. T-tests were used to test the difference between the means of chymotrypsin activity, flow rate, or pH as a function of GI motility phase.

RESULTS AND DISCUSSION

The 50 ml of water given at the beginning of the study was not expected to affect the motility cycle (7). It was observed, however, that during the first 30 min of the study some dogs appeared to empty the volume given immediately. This apparent active phase motility pattern was probably an artifact since other intestinal parameters such as pH and effluent color did not change as would be expected during a true active phase motility pattern. It has been observed that certain dogs can be classified as "quick" gastric emptiers and others as "slow" emptiers. This may explain the active phase artifact. A total volume balance taken at the end of each study revealed that approximately 50 to 400% of the volume given was recovered at the duodenal fistula. Given that water secretion and reabsorption are dynamic processes

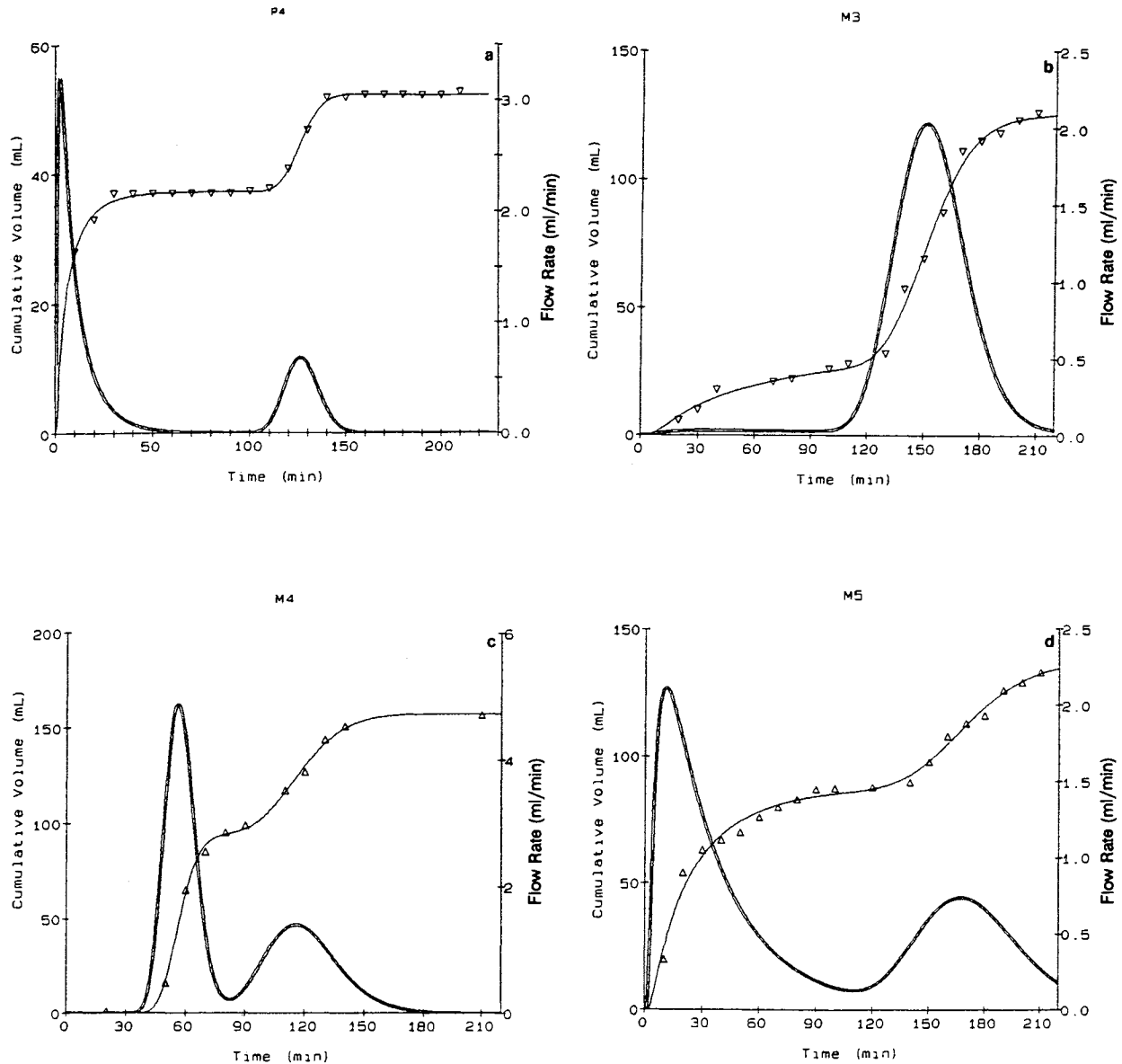


Fig. 1. Four representative studies of the 17 performed in fistulated dogs. Plotted are the fitted curves as cumulative volume (—) volumetric flow rate (—) versus time and the experimental data (∇).

that occur throughout the entire small intestine, this result is not surprising.

Upper GI pH as a Function of Motility Phase

The active and quiescent phase pH data are reported in Table I. The results of the KSL test on the quiescent phase pH data indicate that a log-normal cumulative distribution fit slightly better ($P = 0.013$) than the normal cumulative distribution function ($P = 0.035$), however, the difference between the two is negligible. The active phase pH data were approximately log-normally distributed. The mean active phase pH was found to be 6.4 ± 0.3 , whereas the quiescent phase pH was 7.3 ± 0.3 . The difference between the mean active and the mean quiescent phase pH values is significant ($P < 0.01$). In a study of dogs, Youngberg *et al.* (3) reported

that the fasted-state duodenal pH during the initial period after gastric emptying was 6.1 ± 0.1 . The period immediately after gastric emptying would correspond to late phase II/phase III activity at the duodenal fistula, therefore, the results of Youngberg *et al.* can be considered active phase values. Their results compare favorably with the results reported in Table I for the active phase. The observed active phase pH values were lower than the quiescent phase values, presumably because of the rapid emptying of acidic stomach contents during the active phase. Due to the buffering capacity of the small intestine, however, the pH perturbation observed during the active motility phase in the upper GI is transient and is not expected to be propagated distally. The quiescent phase pH in the upper GI (7.3 ± 0.3) corresponds well with the result of Lui *et al.* (2), who reported the average intestinal pH in dogs to be 7.3 ± 0.1 . Lui

Table I. Active and Quiescent Phase pH Levels in Five Duodenally Fistulated Dogs

Dog	<i>n</i> ^a	Active ^b		Quiescent ^c	
		Mean	SD	Mean	SD
1	4	6.138	0.292	7.380	0.182
2	3	6.473	0.450	7.067	0.292
3	4	5.988	1.129	7.178	0.964
4	2	6.470	0.042	7.710	0.171
5	3	7.177	0.493	7.343	0.866
Overall*		6.399	0.322 ^d	7.329	0.259 ^d

^a Number of studies.

^b Differences among dogs (active phase) not statistically different.

^c Differences among dogs (quiescent phase) not statistically different.

^d Standard error of the mean.

* Differences between active and quiescent values statistically different ($P < 0.01$).

et al. reported that the fasting pH in dogs is approximately one unit higher than in humans. In a recent study of humans, Dressman *et al.* (4) confirmed the finding of Lui *et al.* that the intestinal pH in humans was approximately 6. Dressman *et al.* also reported that the fasting pH in the proximal duodenum in humans is highly variable, whereas the pH in the mid to distal duodenum was found to be much more stable. They did not report their results in terms of GI motility phase, however, the results for the active and quiescent phases in dogs are significantly different, and if the data in Table I are lumped together as a single fasting-state value, it would also show significant variability. Variable and lower duodenal pH during the active phase can affect dosage form performance, especially in the case of pH-dependent enteric-coated dosage forms that would empty during the active phase. Furthermore, the active phase pH lowering effect can influence drug dissolution and absorption leading to variable bioavailability results in dogs and humans.

Volumetric Flow Rate as a Function of Motility Phase

The volumetric flow rate data are reported in Table II. The mean active and quiescent phase flow rates (ml/min) are 1.2 ± 0.2 and 0.28 ± 0.07 , respectively. The flow rate data for both the active and the quiescent phases were approximately log-normally distributed ($P = 0.31$ and $P = 0.24$, respectively) as determined by the KSL test. A normal cumulative distribution was not appropriate for the flow rate data for either phase ($P = 0.96$ and $P = 0.62$, respectively). The results in dogs are consistent with results recently reported in humans by Sinko *et al.* (12), who demonstrated that the distribution of small intestinal transit times in humans was log-normal. Since $t_{res} = (V/Q)$ and the intestinal volume (V) remains relatively constant, it follows that volumetric flow rate (Q) would also be log-normally distributed. The active phase flow rates were consistent among the dogs studied, however, the quiescent phase flow rates among dogs were variable ($P < 0.01$). The variability in quiescent phase flow rates is expected since phase II of the MMC is characterized by intermediate, irregular activity. On the

Table II. Active and Quiescent Phase Volumetric Flow Rates in Five Duodenally Fistulated Dogs

Dog	<i>n</i> ^a	Active ^b		Quiescent ^c	
		Mean (ml/min)	SD	Mean (ml/min)	SD
1	4	1.108	0.574	0.333	0.045
2	3	1.480	0.317	0.480	0.157
3	4	1.040	0.223	0.270	0.138
4	2	1.100	0.255	0.200	0.070
5	3	1.283	0.272	0.103	0.015
Overall*		1.193	0.162 ^d	0.280	0.068 ^d

^a Number of studies.

^b Differences among dogs (active phase) not statistically different.

^c Differences among dogs (quiescent phase) statistically different ($P < 0.01$).

^d Standard error of the mean.

* Differences between active and quiescent values statistically different ($P < 0.01$).

other hand, the active phase flow rates are more consistent among dogs since Phase III is characterized by regular spike activity. As expected, the difference between the active and the quiescent flow rates was significant ($P < 0.01$).

Chymotrypsin Activity as a Function of Motility Phase

A summary of the chymotrypsin data is reported in Table III. The active and quiescent phase chymotrypsin activities are $1.87 \times 10^{-5} \pm 0.53 \times 10^{-5}$ and $1.56 \times 10^{-5} \pm 0.65 \times 10^{-5}$ M, respectively. The chymotrypsin data for the active and quiescent phases were approximately normally distributed ($P = 0.02$ and $P = 0.06$, respectively). The active phase values are not statistically different among dogs, however, the quiescent phase values are variable ($P < 0.01$). Once again, the variability in chymotrypsin levels is expected during phase II of the MMC. The difference between the active and the quiescent phase mean chymotrypsin levels was not statistically significant due to the variability in the means. The variability (expressed as CV% or percentage coefficient of variation) observed in our studies was 62 and 93% for the active and quiescent phases, respectively. Large variability in fasting intestinal chymotrypsin activity has also been observed in other species (12–15). In the upper quarter of rat intestine, Pelot and Grossman (15) observed a CV% of 62% among their mean levels. Rinderknecht *et al.* (13,14) observed an even larger variability in the duodenal chymotrypsin activity in humans in the fasted state (87%). The physiological reason for the high degree of variability in fasting intestinal chymotrypsin activity is that phase II is characterized by irregular spike motility and sporadic pancreatic and biliary secretions.

In a study of humans (13), fasting chymotrypsin activity was reported to be approximately 2 BTEE units/ml. Converting the chymotrypsin concentration units in dogs from moles per liter to BTEE units per milliliter results in chymotrypsin values for the active and quiescent phase of 28.1 and

Table III. Active and Quiescent Phase Chymotrypsin Levels in Five Duodenally Fistulated Dogs

Dog	<i>n</i> ^a	Active ^b		Quiescent ^c	
		Mean (M)	SD	Mean (M)	SD
1	4	2.17×10^{-5}	4.25×10^{-6}	1.75×10^{-5}	5.29×10^{-6}
2	3	2.94×10^{-5}	1.96×10^{-5}	3.76×10^{-5}	2.23×10^{-5}
3	4	1.90×10^{-5}	1.22×10^{-5}	1.01×10^{-5}	4.35×10^{-6}
4	2	9.64×10^{-6}	2.10×10^{-7}	1.20×10^{-5}	4.42×10^{-6}
5	3	9.61×10^{-6}	5.40×10^{-6}	1.86×10^{-6}	1.65×10^{-6}
Overall*		1.87×10^{-5}	0.53×10^{-5d}	1.56×10^{-5}	0.65×10^{-5d}

^a Number of studies.

^b Differences among dogs (active phase) not statistically different.

^c Differences among dogs (quiescent phase) statistically different ($P < 0.01$).

^d Standard error of the mean.

* Differences between active and quiescent values not statistically different.

23.4 units/ml, respectively. Therefore, the fasted-state chymotrypsin activity observed in dogs is approximately a factor of 10 higher than that reported in humans from duodenal aspirates (13,14). The impact of fasted-state serine protease activity on the absorption of peptides and peptide-like drugs can be assessed by performing a calculation on some model peptides. Listed in Table IV are four amino acid analogues that are known substrates of chymotrypsin, along with the kinetic parameters k_{cat} and K_m . These compounds were selected since they possess intestinal metabolic instability characteristics similar to peptides. Based on a mass balance (mixing tank) model for the simultaneous absorption and metabolic/chemical reaction of drugs in the GI tract, the

fraction of substrate that is absorbed and the fraction that is metabolized in the intestine by chymotrypsin are calculated from the following equations (12):

$$F_a = \frac{2 \text{ An}}{1 + 2 \text{ An} + \text{ Da}}$$

$$F_r = \frac{\text{ Da}}{1 + 2 \text{ An} + \text{ Da}}$$

An =

$$P_w^* \text{ Gz}, \quad \text{Gz (mixing tank)} = 2.27, \quad \text{Da} = k_{rxn} t_{res}$$

where $k_{rxn} = k_{cat} E_0/K_m$, Da is the Damkohler number, An is the absorption number, k_{rxn} is the reaction rate constant, k_{cat} is the catalytic rate constant, E_0 is the initial enzyme concentration, K_m is the reaction Michaelis constant, P_w^* is the dimensionless intestinal permeability, Gz is the Graetz number, and t_{res} is the intestinal residence time. The calculations are based on an average small intestinal transit time in humans of 3 hr and chymotrypsin concentrations of 2 units/ml for humans and 28.1 and 23.4 units/ml for the active and quiescent phases in the dog, respectively. It is further assumed that these peptides would be well absorbed ($P_w^* = 1.5$) if they were chemically or metabolically stable in the GI tract. Since it is known that amides are significantly more stable to chymotrypsin hydrolysis than esters, two of each are used for the calculations as best-case and worst-case examples. Most natural peptides are expected to be good substrates for digestive enzymes, whereas it is more difficult to generalize for peptide-like drugs. If they are substrates for chymotrypsin, however, they will probably fall between the extremes selected for the example calculations. As seen in Table IV the two amides (ALYA and ALRA) are estimated to be well absorbed in humans, with a minimal intestinal metabolic component. The corresponding esters (ALYME and ALRME) are considerably less stable than the amides, and, as seen in Table IV, these compounds are estimated to undergo significant intestinal metabolism by chymotrypsin. The absorption component becomes negligible due to the large degree of presystemic/intestinal metabolism. The estimates of intact peptide absorption in dogs are considerably

Table IV. Estimates of Intact Amino Acid Analogue Absorption and Metabolism After Oral Administration to Humans^a

Analogue	k_{cat} (sec^{-1})	K_m (M)	Calculated fraction	
			Metabolized (%) ^b	Absorbed (%) ^c
Acetyl-L-Tyr amide (ALYA)	0.198	0.027	1.7	84.2
Acetyl-L-Try amide (ALRA)	0.033	0.0053	1.5	84.5
Acetyl-L-Tyr methyl ester (ALYME)	116.9	0.00032	99.9	0.1
Acetyl-L-Try ethyl ester (ALRME)	171.9	0.032	92.8	6.2

^a It is assumed that these analogues would be well absorbed ($P_w^* = 1.5$) if stable. Small intestinal residence time is estimated to be 3 hr. Human intestinal chymotrypsin concentrations are taken from the literature. Estimates of absorption in dogs would be significantly lower since the chymotrypsin concentration in dogs is approximately 10 times higher. Chymotrypsin kinetic parameters for the model peptides are given in Refs. 16–18.

^b Fraction metabolized (%) is the calculated percentage of the dose that undergoes presystemic metabolism. For these analogues, presystemic metabolism by chymotrypsin occurs in the intestine.

^c Fraction absorbed (%) is the calculated extent of model peptide absorption into the mesenteric blood supply or portal vein.

Table V. Estimates of Intact Amino Acid Analogue Absorption and Metabolism After Oral Administration to Dogs^a

Analogue	Quiescent phase		Active phase	
	Fraction metabolized (%) ^b	Fraction absorbed (%) ^c	Fraction metabolized (%)	Fraction absorbed (%)
ALYA	15.00	72.86	17.46	70.75
ALRA	13.03	74.54	15.23	72.66
ALYME	99.99	0.01	99.99	0.01
ALRME	99.23	0.66	99.36	0.55

^a It is assumed that these compounds would be well absorbed ($P_w^* = 1.5$) if stable. Dog intestinal chymotrypsin concentrations are taken from Table III. Chymotrypsin kinetic parameters for the model peptides are given in Refs. 16–18.

^b Fraction metabolized (%) is the calculated percentage of the dose that undergoes presystemic metabolism. For these peptides, presystemic metabolism by chymotrypsin occurs in the intestine.

^c Fraction absorbed (%) is the calculated extent of model peptide absorption into the mesenteric blood supply or portal vein.

lower since the chymotrypsin levels are approximately a factor of 10 higher than the levels reported in humans. In the quiescent phase, 70 to 75% of the ALYA and ALRA is estimated to be absorbed, whereas 15 to 18% is predicted to be metabolized. The esters are significantly less stable, with 99% of the “dose” metabolized in the intestine. The complete set of calculations in the dog is summarized in Table V. The example calculations in Table V demonstrate that peptides that are considered stable to chymotrypsin will still undergo significant presystemic metabolism. Further, when intestinal brush border, cytosolic, liver, and systemic metabolism is considered, the bioavailability of even the “stable” peptides could be greatly diminished. Understanding the differences between dogs and humans allows the data obtained in dogs to be used effectively for developing a strategy for reducing intestinal metabolism and bioavailability variability in humans.

Although fasted:fed comparisons were not performed in the current studies, other investigators have found that the ratio of fasted:fed chymotrypsin activity in the duodenum of rats was 0.63. Even though there are no data for chymotrypsin levels in the fed state in dogs or humans, the physiological need for digestive enzymes would be highest in the postprandial state and a pattern similar to that found in rats would seem reasonable. Since pharmacokinetic studies in animals and humans are routinely performed during the fasted state, the present studies give important results for pH and protease levels needed to correlate the results from dog studies to humans.

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