Oral Absorption of Peptides: The Effect of Absorption Site and Enzyme Inhibition on the Systemic Availability of Metkephamid

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In this study the intestinal degradation and absorption of a synthetic pentapeptide, metkephamid, were investigated in the rat by determination of its wall permeabilities in the small and large intestine and the extent and mechanism of its intestinal degradation. The peptide was metabolized in the gut wall through contact with membrane-bound enzymes in the brush border membrane. The extent of metabolic inactivation depended on the intestinal segment investigated and decreased in the axial direction. No metabolism was found in the colon. The dimensionless wall permeabilities (P_w*), determined by single-pass perfusion, were also site dependent. P_w* was highest in the ileum [1.91 ± 0.24, (SE); n = 4], followed by the jejunum (1.64 ± 0.34; n = 4) and the colon (0.67 ± 0.38; n = 4). Based on the permeability data alone and under the assumption of no presystemic metabolism, complete bioavailability would be predicted for metkephamid. However, following oral administration, the mean absolute bioavailability was only 0.22 ± 0.065% (n = 3), indicating the overall dominance of degradation in the absorption process. Thus future strategies in oral peptide delivery should focus on increasing the stability of the peptide in the intestine by modifying the peptide structure and/or delivering the compound to an intestinal segment showing little or no enzymatic degradation.

KEY WORDS: absorption; peptides; metkephamid; bioavailability; degradation; permeability.

INTRODUCTION

The present study addresses the mechanism of intestinal peptide transport and metabolism by determining the rate-limiting steps and their implications for systemic peptide bioavailability. For these investigations, a pentapeptide was chosen as a model compound. Metkephamid (Tyr-D-Ala-Gly-Phe-*N*-Me-Met-NH_2), a synthetically modified derivative of [Met]enkephalin (Tyr-Gly-Gly-Phe-Met), has been tested previously for its bioavailability after oral dosing in rats (1). The serum concentrations of the peptide were below the detection limit of the analytical method, indicating negligible oral availability of the unchanged compound. From a bioavailability point of view, therefore, metkephamid is typical for the problems encountered with other peptides administered by the oral route (2). However, this does not mean that the rate-limiting steps in the overall absorption/metabolism process are identical for all peptides. In most cases these have not yet been identified, making it difficult to overcome the problem of insufficient bioavailability. Similarly, in the case of metkephamid, the potential barriers to the oral availability of this compound were not identified. From pharmacokinetic studies a rapid clearance of the peptide from blood was demonstrated by monitoring serum levels after i.v. dosing, with a terminal elimination half-life of 19 min. To contribute to the assessment of intestinal absorption and metabolism, transport and degradation of metkephamid were studied in the presence of brush border membrane vesicles from enterocytes (3). Intact peptide was transported across the intestinal brush border membrane, suggesting intestinal uptake of the peptide. In addition, considerable metabolism of metkephamid by the abundant brush border aminopeptidases was observed. Metabolic inactivation of the compound occurs by cleavage of the peptide bond of the N-terminal amino acid, leading to the formation of tyrosine and the tetrapeptide D-Ala-Gly-*N*-Me-Met-NH_2 as metabolites (3). Degradation by cytosolic enzymes was negligible (3). Based on the metabolism and transport data, a model for simultaneous transport and metabolism was developed showing that a major fraction of the peptide is metabolized before absorption can occur.

In the present study possible site specificity of intestinal degradation along the longitudinal axis of the intestine was investigated; further, the degradation by luminal gastrointestinal enzymes, binding of the peptide to mucus and the intestinal wall permeability of the peptide were studied. Moreover, the effect of optimized peptide delivery with respect to the site of absorption and enzyme inhibition on the bioavailability of metkephamid were demonstrated.

MATERIALS AND METHODS

Metkephamid, Tyr-D-Ala-Gly-Phe-*N*-Me-Met-$NH_2 \cdot CH_3COOH$ (MW 660.8) was kindly supplied by Ely Lilly (Indianapolis, IN). Puromycin HCl, *o*-phthaldialdehyde, 2-mercaptoethanol, polyethylene glycol 4000, urethane (ethylcarbamate), mucin type III, *N*-acetyltrialanyl methyl ester, and EDTA disodium salt were obtained from Sigma Chemical (St. Louis, MO), 1,2-^{14}C-polyethylene glycol 4000 from New England Nuclear (Boston, MA), liquid scintillation cocktail EcoLite from ICN Biomedicals (Irvine, CA), pepsin, trypsin, α-chymotrypsin, carboxypeptidase A, elastase *N*-benzoyl-L-tyrosine ethyl ester from Fluka (Buchs, Switzerland), and L-leucine-4-nitroanilide, *o*-phthaldialdehyde, *N*-benzoyl-L-arginine-4-nitroanilide hydrochloride, hippuryl-L-phenylalanine, and hemoglobin from Merck (Zürich, Switzerland). All buffer and mobile-phase components were analytical grade and used as received.

Assay Method

HPLC

Metkephamid and tyrosine were assayed by HPLC on a Waters system (Milford, MA) equipped with two pumps (Model 510), a WISP automatic sampler (Model 712), and a

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scanning fluorescence detector (Model 470). UV absorbance was measured by a spectroflow UV detector (Model 773), Kratos (Ramsey, NJ). Data acquisition and integration were provided by a Waters baseline 810 software package (Dynamic Solutions, Ventura, CA).

Assay from Perfusates

Metkephamid and tyrosine samples from enzyme incubation and perfusion solutions were acidified, frozen immediately, and stored at −20°C until analysis. Separation was on a reversed-phase column (LiChrospher 100 RP 8; 5 μm, 4 × 250 mm), with a RP-8 guard column (LiChroCart 4-4; Merck, Schlieren, Switzerland). The mobile phase consisted of 0.01 *M* sodium heptanesulfonate in 50 m*M* phosphate buffer adjusted to pH 4.0 by the addition of phosphoric acid and acetonitrile (68:32, v/v). The flow rate was 1.5 mL/min; the detection wavelength, 210 nm; and the injection volume, 40–80 μL. Metkephamid and tyrosine eluted at 3.5 and 2.2 min, respectively.

Metkephamid Assay from Whole Blood

Blood samples from treated rats were transferred to Eppendorf tubes to which 50 μL heparin (1000 U/mL), 0.05 mmol EDTA, and 32 μmol sodium fluoride had been added and vortexed. The samples were kept on ice during the study. No degradation of metkephamid was observed when blood samples were treated according to this procedure.

Sample Preparation

To 400 μL of each blood sample was added 100 μL of sodium fluoride–EDTA solution containing 32 m*M* NaF, 0.5 m*M* EDTA, and 27 μ*M* 1-phenyl-2-aminopropane sulfate as internal standard. Further, 750 mg of sodium chloride and 300 μL ammonium acetate buffer, pH 9, were added. The mixture was vortexed and extracted four times with 400 μL ethyl acetate. Following centrifugation the organic phases were combined and evaporated under nitrogen at 25°C to dryness. The dried extracts were stored at −20°C and analyzed within 48 hr by reconstituting the residue in 100 μL methanol/water (1:1), adding 100 μL *o*-phthaldialdehyde solution (2.26 mg *o*-phthaldialdehyde/mL solvent; methanol/borate buffer, pH 9.0/mercaptoethanol, 0.45:4.45:0.01, v/v), and reacting for 5 min. After appropriate mixing a 160-μL sample was injected onto the column. The fluorescence of the sample was determined at a maximum excitation wavelength of 330 nm and a maximum emission of 443 nm. The mobile phase consisted of phosphate buffer, pH 6.9 (A), and methanol (B). A stepwise gradient was applied with 37% A and 63% B from 0 to 7.5 min, followed by 30% A and 70% B from 7.6 to 14.5 min. The flow rate was set at 1.5 mL/min. Retention times for metkephamid and the internal standard were 9.5 and 14.1 min. For all assays CVs within days and between days were <5 and <10%, respectively. The minimum detectable concentration of metkephamid in blood was 10 ng/mL. Unknown concentrations of metkephamid were quantified with a standard curve.

Radioactivity

1,2-^{14}C-Polyethylene glycol 4000, a negligibly absorbable and nonmetabolized marker, was used to quantify intestinal water absorption or secretion. A 0.5-mL perfusate sample was mixed with 10 mL of scintillation cocktail and counted using a Beckman LS 9000 counter (Beckman Instruments, Fullerton, CA).

Perfusion Solutions

Perfusion solutions for the estimation of intestinal wall permeabilities contained 29 m*M* sodium acetate, 121.3 m*M* acetic acid, 5 m*M* potassium chloride, 80 m*M* sodium chloride, 2 m*M* puromycin HCl, and 0.01% polyethylene glycol (PEG 4000) traced with labeled PEG 4000. The pH was adjusted to 4.0 to ensure maximum suppression of degrading enzyme activity. For the metabolism studies at physiological pH a buffer composed of 140 m*M* sodium chloride, 10 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES), 5 m*M* potassium chloride, and 0.01% PEG 4000 traced with labeled PEG 4000 and adjusted to pH 6.5 with NaOH was used. Isoosmolarity (290 ± 5 mOsm/kg) was confirmed by a Wescor 5500 vapor pressure osmometer (Logan, UT).

Perfusion Studies

The perfusion studies were performed using the single-pass perfusion technique (4). Male Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA), weighing 300–350 g, were fasted for 15–20 hr prior to the experiment. Anesthesia was induced by an i.m. injection of 1.5 g/kg body weight of urethane. The rats were put on a heating pad and under a heating lamp to maintain body temperature. Segments 4 to 8 cm long of the duodenum, proximal and middle jejunum (approx. 5 and 30 cm distal to the ligament of Treitz), ileum, or colon were cannulated with Tygon tubing following a midline longitudinal incision. For duodenal perfusion studies the bile duct was ligated to prevent bile from entering the intestine. Each segment was straightened as much as possible and placed outside the abdominal incision. Extensive fluid loss of the animal was prevented by covering the surgical area with pads soaked in physiological saline and parafilm. The segments were precleaned by passing 10 mL of plain perfusate buffer through the segment. Then the inlet cannula was attached to a syringe (Becton-Dickinson, Rutherford, NJ), which was placed in a Harvard infusion pump (Model 931; Harvard Apparatus Company, South Natrick, MA). The inlet tubing was thermostated to 37°C by a water bath so that the perfusate entered the intestinal segment at body temperature. Steady-state water and solute transport was reached within 30 min after the initiation of the perfusion. Then the eluate from the intestine was collected in six fractions over a 60-min period.

For studying metkephamid metabolism during perfusion, each segment was sequentially perfused with 10 m*M* MES buffer, pH 6.5, for 60 min and thereafter for 60 min with 150 m*M* acetate buffer, pH 4.0, containing 2 m*M* puromycin HCl. Metkephamid was kept constant at a 2 m*M* concentration. Under these perfusion conditions no metabolism was observed, i.e., no metabolite could be detected in the perfusate. Metabolic stability was also confirmed by experiments with brush border membrane vesicles (BBMVs). Therefore the loss in the perfusion experiments is due entirely to absorption and not to intestinal metabolism. Flow

rates for all studies were 0.191 mL/min. Upon completion of the experiment the lengths of the intestine were measured with a thread. Inlet and outlet concentrations of metkephamid and outlet concentrations of tyrosine were determined by HPLC.

Water Transport Measurements in Perfusion Experiments

1,2-^{14}C-Polyethylene glycol 4000 (sp act, 0.61 mCi/g) was used as nonabsorbable marker and added to the perfusion solution such that the solution specific activity was 0.33 μCi/100 mL (conc. PEG 0.01%). The water flux was calculated as follows:

$$\frac{\text{dpm inlet} - \text{dpm outlet}}{\text{dpm outlet}} \times \frac{100}{\text{length of segment}}$$

based on the assumption that PEG 4000 was not absorbed from the intestine. Water transport below 1%/cm of intestinal length was considered to be normal and perfusion experiments with higher water transport were excluded from the calculation of wall permeabilities and metabolism. Outlet concentrations of metkephamid and its metabolite tyrosine were corrected for net water absorption or secretion.

Brush Border Metabolism Studies

Brush border membrane vesicles (BBMVs) were prepared from rat small intestine by a Mg^{2+} precipitation technique (5). The orientation and shape of the vesicles were confirmed by electron microscopy. Enrichment of the brush border membrane fraction, determined by assay of aminopeptidase N, was 14.1 ± 0.7-fold compared to the homogenate. D-Glucose uptake experiments revealed a functional integrity of the membrane. To investigate the effect of pH on intestinal metabolism, metkephamid was incubated with BBMVs at 37°C in acetate and phosphate buffers at pH's ranging from 3.5 to 7.9. The enzymatic reaction was terminated by addition of 0.2% Triton X-100 in 0.2M perchloric acid. The concentration of tyrosine, the principle metabolite, was determined after 5 min incubation times.

Pharmacokinetic Studies in Rats

Male Sprague–Dawley rats (350- to 470-g body weight) were fasted for 15–20 hr prior to the experiment. Anesthesia was induced by an i.m. injection of 1.5 g/kg urethane. The rats were put on a heating pad and under a heating lamp to maintain body temperature. Rats received either 14 mg metkephamid/kg dissolved in 400 μL saline solution i.v. through the femoral vein or 100 mg/kg dissolved in isoosmolar saline solution or acetate buffer, pH 4, with and without enzyme inhibitor either intragastrally by gavage or into an ileal loop (6). The 15-cm loop was prepared by ligating 20 and 5 cm proximal to the ileocecal junction after fecal matter had been removed. For the intestinal absorption studies 2 mL acetate buffer, pH 4.0, containing 2 m*M* puromycin HCl and 100 mg/kg metkephamid in saline solution was injected quantitatively via the proximal port. As a control experiment the same dose of metkephamid was administered intragastrally. For the i.v. studies the intestinal loop was filled with 2 mL of 2 m*M* puromycin HCl in acetate buffer, pH 4. After the termination of the pharmacokinetic experiment, the intestinal loop was flushed with 20 mL of saline solution and the residual content of peptide was analyzed. Seven or eight blood samples (300–400 μL each) were withdrawn from a jugular vein cannula at timed intervals.

Data Analysis

Estimation of Intestinal Metabolism

The fraction of the initial concentration metabolized per centimeter of perfused intestine was calculated from

$$f_{met} = (C_m - C_m')/C_o \times 1/l$$

where C_m and C_m' represent the outlet concentration of peptide corrected for water transport from perfusions with (C_m) and without (C_m') enzyme inhibition, respectively. C_o is the inlet peptide concentration, and l is the length of the perfused intestinal segment.

Estimation of Intestinal Wall Permeability

The unbiased intrinsic wall permeability P_w* was estimated by a modified boundary layer approach (7). Briefly, the effective resistance P_{eff} to solute transport through the intestinal wall can be written as a sum of individual resistances:

$$1/P_{eff} = 1/P_{aq} + 1/P_w$$

where P_{aq} is the permeability of the aqueous boundary layer and P_w is the intestinal wall permeability. The effective dimensionless permeability P_{eff}* obtained by normalizing permeabilities for the ratio of intestinal radius to solute aqueous diffusivity can be calculated from single pass-perfusions by

$$P_{eff}^* = (1 - C_m/C_o)/4\ Gz$$

where C_o is the inlet solute concentration and C_m represents the outlet concentration and Gz is the Graetz number, the ratio of the mean residence time of a fluid element in the intestine ($t_{res} = \pi R^2 L/Q$) to the mean radial diffusion time ($t_{diff} = 2R^2/D$) of a solute, where R is the inner radius of the intestine, L is the intestinal length, Q is the perfusion flow rate, and D is the aqueous diffusivity of the solute.

$$Gz = \frac{\pi D L}{2\,Q}$$

P_{aq}*, the dimensionless aqueous permeability, is estimated according to the following equation:

$$P_{aq}^* = 1/(A \cdot Gz^{1/3})$$

and

$$\begin{aligned} A &= 10.00\ Gz + 1.01, & 0.004 \leq Gz \leq 0.01 \\ A &= 4.50\ Gz + 1.065, & 0.01 \leq Gz \leq 0.03 \\ A &= 2.25\ Gz + 1.125, & 0.03 \leq Gz \end{aligned}$$

The wall concentration and dimensionless wall permeability are calculated from

$$C_w = C_o(1 - P_{eff}^*/P_{aq}^*)$$
$$P_w^* = P_{eff}^*/[1 - P_{eff}^*/P_{aq}^*]$$

Pharmacokinetic Analysis

Pharmacokinetic parameters were calculated following compartmental data analysis (8). Initial parameter estimates obtained by feathering were refined by nonlinear regression analysis using NONLIN (9). Areas under the blood concentration–time curves (AUC) were calculated according to the linear trapezoidal rule. For the systemic route, total blood clearance (CL_{tot}) was calculated by dividing the dose by AUC. The absolute bioavailability F of metkephamid after oral administration was calculated according to

$$F = \frac{AUC_{po}}{AUC_{iv}} * \frac{D_{iv}}{D_{po}}$$

The fraction of the dose cleared by the first-pass effect f_{fp} was estimated from studies with intestinal loops when enzymatic peptide degradation in the intestine was completely inhibited:

$$f_{fp} = 1 - \frac{AUC_{po}}{AUC_{iv}} * \frac{D_{iv}}{D_{abs}}$$

where D_{abs} is the absorbed dose and is calculated from the difference between the applied dose and the amount of compound recovered at the termination of the experiment.

RESULTS

Metkephamid was metabolized in the gut wall because of contact with membrane-bound enzymes in the brush border of the enterocytes. The enzymatic degradation was strongly pH dependent (Fig. 1). Maximum enzyme activity was found at pH 7–8, which was significantly reduced by lowering the pH in the incubation medium (Fig. 1). In the absorption studies by the single-pass perfusion method the strongly diminished enzymatic degradation of metkephamid at low pH was utilized to ensure peptide stability during the perfusion. No degradation of metkephamid was detected in the presence of the luminal gastrointestinal enzymes pepsin, trypsin, α-chymotrypsin, carboxypeptidase A, and elastase during the 60- to 70-min incubation period (data not shown).

Interestingly, the extent of metabolic inactivation of metkephamid was not constant throughout the entire length of the intestine. It was dependent on the intestinal segment investigated and decreased in the axial direction (Fig. 2A). In the duodenum the metabolic loss of metkephamid averaged 14.9 ± 3.05%/cm intestine perfused (SE; n = 4). The metabolism then gradually decreased from 8.14 ± 2.32%/cm (n = 5) in the proximal jejunum 2–4 cm below the ligament of Treitz, to 3.08 ± 0.51%/cm (n = 4) in the middle jejunum approx. 25 cm below the ligament of Treitz to 1.72 ± 0.31%/

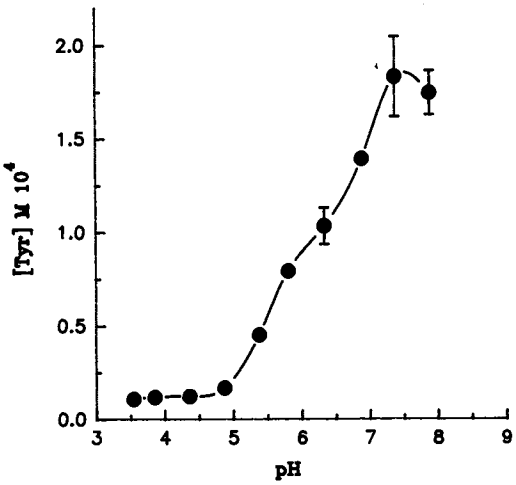

Fig. 1. Effect of pH on the metabolism of metkephamid in the presence of brush border membrane vesicles. The concentration of Tyr, the principal metabolite, was determined after 5 min of incubation. Means ± SE; n = 3.

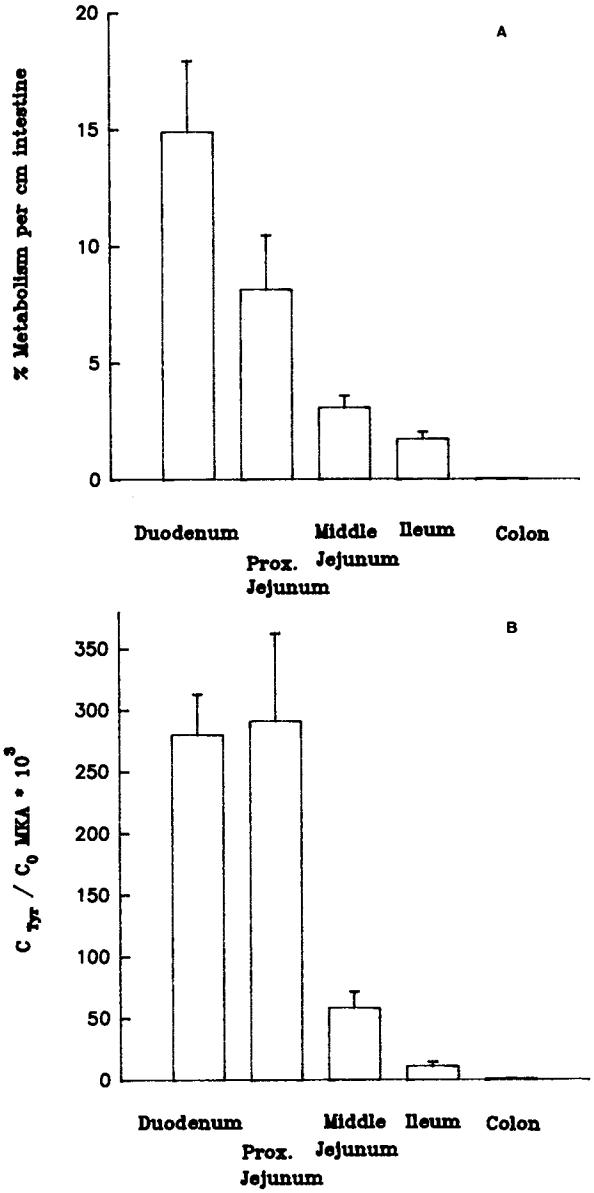

Fig. 2. Metabolism of metkephamid in different intestinal segments of the rat (means ± SE; n = 3–5). (A) Metabolism calculated from loss of parent compound from the perfusate corrected for fraction absorbed and normalized for length of the perfused segment. (B) Metabolism calculated from appearance of Tyr in the perfusate. C_{Tyr} is the concentration of Tyr in the perfusate, C_0MKA is the initial concentration of intact peptide in the perfusion solution.

cm (n = 4) in the ileum. Under the experimental conditions employed, no metabolism was found in the colon. Differences between metabolism rates for the segments investigated were highly significant (P = 0.0006, ANOVA). Statistical analysis of the data by the multiple-comparison procedure (LSD) resulted in the following conclusions: Metabolism in the ileum and colon was significantly lower than in the proximal jejunum and duodenum when comparing the latter two sections. Degradation in the duodenum was significantly higher than in the proximal jejunum ($P < 0.05$). A similar ranking of the metabolic activity of the various intestinal segments was obtained when gut wall metabolism was quantified on the basis of metabolite concentrations in the perfusate (Fig. 2B). Nevertheless, these data should be interpreted with care since metabolite concentrations have not been corrected for absorption. Valid conclusions from these data are to be drawn under the assumption of similar metabolite absorption velocities in the different intestinal segments investigated.

The dimensionless wall permeabilities P_w* of metkephamid, i.e., the effective permeabilities corrected for the permeability of the aqueous boundary layer, were determined by *in situ* perfusion of intestinal segments 6–8 cm long during complete intestinal enzyme inhibition with perfusion rates of 0.19 mL/min. The aqueous diffusion coefficient of metkephamid, calculated using the Hayduk–Laudie correlation (10), was 3.01^{-4} cm^2/min. The intestinal wall permeabilities, expressed as the dimensionless wall permeability P_w* were site dependent (Fig. 3). In the ileum P_w* was highest (1.91 ± 0.24; n = 4), permeability in the middle jejunum was slightly lower (1.64 ± 0.34; n = 4), and the least permeable segment was the colon (0.67 ± 0.38; n = 4). In the middle jejunum and ileum P_w* were not significantly different from each other. However, permeabilities of the peptide in the colon were significantly lower than in the small intestine ($P < 0.05$; LSD).

Pharmacokinetic studies were conducted in four rats at a dose of 14 mg/kg for i.v. administration. A two-compartment body model was fitted to the blood concentration time profiles (Fig. 4). A very rapid distribution half-life of 0.51 ± 0.48 min (n = 4) and an elimination half-life of 6.37 ± 0.76 min (n = 4) were calculated from the parameters of the model equations. Various kinetic parameters following the i.v. dosing are shown in Table I. Metkephamid was rapidly cleared from the blood (73.2 ± 14.3 mL min^{-1} kg^{-1} (n = 4); the volume of distribution at steady-state averaged 417.5 ± 109.3 mL kg^{-1} (n = 4). Following oral administration of the peptide dissolved in saline, only very low concentrations were detected (Fig. 5). The mean absolute bioavailability by this administration route was 0.22 ± 0.065% (n = 3). Bioavailability was increased to 1.79 ± 0.55% (n = 4) when the peptide was administered into an ileal loop in a solution buffered to pH 4 (Fig. 6). An additional increase in the fraction absorbed was observed when the drug was given into an ileal loop and the aminopeptidase inhibition was intensified by the addition of 2 mM puromycin (Fig. 7). An

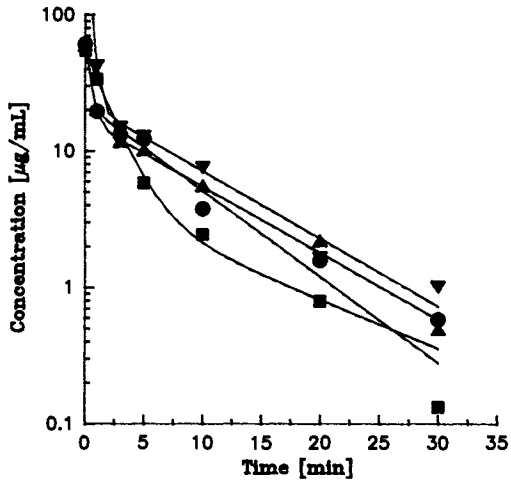

Fig. 4. Semilogarithmic blood concentrations versus time plots of metkephamid following i.v. administration of 14 mg/kg in the femoral vein. The lines are the theoretical individual profiles following curve fitting in accordance with a two-compartment body model.

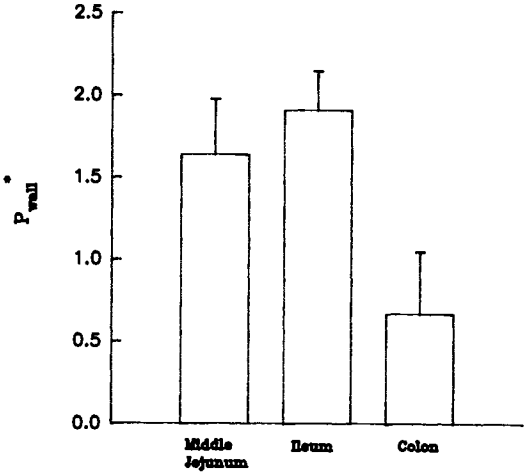

Fig. 3. Dimensionless wall permeabilities P_w* of metkephamid in different intestinal segments (means ± SE; n = 4). No accurate value of P_w* could be obtained for the proximal jejunum, since the metabolism could not be completely inhibited.

Table I. Summary of Pharmacokinetic Parameters After i.v. Administration of Metkephamid (14 mg/kg)[a]

Parameter	Mean ± SE (n = 4)
A (µg/mL)	135.2 ± 91.5
B (µg/mL)	16.3 ± 4.2
α (min^{-1})	2.41 ± 0.71
β (min^{-1})	0.113 ± 0.013
$t_{1/2\beta}$ (min)	6.37 ± 0.76
$AUC_{0\to\infty}$ (mg mL^{-1} min)	233.7 ± 70.2
MRT (min)	5.51 ± 0.83
CL_{tot} (mL min^{-1} kg^{-1})	73.22 ± 14.32
V_c (mL/kg)	189.4 ± 52.6
V_{ss} (mL/kg)	417.5 ± 109.3

[a] A, B—hybrid coefficients; α, β—hybrid disposition rate constants; $t_{1/2\beta}$—terminal half-life; $AUC_{0\to\infty}$—total area under the concentration vs time curve (from data); MRT—systemic mean residence time; CL_{tot}—total (blood) clearance; V_c—volume of distribution of the central compartment; V_{ss}—steady-state distribution volume.

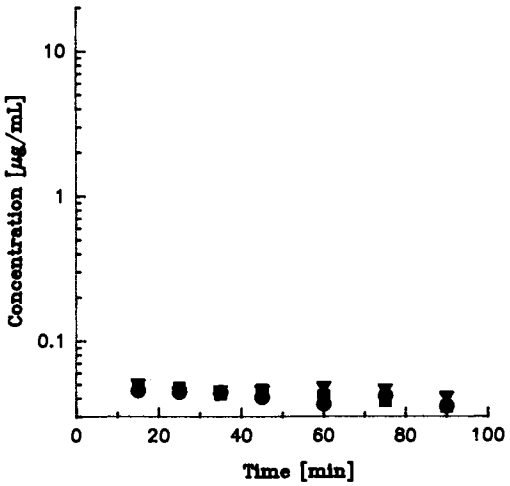

Fig. 5. Blood concentrations of metkephamid following a p.o. dose of 100 mg/kg dissolved in saline by gavage.

absolute bioavailability of 4.5% ± 1.66 (n = 4) was then obtained.

For the pharmacokinetic studies with ileal loops the pharmacokinetic model that described blood concentration with time best was a one-compartment body model with first-order absorption and elimination pathways. Characteristic coefficients of the model equation and the derived parameters are given in Table II. From determination of the residual peptide content in the ileal loop (experiment with buffer pH 4 and aminopeptidase inhibitor puromycin) and the administered dose, the absorbed amounts of peptide were calculated. Using this information and applying Dost's principle of corresponding areas, comparison of the area under the curves following p.o. and i.v. dosing allows an estimate of the presystemic first-pass effect of metkephamid following absorption of the compound from the intestinal segment. The calculated fraction of the peptide dose cleared during the passage from the absorption site to the systemic

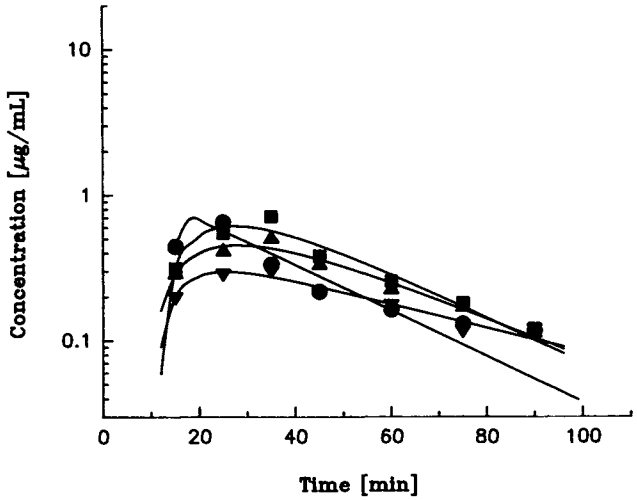

Fig. 6. Blood concentrations of metkephamid in four rats as a function of time after administration of the peptide (100 mg/kg) dissolved in acetate buffer, pH 4, into an ileal loop. Lines represent the predicted values generated by nonlinear regression.

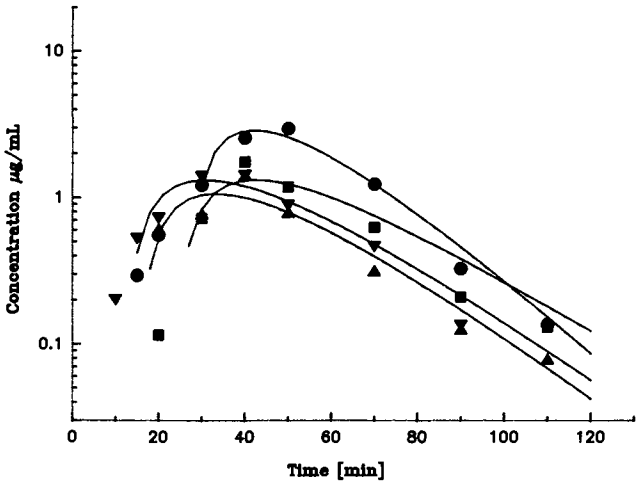

Fig. 7. Blood concentrations of metkephamid in four rats as a function of time after administration of the peptide (100 mg/kg) dissolved in acetate buffer, pH 4, +2 mM puromycin into an ileal loop. Lines represent the predicted values generated by nonlinear regression.

circulation averaged 0.93 ± 0.01 (n = 4). This value indicates that, although increased 20-fold by acidification and enzyme inhibition, still a high fraction of the dose is cleared before the peptide reaches the general circulation.

DISCUSSION

Apparently metabolism by aminopeptidase is the dominating degradation pathway of metkephamid. Similar metabolism of peptides derived from [Met]enkephalin or [Leu]enkephalin in various mucosae has been reported by several authors (11–14), who generally agreed that metabolism of enkephalins by aminopeptidase is the main pathway more important than degradation by endopeptidase 24.11 and dipeptidylaminopeptidase. While replacement of Gly^2 in [Met]enkephalin by D-Ala^2 renders the peptide more stable toward aminopeptidases, it does not completely inhibit its enzymatic cleavage. The pronounced pH dependence of the enzyme-catalyzed hydrolysis of metkephamid may have several distinct underlying causes. For example, a change in the ionization of the substrate could occur or an inactivation of the enzyme outside a certain pH range, since the affinity of the substrate for the binding site on the enzyme may depend on the degree of ionization of certain amino acid side chains (15). In the case of the enzymes responsible for intestinal protein digestion, this pH dependence has been demonstrated, e.g., for chymotrypsin, with a pH optimum of about 7–9 and little enzymatic activity below pH 5 and above pH 9.5 (16). In the case of aminopeptidase N, the enzyme responsible for the metabolism of metkephamid, maximum enzyme activity has been reported in the range of pH 7.5–8.0 using L-Ala as a substrate and at pH 7.6 using Ala-2-naphthylamide (17). Little activity was reported below pH 6.5 and above pH 10.5. The dependence of metkephamid metabolism on the segment perfused must be due to a regiospecific distribution of aminopeptidase N. The site-specific intestinal concentration difference of this enzyme was experimentally verified previously in rat small intestine (18). Total enzyme activity in the brush border membrane

Table II. Summary of Pharmacokinetic Parameters After Oral Administration of Metkephamid (100 mg/kg) (Means ± SE)[a]

Parameter	Mode of administration		
	p.o. (saline)	Ileal loop (acetate buffer, pH 4)	Ileal loop (acetate buffer, pH 4, + 2 m*M* puromycin)
V/F (L kg^{-1})	n.d.[b]	28.1 ± 7.9	23.8 ± 7.9
k_{01} (min^{-1})	n.d.[b]	0.030 ± 0.004	0.055 ± 0.006
k_{10} (min^{-1})	n.d.[b]	0.026 ± 0.014	0.063 ± 0.0045
t_{lag} (min)	n.d.[b]	11.2 ± 0.97	20.0 ± 3.5
$AUC_{0\to\infty}$ (μg mL^{-1} min)	3.59 ± 0.20	29.8 ± 1.71	76.7 ± 15.45
F (%)	0.22 ± 0.065	1.79 ± 0.55	4.6 ± 1.66

[a] V, volume of distribution; F, absolute bioavailability; k_{01}, first-order input rate constant; k_{10}, first-order elimination rate constant; t_{lag}, lag time; AUC, total area under the concentration vs time curve (from data).

[b] The observed concentrations from the p.o. study were at the limit of detection of the analytical method. Due to the scatter in the observed data, no compartmental analysis was performed.

peaked in the proximal small intestine and decreased along the length of the intestine. This is in accordance with known patterns of protein digestion in the mammalian intestine, which indicate that the bulk of protein digestion and absorption occurs in the proximal and mid jejunal regions (19). These findings on aminopeptidase distribution, however, differed from the data reported for rabbits (20) and man (21). The negligible metabolism of metkephamid in the colon indicates a low aminopeptidase activity in the large intestine, however, the hydrolytic potential in the colon based on aminopeptidase distribution has not been determined. Intestinal wall permeability determined by the single-pass perfusion technique in a rat intestinal segment can be correlated with the fraction of the dose absorbed in humans under the assumption that solubility or metabolism factors are not significant (22). From this correlation it can be predicted that drugs with a P_w* above 1.0 will be well absorbed, whereas the availability of compounds with a P_w* of 0.5 is rather limited. The calculated wall permeabilities for metkephamid in the small intestine, ranging from 1.64 (jejunum) and 1.91 (ileum), thus indicate that the peptide is rather readily transported through the intestinal membrane and that membrane permeability does not limit the absorption of the peptide in the small intestine. Higher permeabilities in the distal ileum compared to the proximal segments of the small intestine have also been reported for other peptides, such as oxytocin, antocin II, and carbetocin (23). Our results also confirm the experimental findings on small intestinal wall permeabilities of [Leu]enkephalin and [Leu]-D-Ala2-enkephalin (1.83 ± 0.9%) by Friedman and Amidon (13) and also observations on brush border transport of metkephamid which indicated rapid uptake of the peptide through the brush border membrane of enterozytes (3). Therefore the results of this study show that the molecular weight and physicochemical properties of these oligopeptides in principle are not a limiting factor in their absorption. From the permeabilities in the colon the predicted fraction absorbed based on the correlation would be reduced to 50–60% of the administered dose. The lower permeabilities in the colon may be attributed to the greatly reduced surface area due to the lack of villi in this segment and to a reduced number or radius of aqueous pores. Also, in the case of hydrophilic polyethyleneglycols with various molecular weights, decreased absorption was observed in the colon compared with segments from the small intestine (24). Comparatively poor absorption from the colon has also been shown for 1-deamino-8-D-arginine vasopressin (dDAVP) in rabbits, whereas absorption at the ileal–cecal junction was at its maximum along the GI tract (25). Nevertheless, these observations cannot be generalized since the available information today on site-specific absorption of oligopeptides is still rudimentary.

The pharmacokinetics of metkephamid in rats in this study differs from the results reported by Su *et al.* (1) mainly in the estimation of the elimination half-lives. The half-lives in this study were considerably shorter than reported by Su and co-workers, with 6.3 and 18.7 min for the α and β phase, respectively. However, their estimation of the terminal elimination rate constant was based merely on the two final data points, at 60 and 90 min following injection, leading to considerable uncertainty in the estimate. The very rapid distribution phase at the beginning has not been detected due to their sampling schedule. Therefore the half-life of the α phase in the study by Su *et al.* should represent the β half-life in the present study, and both values are in close agreement (6.3 vs 6.4 min). The rapid clearance of metkephamid from blood is not surprising and resembles the pharmacokinetic behavior of many other peptides that are rapidly eliminated from the body. In the case of metkephamid, the mechanism of clearance is unknown. However, it may be speculated that, assuming negligible renal excretion of unchanged compound, clearance is mainly metabolic in nature and due to aminopeptidase activity. The major clearing organ hence is probably the liver, with considerable contribution from other organs as well. Aminopeptidase activity has also been reported for blood (26) and for many organs such as lung, blood vessel endothelium, lymph nodes, etc. (27). Inhibition of intestinal metabolism led to a pronounced (20-fold) increase in the bioavailability of metkephamid, which supports

the results from the intestinal permeability studies and demonstrates that intestinal metabolism rather than membrane permeability is the issue in the absorption of this pentapeptide. Nevertheless, the absolute bioavailability, even though increased to 4.5%, is still limited due to presystemic elimination following absorption. It should be pointed out, however, that the experimental procedure utilized for this study does not allow us to attribute this extraction to liver metabolism or biliary excretion alone. It should rather be viewed as the sum of the clearances in the blood, in the endothelium, and in the liver. More detailed studies are necessary to investigate the significance of the liver as a clearing organ for peptide drugs.

Our findings suggest that oral administration of some oligopeptides as drugs may become feasible. The proper strategy of delivering a peptide by the oral route should be to increase the stability of the peptide toward enzymatic degradation while retaining its intrinsic activity and/or to deliver the peptide to an intestinal segment showing sufficient intestinal wall permeability and little or no enzymatic degradation. Even if the bioavailability of peptides may not exceed 5%, this may be sufficient as in the case of certain other drugs given perorally, such as the quaternary antimuscarinic spasmolytics and some ergot alkaloids, and in the same range as some intranasal peptide formulations, avoiding local toxicity.

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