Colonic Absorption and Bioavailability of the Pentapeptide Metkephamid in the Rat

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The concept of delivering systemically active peptide drugs to the colon in order to improve their oral absorption requires reasonable peptide permeability of the large intestinal wall and stability against the activity of the colonic microflora. In addition, the role of hepatic extraction needs to be addressed. In this study the absorption of the pentapeptide metkephamid following single pass perfusion of rat ascending colon was investigated by monitoring its disappearance from the large intestine and simultaneous appearance in the portal vein, the hepatic vein and the aorta. In addition its stability against colonic microflora was tested in vitro using pig caecal contents. Metkephamid was absorbed from the large intestine and appeared in the blood circulation; peptide concentrations in the portal vein increased over-proportionally with increasing perfusate concentrations (0.1 − 4.6 mmol/L) from 0.19 μg/mL ± 0.12 (SD, n = 7) to 31.6 μg/mL + 20.65 (SD, n = 4), respectively, and thus suggesting a saturable transport or metabolism. Concentrations in the hepatic vein were significantly lower than in the portal vein, hepatic extraction ratios were 0.35 ± 0.14, 0.61 ± 0.18 and 0.62 ± 0.28 (SD, n = 4) for 0.1, 0.5 and 1.0 mM metkephamid perfusate concentrations, respectively. In the anaerobic colon metabolism model the degradation half-life of the peptide was 14.9 hours, thus, indicating relative stability in the bacterial environment of the colon. The results of the present study encourage further investigations on colonic delivery of peptide drugs.

KEY WORDS: intestinal absorption; colonic delivery; peptides; intestinal microflora.

INTRODUCTION

Metabolic degradation of enzymatically labile peptides in the intestinal tract is currently considered one of the most important barriers that prevent entry of peptides into the systemic circulation. Among the approaches to circumvent this biochemical barrier, modifications of the peptide structure (1–2), coadministration of peptidase inhibitors (3) and restriction of the peptide release to an intestinal site devoid of secreted digestive enzymes (4) has been suggested. From a metabolic activity point of view, there are indications that the colonic mucosa is fairly low in the activity of endogenous digestive enzymes (5,6) that affect peptide stability in the small intestine. This has led to an increasing development of colonic delivery systems for peptide drugs. However, several, mainly biological aspects in the field of colonic peptide delivery remain unclear.

(i) The bioavailability of colonically delivered peptides, being a function of their membrane permeability, metabolic degradation and residence time in the absorbing segment is insufficiently characterized although absorption of a variety of therapeutics from the large intestine has been demonstrated, including beta-blockers, ACE-inhibitors, NSAID's, Xanthin derivatives (7).

(ii) Although there is conclusive evidence that extensive digestion of polysaccharides occurs in the colon, little is known about the digestion of proteins and peptides by colonic bacteria. Only a few species of colonic bacteria, e.g. Bacteroides ruminicola, can utilize proteins as a sole source of carbon and energy, and these species are found only in very low numbers in the colon (8). For this reason it is often assumed, that digestion of protein in the colon is much less extensive than the digestion of polysaccharides. On the other hand, all bacteria produce proteases which are involved in the turnover of cell protein and these proteases could well contribute to the breakdown of therapeutic peptides and proteins in the colon. A recent investigation by Macfarlane et al (9) points out the possibility that considerable bacterial protein fermentation in humans could potentially account for 17% of the short-chain fatty acids found in the caecum and 38% found in the sigmoid/rectum.

(iii) The scarce information available today on the role of hepatic extraction of orally administered peptide and protein drugs indicates that a contribution of the liver to the presystemic elimination by metabolism and/or biliary excretion may be quite variable depending on the structure of the compound investigated (10).

The aim of the present study is to address some of the above mentioned questions utilizing the pentapeptide metkephamid as a model compound. More specifically the investigations were designed to clarify whether

-the peptide is absorbed from the ascending colon and appears intact in the portal vein,

-metkephamid is extracted by a hepatic first-pass effect and appears in the systemic circulation following colonic administration,

-there is significant metabolic peptide degradation in the presence of viable hindgut microflora.

MATERIALS AND METHODS

Materials

Metkephamid (Tyr-D-Ala-Gly-Phe-N-Me-Met-NH_2 · CH_3COOH, mol. wt. 660.8) was kindly provided by Ely Lilly (Indianapolis, IN). O-phthaldialdehyde, 2-mercaptoethanol, urethane (ethylcarbamate) were obtained from SIGMA Chemical (St. Louis, MO). Propionic acid, phosphoric acid, potassium chloride, calcium chloride, magnesium chloride, sodium bicarbonate, monobasic sodium phosphate, sodium acetate were obtained from Fluka (Buchs, Switzerland), methanol from Romil Chemicals, Loughborough, England. Phenol red solution was purchased from E.

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Merck (Zürich, Switzerland). All buffer and mobile phase components were analytical grade and used as received.

Assay Method

Metkephamid was assayed by HPLC on a Merck-Hitachi system (Dietikon, Switzerland) equipped with a L-6200A gradient pump, a L-4250 UV-VIS detector, a F-1050 fluorescence spectrophotometer, an AS-2000 autosampler and a D-2500 chromato-integrator. The methods for quantification of the peptide from perfusate and blood have been published previously (5).

Sodium and potassium in the intestinal perfusion solutions were quantitatively determined by flame photometry (Instrumentation Laboratory Flame Photometer 243, Instrumentation Laboratory Inc., Lexington MA, USA). Chloride was quantified argentometrically using a chloride analyzer (model 925, Corning Ltd., Halstead, GB). The volume marker phenol red was analyzed at 546 nm using a photometer (model 4010, Boehringer Mannheim, Mannheim, FRG) following dilution of a 0.15 mL sample with 0.5 mL 1M sodium hydroxide solution.

Perfusion Solutions

Perfusion solutions for colonic perfusion experiments consisted of a buffer prepared according to Argenzio et. al [11]. It was composed of 20 mM potassium chloride, 2.5 mM calcium chloride, 2.5 mM magnesium chloride, 20 mM monobasic sodium carbonate, 30 mM monobasic sodium phosphate, 60 mM sodium acetate, 10 mM propionic acid and 56.4 mM phenol red and metkephamid (0.1 mM–4.6 mM). The pH of the buffer was adjusted to 5.5 with phosphoric acid. 15 minutes prior to perfusion, the buffer was oxygenated with O_2. Perfusion solutions were isoosmotic.

Perfusion Studies

Perfusion studies were performed using the single-pass perfusion technique. Male rats, strain SIVZ-50 (Institut für Zuchthygiene, Veterinärmedizinische Fakultät der Universität Zürich), weighing 280–320 g, were fasted 15–20 h prior to the experiment. Anaesthesia was induced by an i.m. injection of 1.5 g/kg body weight of urethane. The rats were put on a heating pad to maintain body temperature. The peritoneum was opened by a midline incision. Segments of the ascending colon, about 4–6 cm in length, starting approximately 1 cm caudal of the ileocaecal valve, were cannulated with a silicon tube (OD: 4mm, ID: 2mm) following a midline longitudinal incision. Blood supply to the perfused segments was maintained during the study. The segments were thoroughly cleaned of faecal matter by passing an appropriate volume of plain perfusate buffer through the segment. Perfusion of the colon was from cranial to caudal. The inlet cannula was attached to a 50 mL syringe (Becton-Dickinson, Basel, Switzerland) which was placed on a perfusion pump (Perfusor^R VI, Braun Melsungen AG, Neuhausen, Switzerland). Perfusion solution was delivered continuously at a rate of 0.2 mL/min for 90–120 minutes through the segment. The inlet tubing was thermostated at 39°C by a water bath so that the perfusate entered the intestinal segment at body temperature. After the surgery the intestinal segment was placed back into the abdominal cavity which was then closed with wound clips to prevent water loss from the animals' body. Care was taken to avoid any kinks. Initially the eluate from the colonic segments was collected in 15 minute intervals to determine the time necessary to reach a steady state flow in terms of peptide, water and electrolyte absorption. In later studies, the eluates from t = 0 to t = 30 min were discarded and from t = 30 min to t = 90 min were combined and assayed and compared to peptide and electrolyte concentration in perfusion buffers prior to absorption. After 90 min of perfusion, 1.0 mL blood samples were taken from the portal vein and the aorta abdominalis of the animal for all perfusate concentrations of metkephamid, whereas samples from the hepatic vein were available only at 0.1, 0.5 and 1.0 mM perfusate concentrations. Samples were immediately stored on ice and processed as described previously (5). Thereafter the colonic segment perfused was removed from the animal, its length was measured and the segment was dried at 50°C for 24 hours to determine the tissue dry weight.

Peptide Stability in the Presence of Hindgut Anaerobic Microflora

Metkephamid stability in the presence of anaerobic and metabolically functional microflora was evaluated at 1 mM and 0.1 mM peptide concentrations in the incubation fluid using the Positec (Colon Simulation Technique) apparatus described by Breves et al. (12, 13). Briefly, hindgut contents were obtained from caecally fistulated pigs and gauze filtrated caecal fluid was used to start the system. Nylon bags containing freeze-dried particles from the caecum were introduced into the incubation vessels as a pre-digested substrate to be fermented. In each experiment equilibration for 8 days was followed by 6–8 sampling days, with volatile fatty acids (VFA) and gas production, microbial protein synthesis and digestibilities of organic matter and fibrous carbohydrates being measured.

Data Analysis

Water absorption (mL/100 mg dry weight · h) was calculated from the difference in concentrations of the volume marker phenol red in the perfusion solutions before and after single-pass perfusion, and was then calculated as:

$$\textit{Water absorption } W = \frac{(1 - F) \times PR \times 100 \times SI}{DW}$$

where F is the ratio of the absorbance at 546 nm of the perfusate solution before and after perfusion of the isolated gut segment, PR is the perfusion rate (mL/min), SI is the sampling interval (min) and DW is the dry tissue weight of the perfused segment (mg). The solute absorption (μmol/100 mg dry weight · h) from the analysis of perfusate samples was corrected for net water absorption/secretion and was calculated according to

$$\textit{Solute absorption } S = \frac{(C_0 - (C_m xF)) \times PR \times 100 \times SI}{DW}$$

where C_m and C_0 represent the outlet and inlet concentrations (μmol/L) of the solute respectively. Positive values for

W and S indicate apparent absorption and negative values secretion into the colonic segment.

Hepatic organ clearance was calculated according to a physiological flow model for drug clearance and expressed as extraction ratio ER:

$$ER = \frac{Q(C_{PV} - C_{VH})}{QC_{PV}} = \frac{C_{PV} - C_{VH}}{C_{PV}}$$

where the term Q denotes blood flow rate through the organ (mL/min) and the terms C_{PV} And C_{VH} denote drug concentrations in the portal vein and the hepatic vein, respectively (μg/mL).

RESULTS

The time course of absorption of water, sodium and chloride from the proximal colon is depicted in Fig. 1. Absorption of chloride was observed during the entire course of the experiment whereas sodium and water transport changed from initial secretion in the first 15 minute interval to absorption thereafter. From these data the functional integrity of the perfused segment during the perfusion experiment may be derived. Significant metkephamid absorption from the perfused rat colon could also be demonstrated (Fig. 1). Metkephamid absorption was at steady-state levels already during the first 15 minute interval.

In order to further investigate the transport mechanism through the colonic epithelium, absorption was quantified at different peptide concentrations in the perfusate. The effect of perfusate concentration on peptide colonic absorption by comparison of perfusate concentrations prior to and after perfusion is shown in Fig. 2. In the investigated concentration range from 0.1 mM to 4.6 mM metkephamid, a linear relationship was found between peptide absorbed and perfusate concentration. This observation is indicative of a diffusive non-saturable mechanism of peptide absorption.

All absorbed material from the colon appears in the portal vein and passes through the liver before entering the systemic circulation. Solute absorption from the colon may therefore be demonstrated by monitoring the concentration of the intact molecule in the vessel. Steady state concentrations of metkephamid in the portal vein following perfusion of the ascending colon as a function of peptide concentration in the perfusion solution is depicted in Fig. 3. Interestingly, peptide concentrations in the vessel increased overproportionally with increasing perfusate concentrations (0.1–4.6 mmol/L) from 0.19 μg/mL ± 0.12 (SD, n = 7) to 31.6 μg/mL ± 20.65 (SD, n = 4), respectively suggesting a saturable transport or metabolism step which was not detectable by analysis of the absorption rates as a function of the luminal MKA concentrations (see Fig. 2).

Concentrations of metkephamid in the hepatic vein were significantly lower than in the portal vein indicating a hepatic extraction of the peptide by metabolism and/or bili-

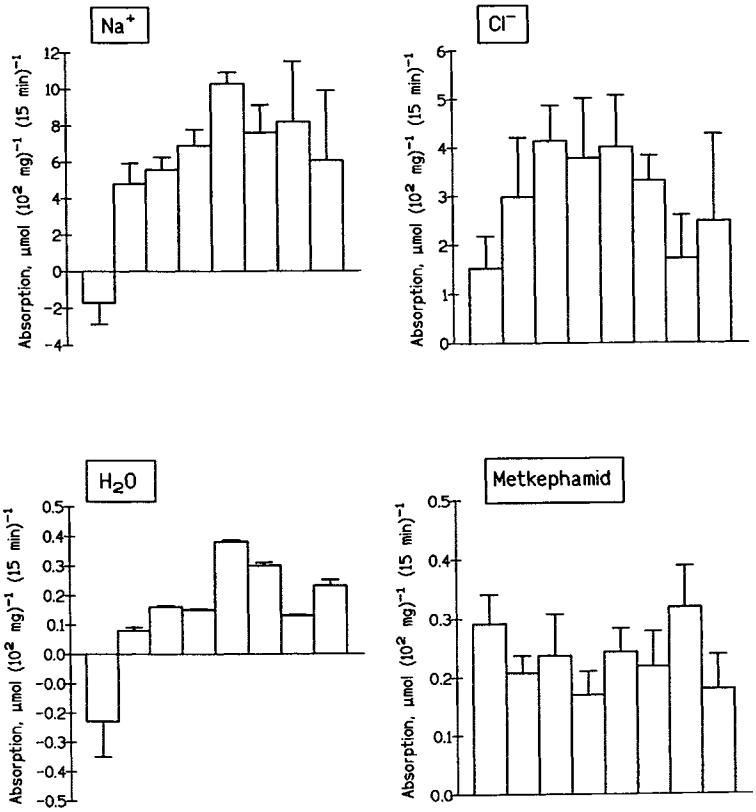

Figure 1: Time course of electrolyte, water and metkephamid absorption by the perfused proximal colon. Values are means ± SEM for 4 animals. The concentration of the peptide in the perfusion solution was 4.5 mM; Each bar represents a time period of 15 minutes.

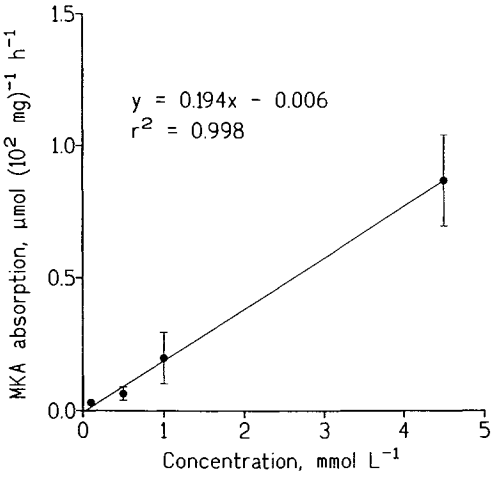

Figure 2: Absorption of the pentapeptide metkephamid from the proximal colon as a function of peptide concentration in the perfusate solution. Values are means ± SEM for 4–7 animals for each concentration. Absorption was calculated from the concentration of the peptide in the perfusion solution before and after perfusion and is corrected for water absorption.

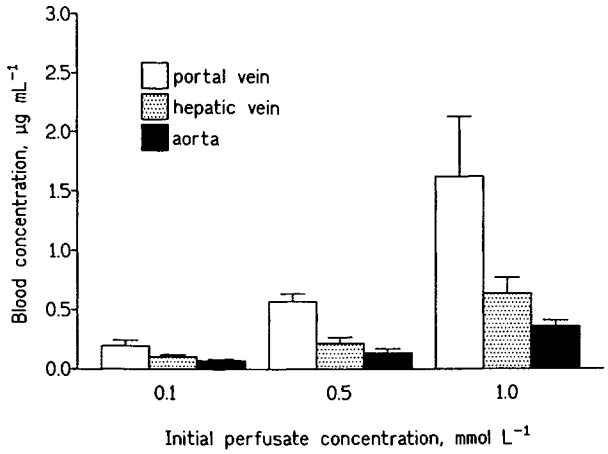

Figure 4: Steady state concentrations of metkephamid in the portal vein, the hepatic vein and the aorta abdominalis following colonic perfusion of metkephamid solutions at 0.1, 0.5 and 1 mM concentrations (means ± SEM, n = 5–7).

ary elimination for 0.1, 0.5 and 1 mM metkephamid perfusate concentrations (Fig. 4). The extraction ratios were 0.35 ± 0.14 (SD, n = 4), 0.61 ± 0.18 (SD, n = 5) and 0.62 ± 0.28 (SD, n = 4) for 0.1, 0.5 and 1.0 mM perfusate concentrations, respectively. In the investigated concentration range there was no indication of a saturability of the hepatic extraction indicating a marked capacity of this elimination pathway. Markable concentrations of the peptide were also found in the systemic circulation. The significantly lower MKA concentrations in the aorta abdominalis compared to the hepatic vein, may be attributed to the distribution of the compound in its systemic distribution volume and also probably reflects organ specific metabolism. Similarly to hepatic vein concentrations, levels of metkephamid in the aorta rose according to a parabolic rather than a linear function with peptide concentration in the colonic perfusate (Fig. 5). Steady state levels were 0.068 µg/mL ± 0.011 (SD, n = 5), 0.134 µg/mL ± 0.036 (SD, n = 5), 0.358 µg/mL ± 0.050 (SD, n = 5), 4.31 µg/mL ± 0.672 (SD, n = 4) for 0.1, 0.5, 1.0 and 4.6 mM metkephamid perfusate concentrations, respectively.

The metabolism of metkephamid by anaerobic colonic bacteria was investigated using the anaerobic colon metabolism model. The incubation system has been validated as a suitable in vitro method for measuring qualitative and quantitative parameters of microbial hindgut metabolism (12, 13). Metabolism of metkephamid by anaerobic colonic microflora was negligible and appears not to be rate limiting in the overall bioavailability of the peptide. As shown in Fig. 6 peptide concentrations decreased in the colon metabolism vessel mainly due to fluid turnover which was necessary to remove end products of hindgut metabolism. The rate con-

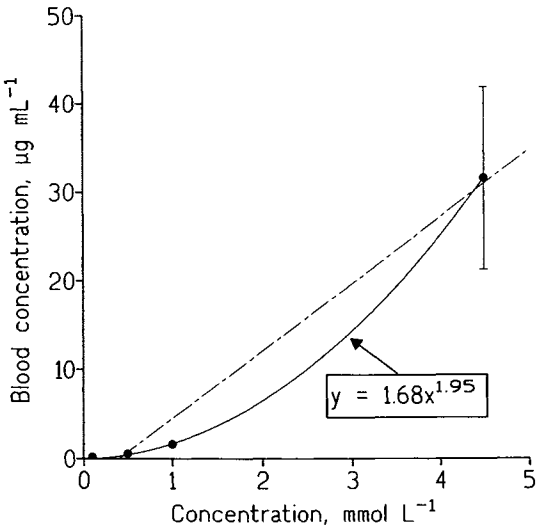

Figure 3: Steady-state concentrations of metkephamid in the portal vein following perfusion of the ascending colon as a function of peptide concentration in the perfusion solution (means ± SEM, n = 4–7 for each concentration). The nonlinear correlation may be due to a saturable metabolism of the peptide in the vascular endothelium.

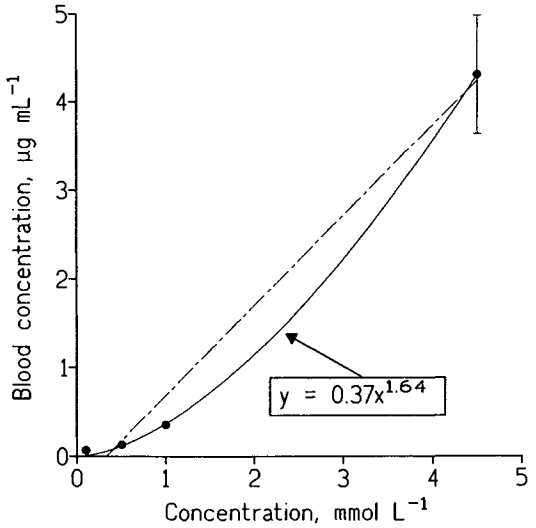

Figure 5: Steady-state concentrations of metkephamid in the aorta following perfusion of the ascending colon as a function of peptide concentration in the perfusion solution (means ± SEM, n = 5–7).

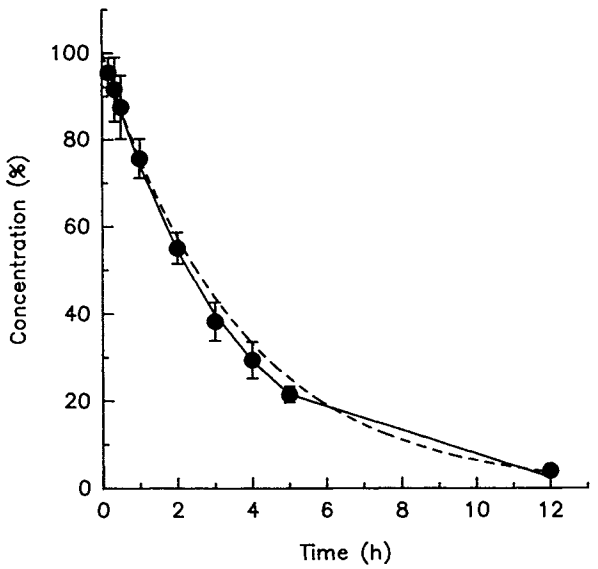

Figure 6: Time course of metkephamid concentration during incubation with colonic microflora in the anaerobic colon metabolism model. Values are means ± SEM, n = 4. The dashed line represents the natural disappearance of metkephamid due to fluid turnover in the vessel.

stant of fluid turnover in each vessel was 0.275 h^{-1}. Rate constants of metkephamid disappearance were investigated at 0.1 and 1 mM concentrations and were found to be concentration independent. The overall disappearance rate-constant of metkephamid due to metabolism and fluid turnover was 0.3216 h^{-1} ± 0.029 (SD, n = 4). By subtraction of the rate constant for fluid turnover from the overall peptide disappearance rate constant, the kinetics of peptide metabolic degradation can be calculated. The degradation rate constant averaged 0.0466 h^{-1} which corresponds to a half-life of 14.9 hours. Consequently it may be concluded that the peptide is relatively stable in the bacterial environment of the colon.

DISCUSSION

The colon is a versatile organ which serves several functions, the most important being the maintenance of fluid and electrolyte balance by absorption of sodium and chloride ions as well as water, propulsion and defecation of waste material. Interest in the absorptive functions of the different segments of the gastrointestinal tract and especially of the colon for therapeutic drugs has evolved from several underlying causes, e.g. oral controlled release formulations that deliver drugs at a constant rate along the entire gut including the large intestine where dosage forms may reside for 5 to 10 hours or longer (4). Another motivation for investigating colonic absorption and metabolism is to use this organ as an entry site for orally administered systemically acting drugs sensitive to conditions in the upper gastrointestinal tract, such as the acidic gastric environment or enzymatic activities in the small intestine. Proteins and peptides are the most prominent drugs that fall into this class. The colon, although known to metabolize and, as in the case of phase II metabolites, to bioactive a variety of different drugs belonging to many different classes (14), presents a less hostile environment to proteins and peptides because proteolytic activity, as compared to that in the small intestinal tract, is significantly reduced (15, 16). Although the principal effect of fermentation in the colon is to salvage energy from dietary carbohydrates, the lumen of the large intestine represents a proteolytic environment. In the large bowel proteins and possibly peptides may be degraded by the microflora to a variety of end products including organic acids, carbon dioxide, ammonia and also potentially toxic metabolites such as amines, phenols and indols (17). The various nitrogen-containing substrates also serve for the synthesis of bacterial protein. One of the reasons, why the proteolytic activity of pancreatic enzymes is lower in the large intestine as compared to the small intestine is that these proteases themselves are extensively broken down and also adsorbed onto the epithelial surface or bound to particulate materials in the gut during transit of the chyme through the large intestine (16, 18, 19). Therefore large intestinal proteolysis, which is mainly catalyzed by serine, cysteine and metalloproteases of bacterial origin, leads to both quantitatively and qualitatively different products and degradation kinetics than in the small intestine. In the case of the enzymatically stabilized pentapeptide metkephamid, in vitro degradation in the anaerobic colon metabolism model due to bacterial metabolism indicates relatively good stability of this peptide. It remains to be investigated, whether other therapeutically interesting peptide drugs are also resistant to bacterial metabolism. Such studies are currently undertaken. Although comparatively little is known about the mechanisms that control protein and peptide breakdown in the colon, structure, solubility and the transit time through the gut are likely to be important. Also it is often difficult to generalize in vitro observations obtained with cultures of intestinal bacteria in order to predict their activities in the more complex environment of the colonic ecosystem. However, in vivo studies of large intestinal peptide stability lack feasibility and, therefore, the anaerobic colon metabolism model offers a convenient method to assess the stability in the large intestine.

Although the colon may be a preferable site for peptide delivery because of the diminished activity of proteases, this fact alone does not lead to effective bioavailability. Until recently, the scientific opinion suggested that most types of drugs are poorly absorbed from the colon. On the other hand, various reports have shown that the absorption of some therapeutics from the colonic region may be equivalent to small intestinal absorption both in terms of rate and extent (20, 21). In the case of peptide drugs, it has been known for some time that peptide (and proteins too) can cross the intestinal wall, albeit in low amounts. Also remarkable differences in permeabilities and absorption have been reported between proximal and distal intestinal segments in vitro with oxytocin and vasopressin analogs (22) and also in in vivo studies with 1-deamino-8-D-arginine vasopressin (23). Also in the case of the actively transported octapeptide octreotide, site-preferential absorption was observed with maximum absorption efficiencies in the proximal jejunum (24). To date only very few studies have been reported in which a peptide or protein has been administered intracolonically. For human calcitonin, an absolute bioavailability of 0.12% was calculated after intracolonic administration in humans (20). In the case of insulin, a hypoglycaemic response resulted from intracolonically administered insulin in rats (25).

The results from the present study allow the conclusion that a peptide drug may be absorbed from the colon in principle. Absolute bioavailability of metkephamid in the present experiments may be calculated based on the well known relationship between total peptide clearance and systemic steady state concentration which upon multiplication equals the zero-order infusion rate. The total systemic clearance of metkephamid in the rat has been determined recently after intravenous administration as 73.22 mL min^{-1} kg^{-1} (5). The elimination half-life in this study was 6.37 min. The steady state concentration of metkephamid in the aorta following 90 minutes colonic perfusion has been measured and therefore the zero order input rate constant into the systemic circulation may be calculated. Division of this input rate by the perfusion rate (amount drug per time interval) yields the fraction of the perfused dose absorbed. For the 4 concentrations, an average fraction of 0.09 which equals a bioavailability of 9% of the drug administered was calculated. If however this bioavailability can also be found upon single dose administration remains to be clarified.

For the apparent nonlinearity of the portal concentrations of metkephamid as a function of MKA concentration in the perfusion solutions two explanations may be given:

(i) A nonlinearity e.g. due to a saturable absorption step as a result of the existence of an apically polarized transport system which might share some common features with P-glycoprotein. The effect of this system has been shown by Burton et al. (26) who found that for certain peptides transport in the apical to basolateral direction is hindered, whereas flux in the basolateral to apical direction is increased. In our studies this seems to be unlikely, since involvement of a saturable transport step should also have been reflected by the apparent absorption rates of metkephamid as a function of MKA concentration in the perfusion solutions.

(ii) A saturable metabolic degradation of the peptide due to aminopeptidase activities. The major clearing organ hence is most probably the liver with considerable contribution from other organs as well. Aminopeptidase activities are known to exist in blood and many organs such as lung, blood vessel endothelium, lymph nodes and others.

In summary, our data clearly demonstrate colonic absorption of intact pentapeptide metkephamide. With respect to blood concentrations, highest values were found in the portal vein, followed by the hepatic vein and the aorta abdominalis. MKA was not substantially degraded by the colon microorganisms. Therefore the results of the present study encourage future investigations on colonic delivery of peptide drugs.

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