Rat Jejunal Permeability and Metabolism of μ-Selective Tetrapeptides in Gastrointestinal Fluids from Humans and Rats

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Purpose. To study intestinal transport and metabolism of three new μ-selective tetrapeptide enkephalin analogues, LEF537, LEF553 and TAPP. These peptides are stabilized against enzymatic hydrolysis by having a D-aminoacid in position 2 and a blocked COOH-terminal.

Methods. We used a single-pass perfusion technique to study the transport of the peptides in rat jejunum. To reduce luminal and/or brush-border metabolism during the perfusion we used protease inhibitors (Pefabloc® SC, bestatin and thiorphan). The rate of metabolism was studied by incubations in rat jejunal homogenate, rat jejunal fluid and human gastric and jejunal fluid with and without these inhibitors.

Results. The jejunal permeabilities (P_{eff}) of the peptides were 0.43–$0.78 \cdot 10^{-4}$ cm/s without inhibitors and 0.09–$0.45 \cdot 10^{-4}$ cm/s in presence of the inhibitors. All three peptides were rather rapidly degraded by enzymes in rat jejunal homogenate with half-lives of between 11.9 ± 0.5 and 31.7 ± 1.5 min. The addition of inhibitors to the homogenate prolonged the half-lives substantially for LEF553 (167 ± 35 min) and TAPP (147 ± 2 min), but only slightly for LEF537 (16.4 ± 0.5 min). LEF553 and TAPP were both hydrolyzed in rat and human jejunal fluid, while LEF537 was metabolized less in these fluids. When LEF553 and TAPP were incubated with intestinal fluid in the presence of inhibitors, metabolism was almost completely inhibited. There was no metabolism for any of the peptides in human gastric juice.

Conclusions. The replacement of the terminal free carboxylic group with an amide group did not increase the stability of the peptides in jejunal tissue enough to allow successful oral drug delivery.

KEY WORDS: enkephalin analogues; oral drug delivery; peptide absorption; intestinal perfusion; intestinal metabolism; protease inhibitors.

INTRODUCTION

Although peptides are very potent drugs, their *in vivo* potency after oral administration is often low due to low and variable bioavailability. This is often due to unfavorable physicochemical properties such as high hydrogen bond number, which gives a low permeability (1,2). Instead, carrier-mediated transport by the intestinal oligopeptide carrier might be a preferable route of entry into the body as demonstrated for peptide-like drugs such as β-lactam antibiotics. This is possible for di- and tripeptides structures but it has not been fully clarified whether tetrapeptides are substrate or not (3–5). The second major limitation of oral peptide delivery is extensive hydrolysis by gastrointestinal proteases/peptidases. Potential enzymatic sites are the intestinal lumen by action of proteases secreted from the pancreas, the brush-border membrane, the cytosol and lysosomes of the enterocyte, the liver and the blood. Previous reports have also demonstrated that peptides can be transported in the opposite direction by efflux systems such as P-glycoprotein (6,7). For example Met-enkephalin analogues carrying positive charges may be secreted by the multidrug transporter (8).

The tetrapeptides LEF537 (Tyr-D-Arg-Phe-Nva-NH_2), LEF553 (Tyr-D-Arg-Phe-Phe-NH_2) and TAPP (Tyr-D-Ala-Phe-Phe-NH_2) are μ-selective synthetic analogues of enkephalin (Fig. 1a–b). They have potent antinociceptive effects after i.v. or s.c. administration in *in vivo* inflammatory tests compared to the much lower potencies seen in centrally mediated thermal tests in rats and mice (9). The bioavailability of LEF553 in rats following oral absorption is about 0.5% (Ekström et al., unpublished data), which is similar to the reported bioavailability of 0.22% in rats for another enkephalin analogue, metkephamid (10). In the Caco-2 model it has been shown that Met-enkephalin and analogues are low permeability compounds (8).

In the present study we investigated the transport and metabolism of the three new μ-selective tetrapeptides in rat jejunum. The *in situ* disappearance rate was studied with and without addition of inhibitors. The rate of metabolism was also investigated in human gastric and jejunal fluid. In separate jejunal perfusions the permeability (P_{eff}) of two other passively absorbed compounds, metoprolol and atenolol, was determined as reference permeabilities.

MATERIALS AND METHODS

Chemicals and Perfusion Solution

The three peptides LEF537 (Tyr-D-Arg-Phe-Nva-$NH_2 \cdot 2$ HCl), LEF553 (Tyr-D-Arg-Phe-Phe-$NH_2 \cdot 2$HCl) and TAPP (Tyr-D-Ala-Phe-Phe-$NH_2 \cdot$HCl) (Fig. 1a–b) were synthesized at BioChem Therapeutics (Montreal, Canada). Two inhibitors of brush-border peptidase activity, bestatin (aminopeptidase N) and thiorphan (endopeptidase 24-11), were obtained from Sigma Chemical Co. (St. Louis, USA), and one inhibitor of serine proteases, Pefabloc® SC, from Boeringer Mannheim GmbH (Germany).

The perfusion solution (pH 6.5, about 290 mOsm/kg) contained 1 g/l PEG 4000, 48 mM NaCl, 5.4 mM KCl, 28 mM Na_2HPO_4, 43 mM NaH_2PO_4, 35 mM mannitol and 10 mM D-glucose. ^{14}C-PEG 4000, ^{3}H-D-glucose (2.5 and 10 μCi/l, respectively, Amersham Labs., Buckinghamshire, UK) and antipyrine (Sigma Chemical Co.) were added to the solution as markers for water flux, active and passive transport, respectively. Metoprolol was obtained from Astra Hössle AB (Mölndal, Sweden) and atenolol from Diamalt GmbH (Raubling, Germany). All the other chemicals were of analytical grade.

Jejunal Perfusion Studies

A previously validated *in situ* single-pass perfusion technique (11,12) at a flow rate of 0.2 ml/min was used to study the transport and metabolism of the three tetrapeptides in rat jejunum. Male Sprague-Dawley rats (B&K, Sollentuna, Sweden), weighing 270–300 g, were fasted for 15–20 h prior to

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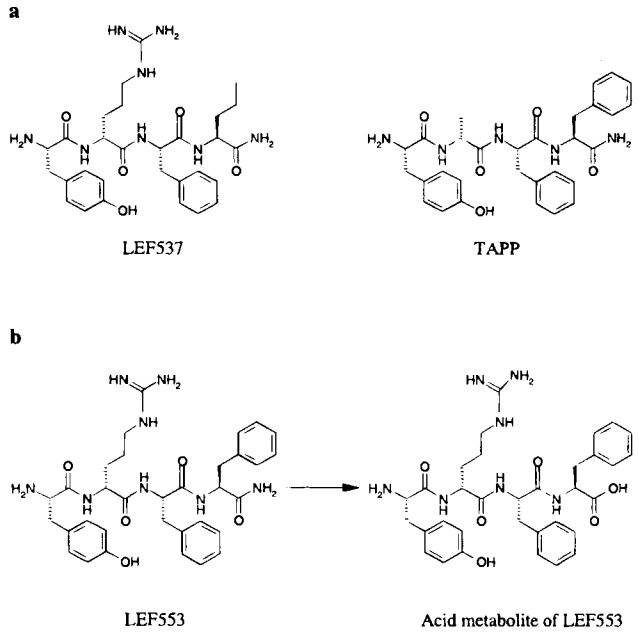

Fig. 1 (a) Structures of LEF537 and TAPP. (b) Structures of LEF553 and the acid metabolite of LEF553.

the experiment. The anesthesia and surgery were performed as described elsewhere (11). Each perfusion experiment was divided into two 90-min periods. In period 1 (P1, 0–90 min) the perfusion solution contained one of the peptides, 0.1 mg/ml, and in period 2 (P2, 90–180 min) the three protease inhibitors were added to the solution at the following concentrations: Pefabloc® SC 1.2 mM, thiorphan 9 μM and bestatin 80 μM (13–15).

The perfusion started by rinsing the segment with saline (37°C), the inlet tube was filled with perfusion solution and in both periods perfusate leaving the jejunal segment was collected at 15-min intervals at steady state. The outlet perfusate was weighed and frozen immediately (−20°C).

In separate perfusion experiments the jejunal P_{eff} of metoprolol and atenolol were determined in male Sprague-Dawley rats (CD®BR, Charles River, Uppsala, Sweden). Atenolol, metoprolol, antipyrine and D-glucose are considered to be stable in intestinal perfusate (11,16).

Preparation of Gastrointestinal Fluids and Mucosal Homogenate for Metabolism Studies

The intestinal fluid was obtained by rinsing jejunal segments (10 cm) of male Sprague-Dawley CD®BR rats using the phosphate buffer including electrolytes, D-glucose and mannitol (pH 6.5, 290 mOsm/kg). A total volume of approximately 2.5 ml for each jejunal segment was collected. Intestinal homogenate was prepared at 6°C by removing a 35 cm segment about 8 cm from the stomach, which was considered to be jejunum (17). The intestine was carefully washed with ice-cold saline and then opened. The mucosa was removed by scraping the epithelial layer and then homogenized (Potter-Elvehjem) at 6°C in phosphate buffer (1 g tissue/5 ml buffer). Human gastric and jejunal fluids were collected using the Loc-I-Gut technique (18). The study was approved by the Ethics Committee of the Medical Faculty, Uppsala University and followed the convictions of the Declaration of Helsinki. The gastrointestinal fluids were stored at −80°C and the homogenate at −20°C until they were used. The protein concentration in the different media was determined by the method of Lowry et al. (19). The study was approved by the Animal Ethics Committee in Uppsala (C352/95).

Metabolism Studies

The rate of metabolism was examined by incubations in human gastric juice, jejunal fluid from humans and rats, and rat jejunal homogenate both with and without protease inhibitors at the following concentrations: Pefabloc® SC 1.2 mM, thiorphan 10 μM, bestatin 100 μM (13–15). The incubation medium consisted of 1 ml of rat jejunal fluid or homogenate, 0.8 ml peptide solution in phosphate buffer (pH 6.5) and 0.2 ml phosphate buffer including the inhibitors. When incubating with human fluids the medium consisted of 1 ml fluid, 0.8 ml peptide solution in NaCl and 0.2 ml NaCl including the inhibitors. The total incubation volume was in every case 2 ml and the final concentration of the peptides was 0.1 mg/ml (LEF537 0.17 mM, LEF553 0.16 mM, TAPP 0.18 mM). The protein concentrations in the final incubation mixtures were 2.2, 2.2, 0.5 and 11 mg/ml in human gastric juice, human jejunal fluid, rat jejunal fluid and rat jejunal homogenate, respectively. The incubation mixture was preincubated at 37°C for about 15 min, while being gently shaken, before the peptide solution was added. Samples (100 μl) were withdrawn and mixed with 100 μl of 50% acetic acid to stop the enzymatic reaction. The mixture was centrifuged for 5 min at 10,000 rpm (Force 7, Denver Instrument Company, USA) and 100 μl of the supernatant was then diluted with 1000 μl mobile phase and injected onto the HPLC column. These chemical assays were performed on line, i.e. directly after the enzymatic process was stopped.

Chemical Assays

All peptides were assayed by an HPLC-method where the separation was achieved on a reversed-phase column (Symmetry C8 3.9 × 150 mm, Waters) with a guard column (Symmetry C8 3.9 × 20 mm, Waters). The mobile phase consisted of a phosphate buffer (pH 2.0, I = 0.05) with 11, 16.5 and 25 v/v % acetonitrile for LEF537, LEF553 and TAPP, respectively. The flow rate was 1.0 ml/min, the UV-detection wavelength 220 nm, and the injection volume 20 μl. The limit of quantitation (LOQ) for LEF537, LEF553 and TAPP and the variability at LOQ (CV%, n = 6) were 4.3 (0.6%), 4.3 (3.0%) and 4.6 (2.9%) μg/ml, respectively. Antipyrine, atenolol and metoprolol were assayed by validated HPLC methods (20,21). Concentrations of ^{3}H-D-glucose and ^{14}C-PEG 4000 were determined by liquid scintillation counting (Mark III, Searle Analytical Inc., USA). The pH and the osmolality were measured by a pH-meter (Metrohm 632) and an osmometer (Vescor 5500), respectively.

Data Analysis

All calculations in the perfusion experiment were made from the steady state concentrations of the outlet perfusate. The net water flux during steady state was calculated as described

elsewhere (11,20). The effective jejunal permeability (P_{eff}) was calculated according to equation 1:

$$P_{eff} = \frac{Q_{in} \cdot \ln (C_{in}/C_{out})}{2\pi rL} \quad (1)$$

where Q_{in} is the perfusion flow, C_{in} and C_{out} are the inlet and outlet (fluid transport-corrected) concentrations of the compounds, respectively, and $2\pi rL$ is the mass transfer surface area, where L is the segment length of 10 cm and r the radius of 0.18 cm.

The half-lives ($t_{1/2}$) for the disappearance of the peptides in the various gastrointestinal media were calculated from the degradation rate constants obtained by linear regression from the initial linear part (30 min) of first-order plots of peptide concentration versus time. For the incubations in human gastric juice, the calculations were based on data up to 60 min.

Student's paired t-test (2-tailed) was used to investigate differences in P_{eff} between the two periods for antipyrine, D-glucose metoprolol and atenolol. The permeability data and half-lives are presented as mean ± S.D.

RESULTS

Jejunal Perfusion Studies

The P_{eff} of LEF537, LEF553 and TAPP, determined by *in situ* perfusion in rat jejunum without inhibitors (P1), were between 0.43 ± 0.19 and 0.78 ± 0.23·10^{-4} cm/s, (Table I, Fig. 2). When the inhibitors were added (P2), the P_{eff} values decreased to 0.09 ± 0.02 and 0.31 ± 0.02·10^{-4} cm/s for LEF553 and TAPP, respectively. For LEF537, the P_{eff} in P2 was still rather high at 0.45 ± 0.23·10^{-4} cm/s.

The peptides were stable in the perfusion solution during the perfusion experiment and no adsorption to the plastic tubes could be detected. The mean recoveries of the non-absorbable marker molecule PEG 4000 (n = 9) during steady state in P1 and P2 were 94 ± 5 and 94 ± 4 %, respectively. The net water fluxes were 0.012 (P1) and 0.019 ml/h/cm (P2). The P_{eff} values for the functional viability marker molecules antipyrine and D-glucose during steady state are presented in Table I together with the P_{eff} values for metoprolol and atenolol. The P_{eff} values for antipyrine and D-glucose decreased by 18% and 22%, respectively, in P2 compared to P1 ($p < 0.05$). For metoprolol and atenolol, no significant difference was discovered (n = 6), although the P_{eff} values tended to decrease for both the β-blockers in P2 (Table I).

Table I. Physicochemical Properties and Permeability Coefficients (P_{eff}), Mean±S.D., Obtained by Jejunal Perfusion in Rats *In Situ*

Compound	MW[a]	HB[b]	Conc. (mM)	P_{eff} (* 10^{-4} cm/s) Period 1	Period 2
LEF537	583	17	0.17	0.78±0.23	0.45±0.23
LEF553	631	17	0.16	0.43±0.19	0.09±0.02
TAPP	546	13	0.18	0.68±0.18	0.31±0.02
Antipyrine	188	3	1.05	1.36±0.22	1.12±0.22
Metoprolol	267	4	0.58	0.44±0.07	0.33±0.25
Atenolol	266	7	0.83	0.08±0.07	0.02±0.02
D-glucose	180	10	10	1.31±0.23	1.02±0.22

[a] MW: molecular weight.
[b] HB: calculated number of hydrogen bonds according to Stein (25).

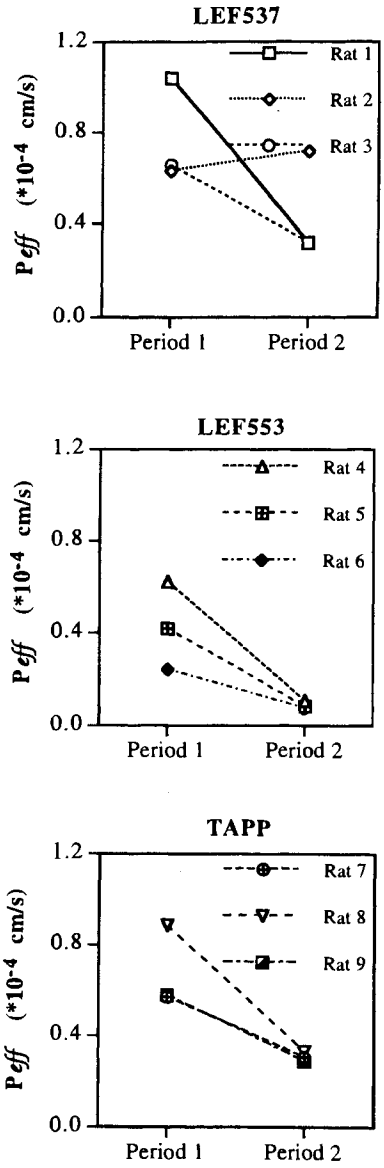

Fig. 2. Individual P_{eff} in the rat jejunum for the three peptides LEF537, LEF553 and TAPP without inhibitors in period 1 and with the inhibitors Pefabloc® SC (1.2 mM), bestatin (80 μM) and thiorphan (9 μM) in period 2.

Metabolism Studies

All three peptides were rather rapidly degraded by enzymes in the rat jejunal homogenate. When no inhibitors were added, their *in vitro* half-lives ranged between 11.9 ± 0.5 and 31.7 ± 1.5 min (Table II). The addition of inhibitors to the jejunal homogenate considerably prolonged the half-lives for both LEF553 (167 ± 35 min) and TAPP (147 ± 2 min). However, the half-life of LEF537 only increased slightly from 11.9 ± 0.5 to 16.4 ± 0.5 min. LEF553 and TAPP were both hydrolyzed in rat and human jejunal fluid, while LEF537 was metabolized less in these fluids (Table II). When the peptides were incubated with intestinal fluid from both species in the presence of inhibi-

Table II. Half-lives (Mean±S.D.) of the Three Peptides in Rat Jejunal Homogenate, Rat Jejunal Fluid, Human Gastric Juice and Human Jejunal Fluid in the Absence and Presence of the Inhibitors Pefabloc® (1.2 mM), Bestatin (100 μM) and Thiorphan (10 μM) at 37°C

	Half-life[a] (min)			
	Rat		Human	
Peptide	Homogenate[b]	Jejunal fluid[c]	Gastric juice[d]	Jejunal fluid[e]
LEF537	11.9±0.5	>180	>180	76.7±3.3
LEF537 with inhibitors	16.4±0.5	>180	n.d.	>180
LEF553	31.7±1.5	42.1±1.8	>180	3.68±0.02
LEF553 with inhibitors	167±35	>180	n.d.	>180
TAPP	26.8±3.9	38.3±3.5	>180	17.1±0.4
TAPP with inhibitors	147±2	>180	n.d.[f]	>180

[a] Calculated from first-order rate constants from two to four experiments.
[b–e] The protein concentrations in the different incubation mixtures were 11[b], 0.5[c], 2.2[d] and 2.2[e] mg/ml, respectively.
[f] n.d. = not determined.

tors, the metabolism was almost completely inhibited. There was no metabolism or degradation for any of the peptides in human gastric juice or for LEF553 in 10 or 100 units of pepsin at pH 2. The metabolism rates for the peptides in the different gastrointestinal media from both rats and humans are shown in Figure 3.

A metabolite for each of the peptides, formed in rat jejunal fluid and homogenate, was detected in the HPLC-chromatograms (Fig. 4.). Each metabolite had a longer retention time than the parent compound. We used a reference compound in the HPLC-assay to support that the metabolite of LEF553 is most likely the acid form, formed by hydrolysis of the COOH-terminal amide group (Fig. 1b).

DISCUSSION

In a rat perfusion model, the effective permeability is based on disappearance of the drug from the intestinal segment and is considered to reflect transport across the complex apical enterocyte membrane (22). We have shown that rat P_{eff} values, obtained by perfusions, correlate with high accuracy to both P_{eff} and extent of absorption *in vivo* in humans (11). For peptides that are metabolized by luminal and/or brush-border enzymes, P_{eff} is influenced by metabolic considerations. In order to obtain more accurate determinations of P_{eff}, we added three different protease inhibitors in the second perfusion period. During the first period, P_{eff} for all three peptides were rather high (0.43–

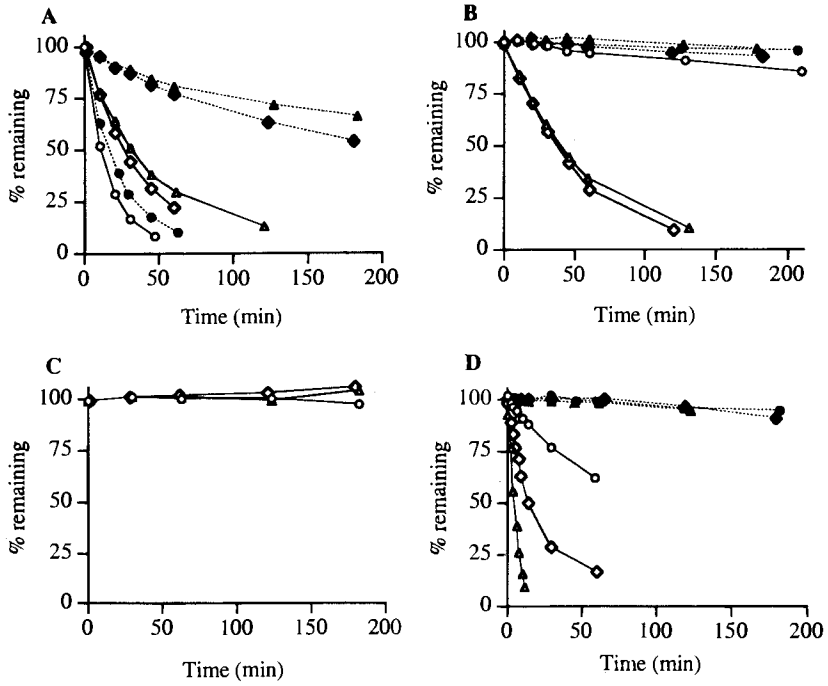

Fig. 3. Metabolism of LEF537 (circles), LEF553 (triangles) and TAPP (squares), 0.1 mg/ml, in the absence (open symbols) and presence (filled symbols) of the inhibitors Pefabloc® SC (1.2 mM), bestatin (100 μM) and thiorphan (10 μM) in A rat jejunal homogenate, B rat jejunal fluid, C human gastric juice and D human jejunal fluid. The results are mean values of two to four incubation experiments.

$0.78 \cdot 10^{-4}$ cm/s) considering their size and hydrophilic character, which suggests that luminal and/or brush-border metabolism occurred during the perfusion. Most of the metabolism of LEF553 and TAPP was probably inhibited during the second perfusion period (P2) as the P_{eff} values decreased by 79% and 54%, respectively. This was supported by the 80% decrease of the metabolism in the homogenate for both peptides. The P_{eff} for LEF553 during protease inhibition ($0.09 \cdot 10^{-4}$ cm/s) presumably mostly reflects transmucosal transport, which thus classifies LEF553 as low-intermediate P_{eff} compound (11). If the three times higher P_{eff} of TAPP is due to true transport across the jejunal barrier, it might be explained by the more lipophilic properties of the peptide and smaller number of hydrogen bonds (Table I). However, it cannot be ruled out that metabolism might still contribute to the disappearance rate during the perfusions, despite enzymatic inhibition. The P_{eff} of LEF537 was less affected by the inhibitors during P2, although the observed decrease in P_{eff} was 42%. The most plausible explanation for this is less efficient enzyme inhibition, which was also supported by lack of enzyme inhibition in rat jejunal homogenate.

Another explanation to the relatively high observed P_{eff} could be that the peptides are carrier-mediated transported. If so, we might also see a decreased P_{eff} in P2 as bestatin has shown to be a substrate for that carrier (23). However, as the peptides transported by this carrier require a free COOH-terminal carboxyl acid and that most tetrapeptides do not seem to be transported (24), the apical to basolateral transport of these tetrapeptides are more likely passive in nature. On the contrary, as the peptides are positively charged they might be substrates for some apical efflux system, which have been shown for similar peptides, e.g. metkephamid, in Caco-2 cells (8).

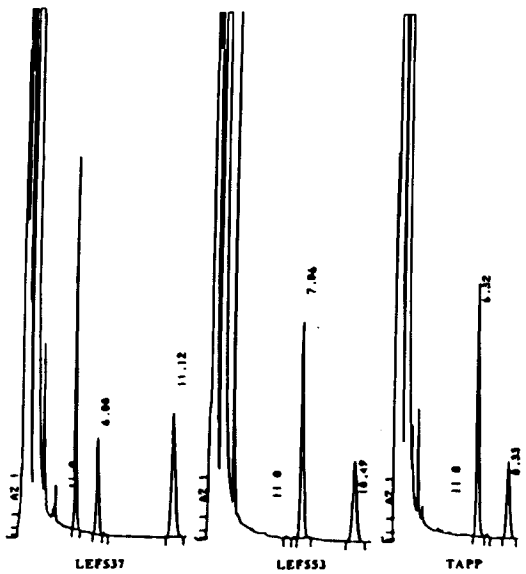

Fig. 4. Chromatograms for LEF537 (retention time, t_{ret} 6.08 min), LEF553 (t_{ret} 7.06 min) and TAPP (t_{ret} 6.32 min) after 20 min incubation in rat jejunal homogenate. One metabolite with a longer retention time (LEF537 11.12 min, LEF553 10.49 min and TAPP 8.33 min) than the parent compound is visible for each peptide. By using a reference compound, the metabolite of LEF553 was identified as the acid formed by hydrolysis of the COOH-terminal amide group.

A decrease in P_{eff} was also observed in the second perfusion period for D-glucose (22%) and antipyrine (18%). This decrease in both passive and active transport may contribute to the lower P_{eff} observed for the peptides in P2. However, it is unlikely that this is the only explanation, since the decrease in P_{eff} for the peptides when the enzyme inhibitors were added was greater. The lower P_{eff} values for the marker molecules in P2 suggest that the intestinal barrier function changes with time; accordingly it is probably best to perform rat perfusion studies in a single period of about 100 min.

Degradation of these three tetrapeptides varied in various gastrointestinal media from both rats and humans. All three peptides were stable in human gastric juice (pH 1.8), which suggests that they are stable in the stomach during fasted state. However, in human jejunal fluid collected in the fasted state, the metabolism was more pronounced for LEF553 and TAPP than for LEF537. Also in rats the metabolism in jejunal fluid was greater for LEF553 and TAPP than for LEF537. While in contrast, the metabolism in jejunal homogenate was faster for LEF537 than for the other two peptides. This difference in metabolism was further demonstrated when the inhibitors were added, as they only had a slight effect on the metabolism of LEF537. The metabolism of TAPP and LEF553 in homogenate was inhibited to about the same extent (about 80%). LEF537 and LEF553 have an arginine as the second amino acid and are hydrophilic. They also have the same number of hydrogen bonds and similar molecular weights (Fig. 1a–b., Table 1). LEF537 differs in its structure since it has a norvaline where the other two peptides have phenylalanine. This makes LEF537 even more hydrophilic which clearly affects its metabolism (Table II, Fig. 3.). The putative acid metabolite, formed by COOH-terminal amide hydrolysis of the peptides in rat jejunal fluid and homogenate, but not in human jejunal fluid, is also indicative of species differences in the intestinal metabolism.

It has been reported that membrane-bound aminopeptidases, located in the brush-border fraction of the enterocyte, are the main enzymes which cleave the N-terminal peptide bond of a pentapeptide, metkephamid (10). In addition to metabolism of these three peptides by aminopeptidases, cytosolic enzymes may also contribute to the observed metabolism in intestinal homogenate, although metkephamid degradation by cytosolic enzymes has been reported to be negligible (10).

This study emphasizes the importance, and also the difficulties, of controlling the metabolism of peptides by luminal and brush-border peptidases in order to determine the rates and mechanisms of pure membrane transport using *in situ-in vivo* intestinal perfusion models. The three peptides LEF537, LEF553 and TAPP are readily metabolized in rat jejunal homogenate and are not completely inhibited by the mixture of the three inhibitors *in vitro*. It is therefore not possible to reach any conclusions about the extent of transport across the apical membrane of the enterocyte (i.e. the extent of absorption) from the perfusion experiments. The replacement of the terminal free carboxylic group with an amide group did not increase the stability of the peptides in jejunal tissue enough to allow successful oral drug delivery. Finally, this study also shows that the peptide structure is crucial for the metabolic mechanism(s) by luminal and brush-border enzymes, as exemplified by the substitution of phenylalanine (LEF553) with norvaline (LEF537). Future research will focus on optimising chemical structure and improved understanding of enzyme inhibition.

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