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Comparison of the ex-vivo oral mucosal permeation of tryptophan-leucine (Trp-Leu) and its myristoyl derivative

F. Veuillez [a], A. Ganem-Quintanar [a], J. Deshusses [b], F. Falson-Rieg [a,c], P. Buri [a,b,*]

[a] *Pharmapeptides, Centre interuniversitaire de recherche et d'enseignement, 74166 Archamps, France*
[b] *Section de Pharmacie, Université de Genève, Quai Ernest-Ansermet 30, 1211 Genève 4, Switzerland*
[c] *ISPB, Université Claude Bernard, Lyon I, Av. Rockefeller 8, 69373 Lyon, France*

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Abstract

The ex vivo permeation of a model peptide, tryptophan-leucine (Trp-Leu), was studied using two different regions of pig oral mucosa, the hard palate and the cheek. In order to increase the mucosal absorption of Trp-Leu, a lipophilic derivative was synthesized by acylation of the N-terminal amino group of Trp-Leu with myristic acid. The purified Trp-Leu derivative (Myr-Trp-Leu) was more lipophilic than the parent Trp-Leu as observed by HPTLC (R_f's values of 0.41 and 0.81, respectively). Measurement of partition coefficients in n-octanol/phosphate buffer pH 7.4, gave K_p values of -0.68 and 1.04 for Trp-Leu and Myr-Trp-Leu, respectively. The native Trp-Leu was unable to pass through the keratinized layer of palatal mucosa, and after 24 h only 12% had passed through the buccal mucosa to the receptor compartment. The higher lipophilicity of the acylated peptide, meant that it was not easily transported across the oral mucosal barrier but accumulated in the tissue, founding 25 and 70% of the original amount in the palatal and buccal mucosae, respectively. Both, Trp-Leu and Myr-Trp-Leu were found to be stable in palatal and buccal mucosae.

Keywords: Tryptophan-leucine; Acylated peptide; Oral mucosal permeation; Myristic acid; Chemical modification; Lipophilicity

1. Introduction

Oral mucosa has been widely studied not only as a local, but as a systemic drug delivery site. One of the most important advantages afforded

* Corresponding author. Tel.: +41 22 7026568; fax: +41 22 7026567.

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by delivering drugs via the oral mucosa is that they are able to bypass gastrointestinal and hepatic drug metabolizing enzymes. Of particular interest is the delivery of peptides via the oral mucosa, however, this is not always feasible, because of the low bioavailability of buccally or sublingually administered peptides.

To increase the oral mucosal absorption of peptides, factors such as molecular size, lipophilicity, charge, conformation and stability have to be considered. The majority of peptides are hydrophilic in nature with a very low partition coefficient; many have molecular weights exceeding 600, considerably larger than most conventional drugs. The intraoral absorption of peptides and proteins is via a passive transport mechanism. To improve passive diffusion across the membrane, it is usually necessary to reduce their size, increase their lipophilicity and/or use chemical enhancers.

However, even if they are not too large, their absorption may be limited due to their low lipophilicity (Muranishi and Yamamoto, 1994). Various absorption enhancers such as surfactants, chelating agents, bile salts and fatty acids have been recently utilized to improve the oral mucosal absorption of different peptides and proteins (Ishida et al., 1981; Siegel and Gordon, 1985; Aungst and Rogers, 1988; Aungst et al., 1988; Nakada et al., 1988; Aungst and Rogers, 1989; Nakada et al., 1989; Aungst, 1994; Steward et al., 1994; Zhang et al., 1994). However, limitations such as local irritation of the mucosa and non-selective absorption are often cited as drawbacks in the use of permeation enhancers.

A potentially useful approach to solve these problems, may be the chemical modification of peptides to provide a significant improvement in lipophilicity using naturally occurring fatty acids (Muranishi and Yamamoto, 1994). The introduction of acyl chains to amino-acid side at the terminal positions, has been performed in order to increase lipophilicity and improve stability of a wide range of molecular weights peptide or protein-like substances. Among them, thyrotropin-releasing hormone (Muranishi et al., 1991; Yamada et al., 1992; Muranishi and Yamamoto, 1994), insulin (Hashimoto et al., 1989; Hashizume et al., 1992; Muranishi et al., 1992; Muranishi and Yamamoto, 1994), tetragastrin (Muranishi et al., 1992; Tenma et al., 1993; Muranishi and Yamamoto, 1994), lysozyme (Muranishi and Yamamoto, 1994), dextrans and bovine serum albumin (Sezaki et al., 1992), have been studied. Most of these permeability experiments have been carried out using intestinal membrane, however, the same principle may be applied to the oral mucosa.

In this study we have determined some of the transport properties of chemically modified peptides for oral mucosal peptide delivery. Tryptophan-leucine (Trp-Leu) was chosen as a small and enzymatically stable model peptide. We synthesized a chemically modified peptide analogue by attaching a myristoyl group to the amino terminal of the peptide molecule. Then, we investigated whether the more lipophilic derivative (myristoyl-tryptophanyl-leucine) could be used to improve the absorption of Trp-Leu in the palatal and buccal mucosa.

2. Materials and methods

2.1. Materials

2.1.1. Animals

Palatal tissue and buccal mucosa (cheek) were removed from pigs at the slaughterhouse immediately after death and were transported in 0.9% saline solution. After removal of the submucosal tissue, mucosae were frozen at -20°C before use (no more than 4 weeks).

2.1.2. Reagents

All reagents were of analytical grade. Tryptophan-leucine (Trp-Leu) was purchased from Sigma (St. Louis MO). Chloroform (HPLC), methanol (HPLC), ethanol, n-octanol, di-sodium hydrogen phosphate, potassium di-hydrogen phosphate and sodium chloride, were all purchased from Fluka (Buchs, Switzerland). Water was passed through a Milli-Q water purification system before use (Millipore, Bedford, MA). Carboxypeptidase A (EC 3.4.17.1) and aminopeptidase (EEC no. 232-874-6) were obtained from Sigma (St. Louis MO).

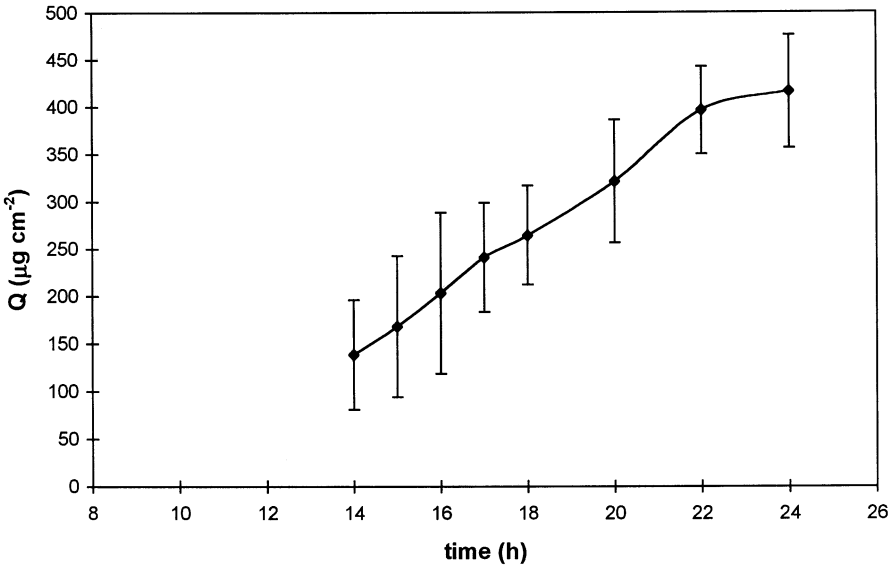

Fig. 1. Permeation of Trp-Leu through buccal mucosa ($n = 6 \pm$ S.D.).

2.1.3. High performance thin-layer chromatography (HPTLC)

High performance, silica gel 60 thin-layer chromatography plates, 10×20 cm without concentration zone, were obtained from Merck (Darmstadt, Germany).

2.2. Experimental procedures

2.2.1. Synthesis of the tryptophan-leucine derivative

A lipophilic derivative of tryptophan-leucine (Trp-Leu) was synthesized according to the method reported by Veuillez et al. (1997) by chemical attachment of myristic acid to the N-terminal amino group.

2.2.2. Determination of Trp-Leu and Myr-Trp-leu solubility

An excess of Trp-Leu or Myr-Trp-Leu was added to the vehicle, which consisted in a 30:70 (v/v) mixture of ethanol:0.02 mM phosphate buffer pH 7.4 (Ph. Helv. VII). The mixture was shaken at 37 ± 1°C for 24 h. The suspension was centrifuged and the supernatant was filtered through a membrane filter (0.45 μm, polypropylene micro-centrifuge tube filters, Whatman, Maidstone, UK). The concentration of Trp-Leu or Myr-Trp-Leu was determined by UV spectrophotometry at 274 ($a = 33.40$ l/g/cm) and 280 nm ($a = 12.56$ l/g/cm), respectively.

2.2.3. Measurement of the apparent partition coefficient

The partition coefficients of Trp-Leu and Myr-Trp-Leu were determined in n-octanol/0.02 mM phosphate buffer pH 7.4. Trp-Leu or Myr-Trp-Leu (300 μg/ml) were dissolved in phosphate buffer or n-octanol, respectively. The solutions were shaken for 24 h with an equal volume of the corresponding aqueous or organic phase. Phase separation was achieved by centrifugation and the concentration in both solutions was measured by spectrophotometry at 274 nm for Trp-Leu and at 280 nm for Myr-Trp-Leu.

2.2.4. Permeation experiments

The permeation of Trp-Leu and Myr-Trp-Leu was investigated using buccal (cheek) and hard palate pig mucosae. The tissue was mounted in a modified Franz diffusion cell (exposed area: 0.78 cm^2; Friend, 1992). The receptor consisted of 2 ml of a 30:70 v/v mixture of ethanol:0.02 mM phosphate buffer, pH 7.4 (Ph. Helv. VII). The device

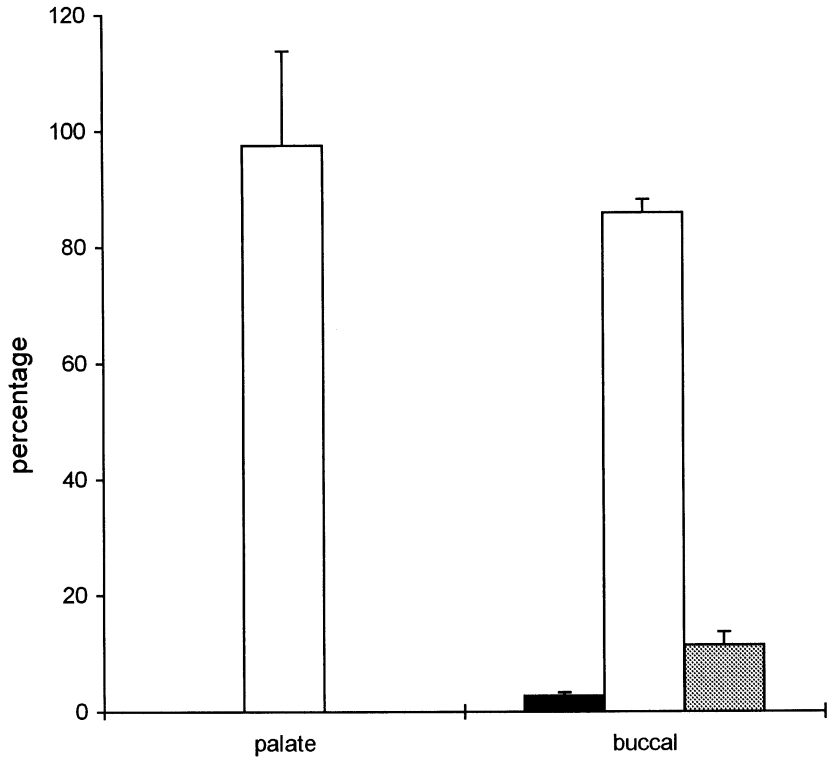

Fig. 2. Percentage of Trp-Leu after 24 h in contact with oral mucosa ($n = 6 \pm$ S.D.): (■) tissue; (□) donor; (▩) receptor.

was maintained at 37°C by a circulating water pump and was constantly stirred with a teflon-coated magnetic bar. The tissue was equilibrated for 30 min with the receptor medium, before 0.5 ml of a solution of Trp-Leu or Myr-Trp-Leu (6 mg/ml in ethanol:phosphate buffer pH 7.4, 30:70 v/v), was added to the donor compartment. In order to prepare the solution of Myr-Trp-Leu, the peptide was, at first, solubilized in pure ethanol, and then, the necessary volume of buffer was added. A clear solution was obtained. Aliquots (100 μl) of the receptor fluid were withdrawn periodically from 14 to 18 h (each hour) and then from 18 to 24 h (every 2 h), replacing them with an equivalent volume of fresh solution. Samples were stored frozen until High Performance Thin-Layer Chromatography (HPTLC) assay. The results were corrected for dilution effect. At the end of the experiment, the amount of Trp-Leu or Myr-Trp-Leu remaining in the donor compartment and in the tissue were quantified by HPTLC.

2.2.5. Determination of the amount of Trp-Leu and Myr-Trp-Leu in the tissue

2.2.5.1. Trp-Leu. At the end of the experiment, once the tissue was carefully rinsed on both sides with the ethanol:phosphate buffer solution, it was finely divided and soaked twice during a period of 24 h, first, in a hypertonic saline solution (NaCl 20% w/v), and then in distilled water.

2.2.5.2. Myr-Trp-Leu. The tissue was rinsed as above, and dipped in chloroform for 24 h. It was then finely divided and extracted once in chloroform for 24 h at room temperature. In each case, the extraction suspensions were centrifuged and the supernatant filtered through a membrane filter (0.45 μm, polypropylene micro-centrifuge tube filters, Whatman, Maidstone, UK). The stratum corneum of the palatal samples was removed prior to the extractions with pliers, and subjected to the corresponding treatment for Trp-Leu or Myr-Trp-Leu.

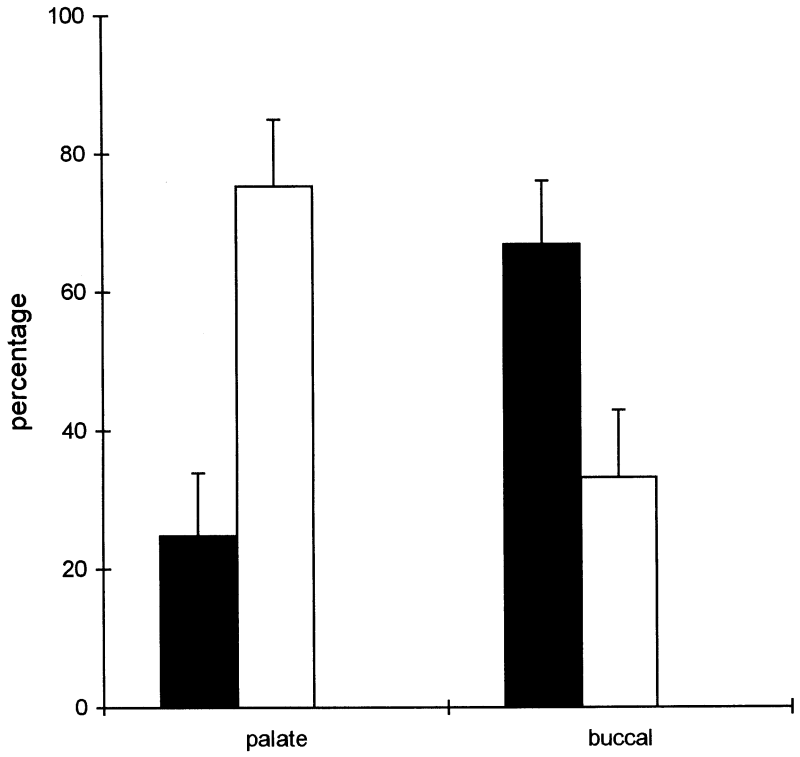

Fig. 3. Percentage of Myr-Trp-Leu after 24 h in contact with oral mucosa ($n = \pm$ S.D.): (■) tissue; (□) donor.

2.2.6. HPTLC

Two microliters of the samples of Trp-Leu or Myr-Trp-Leu, were spotted on HPTLC plates using the CAMAG Linomat IV (CAMAG, Muttenz, Switzerland). Standard solutions of increasing concentrations were applied on each plate for mass calibration. Separation was carried out, for both substances, in developing chambers (CAMAG) saturated at room temperature with chloroform:methanol:water (16:6:1). The solvent front was allowed to migrate to 5.0 cm above the origin. The plates were dried at 45°C for 20 min and scanned (in the Refl-Abs mode) with a CAMAG TLC Scanner II at 274 nm for Trp-Leu (limit of quantification: 60 ng) and at 280 nm for Myr-Trp-Leu (limit of quantification: 35 ng). Quantifications were obtained by integration of the signals with a CAMAG SP4290 integrator.

2.2.7. Enzymatic degradation of the peptides

Susceptibility to enzymatic degradation of both peptides was determined using a procedure adapted from Tate (1985) where the buffer and substrate solution were prepared in a solution of 30% ethanol. The peptides were used at 1.6 mM and incubated for 10 and 20 min in the presence of three units of each enzyme. In contrast to the original method, the ninhydrin reaction was performed in phosphate buffer (at pH 6.0) instead of citrate, which interfered with the colorimetric assay used.

3. Results and discussion

In order to compare the permeation of Trp-Leu and Myr-Trp-Leu, two regions of the oral mucosa where chosen, the palate and the cheek (buccal mucosa). These two mucosae have different structural and compositional characteristics, the most

important difference being the presence of a keratinized layer on the hard palate surface, similar in its pattern of maturation to the epidermal stratum corneum. In contrast, in the buccal mucosa, the absence of a keratinized layer makes the tissue more permeable than its keratinized counterparts. Myr-Trp-Leu is poorly soluble in water and it was therefore necessary to use a 30:70 (v/v) ethanol:phosphate buffer mixture, to have a clear solution (solubility = 4.34 mg/ml). The same solvent was used for Trp-Leu (solubility = 12.38 mg/ml). This percentage of ethanol was shown to enhance Trp-Leu permeation through buccal mucosa (Fig. 1), as compared to a pure aqueous solution where no peptide was found in the receptor chamber. As shown, using the ethanolic solution, after a long lag time of 9 h, a constant flux of 28.97 $\mu g/cm^2/h$, was observed between 14 and 22 h. It is worth noting that the transport of Trp-Leu with this solvent was only possible in the buccal mucosa. In this study, the results demonstrated that under the chosen experimental conditions, Trp-Leu was unable to pass through the keratinized tissue. Even after being in contact with the palate for 24 h, no penetration could be detected. The extraction from the tissue did not reveal the presence of Trp-Leu and the entire applied amount was found in the donor compartment. Fig. 2 shows the distribution of Trp-Leu at the end of the experiment (24 h) for the two mucosae. In buccal mucosa, at 24 h, only 12% of the initial amount of Trp-Leu had passed into the receptor chamber, and about 3% remained in the tissue. Such low absorption of Trp-Leu was mainly attributed to its low lipophilicity ($\log K_{p_{oct/water}} = -0.68 \pm 0.01$; R_f in HPTLC = 0.41).

Myr-Trp-Leu due to its long acyl chain, was shown to be a more lipophilic product ($\log K_{p_{oct/water}} = 1.04 \pm 0.26$; R_f in HPTLC = 0.81). This resulted in an accumulation in the oral tissue, as shown in Fig. 3. Uptake was greater in the buccal tissue as compared to the palatal mucosa (70 and 25%, respectively) and this could be attributed to the keratinized surface layer present on the palate, being an efficient permeability barrier. However, the results showed that only about $2.2 \pm 0.8\%$ of Myr-Trp-Leu remained in the stratum corneum. This great affinity between Myr-Trp-Leu and the tissue, probably resulted in strong interactions with the membrane since nothing was detected in the receptor chamber using HPTLC. However, even if it is desirable for certain products that they pass through the epithelium into the receptor compartment, for others, the formation of a depot in the membrane, from which the drug can be slowly delivered, may also be an interesting possibility.

It is important to mention that the two products, Trp-Leu and Myr-Trp-Leu, were both, stable in the palatal and buccal mucosae. The experiments described here showed that the total recovery from the three compartments (receptor, tissue and donor) was close to 100%. The migration in HPTLC always corresponded to the Trp-Leu and Myr-Trp-Leu standards. No peaks were observed at the corresponding retention times of Trp (or Trp-Leu when Myr-Trp-Leu was the studied substance), suggesting that both, Trp-Leu and Myr-Trp-Leu were stable in the oral mucosa for at least 24 h. The absence of degradation observed was not due to an effect of ethanol in the compartments. To test such an hypothesis, experiments (not shown here) where both peptides were incubated in the presence of mucosa homogenates were performed. As in the permeation experiments, recovery of the peptides after being incubated with mucosal homogenates in the presence of ethanol showed that the tissue is unable to hydrolyse them. This inability is not due to the presence of ethanol since rapid hydrolysis by carboxypeptidase of both peptides were observed in the presence of ethanol and of the free Trp-Leu by aminopeptidase. These results suggest that an optimal lipophilicity for improving the oral mucosal permeability of Trp-Leu may be required. As an hypothesis for further work, it could be interesting to test the properties of analogues with shorter acyl chains. Such modifications could result in the decrease of the deposition in the tissue and in the promotion of the transport to the receptor compartment.

4. Conclusions

The present study demonstrated that Trp-Leu was able to pass through the non-keratinized buccal mucosa, when a mixture of ethanol:phosphate buffer pH 7.4 (30:70 v/v) was used as vehicle, however, it was unable to penetrate into the keratinized hard palatal tissue. Acylation of Trp-Leu with myristic acid, increased the lipophilicity of the product, which resulted in a greater affinity of Myr-Trp-Leu to the membrane components and its accumulation in the tissue. These results suggested that an optimal lipophilicity (probably acylation with a shorter-chain fatty acid) may improve the passage of the product through the oral mucosa, to the receptor.

Accumulation in the tissue could however present, in some cases, new properties that could be exploited. In order to be able to consider the accumulation of the drug in the mucosal tissue as a possible means to a slow release process, we have to be able to manipulate lipophilicity of the peptide derivatives by choosing appropriate acylating groups of different hydrophobicity or by selecting specific groups that could be cleaved by enzymes present in the mucosal tissue. Such an approach could lead to the creation of new classes of peptides whose properties could reduce the need for permeation enhancers.

References

Aungst, B.J., 1994. Site-dependence and structure effect relationships for alkylglycosides as transmucosal absorption promoters for insulin. Int. J. Pharm. 105, 219–225.

Aungst, B.J., Rogers, N.J., 1988. Site dependence of absorption promoting actions of laureth-9, Na salicylate, Na_2EDTA, and aprotinin on rectal, nasal, and buccal insulin delivery. Pharm. Res. 5, 305–308.

Aungst, B.J., Rogers, N.J., 1989. Comparison of the effects of various transmucosal absorption promoters on buccal insulin delivery. Int. J. Pharm. 53, 227–235.

Aungst, B.J., Rogers, N.J., Shefter, E., 1988. Comparison of nasal, rectal, buccal, sublingual and intramuscular insulin efficacy and the effects of a bile salt absorption promoter. J. Pharmacol. Exp. Ther. 244, 23–27.

Friend, D.R., 1992. In vitro skin permeation techniques. J. Control. Rel. 18, 235–248.

Hashimoto, M., Takada, K., Kiso, Y., Muranishi, S., 1989. Synthesis of palmitoyl derivatives of insulin and their biological activities. Pharm. Res. 6, 171–176.

Hashizume, M., Douen, T., Murakami, M., Yamamoto, A., Takada, K., Muranishi, S., 1992. Improvement of large intestinal absorption of insulin by chemical modification with palmitic acid in rats. J. Pharm. Pharmacol. 44, 555–559.

Ishida, M., Machida, Y., Nambu, N., Nagai, T., 1981. New mucosal dosage form of insulin. Chem. Pharm. Bull. 29, 810–816.

Muranishi, S., Sakai, A., Yamada, K., Murakami, M., Takada, K., Kiso, Y., 1991. Lipophilic peptides: Synthesis of lauroyl thyrotropin-releasing hormone and its biological activity. Pharm. Res. 8, 649–652.

Muranishi, S., Murakami, M., Hashidzume, M., Yamada, K., Tajima, S., Kiso, Y., 1992. Trials of lipid modification of peptide hormones for intestinal delivery. J. Control. Release 19, 179–188.

Muranishi, S., Yamamoto, A., 1994. Modification of peptides for intestinal delivery. In: Crommelin, D.J.A., Midha, K.K., Nagai, T. (Eds.), Topics in Pharmaceutical Sciences. Medpharm Scientific, Stuttgart, pp. 373–382

Nakada, Y., Awata, N., Nakamichi, C., Sugimoto, I., 1988. The effect of additives on the oral mucosal absorption of human calcitonin in rats. J. Pharmacobio-Dyn. 11, 395–401.

Nakada, Y., Awata, N., Ikuta, Y., Goto, S., 1989. The effect of bile salts on the oral mucosal absorption of human calcitonin in rats. J. Pharmacobio-Dyn. 12, 736–743.

Sezaki, H., Takakura, Y., Hashida, M., 1992. Chemical modification and disposition of proteins and peptides: biopharmaceutical aspects. In: Crommelin, D.J.A., Midha, K.K. (Eds.), Topics in Pharmaceutical Sciences. Medipharm Scientific, Stuttgart, pp. 47–56.

Siegel, I.A., Gordon, H.P., 1985. Effects of surfactants on the permeability of canine oral mucosa in vitro. Toxicol. Lett. 26, 153–157.

Steward, A., Bayley, D.L., Howes, C., 1994. The effect of enhancers on the buccal absorption of hybrid (BDBB) α-interferon. Int. J. Pharm. 104, 145–149.

Tate, S.S., 1985. Microvillus membrane peptidases that catalyse hydrolysis of cysteinylglycine and its derivatives. In: Colowick, S.S., Kaplan, N.O. (Eds.), Methods in Enzymology, vol. 113. Academic Press, New York, p. 473.

Tenma, T., Yodoya, E., Tashima, S., Fujita, T., Murakami, M., Yamamoto, A., Muranishi, S., 1993. Development of new lipophilic derivatives of tetragastrin: Physicochemical characteristics and intestinal absorption of acyl-tetragastrin derivatives in rats. Pharm. Res. 10, 1488–1492.

Veuillez, F., Tissot, P., Buri, P., Deshusses, J., 1997. Modification of peptide properties upon acylation in scope of enhancing transmucosal delivery. Proc. Int. Symp. Control. Rel. Bioact. Mater. 24, 431–432.

Yamada, K., Murakami, M., Yamamoto, A., Takada, K., Muranishi, S., 1992. Improvement of intestinal absorption of thyrotropin-releasing hormone by chemical modification with lauric acid. J. Pharm. Pharmacol. 44, 717–721.

Zhang, J., Niu, S., Ebert, C., Stanley, T.H., 1994. An in vivo dog model for studying recovery kinetics of the buccal mucosa permeation barrier after exposure to permeation enhancers: apparent evidence of effective enhancement without tissue damage. Int. J. Pharm. 101, 15–22.