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Permeation of a myristoylated dipeptide across the buccal mucosa: topological distribution and evaluation of tissue integrity

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Abstract

The ex vivo permeation of an acylated model dipeptide, Myristoyl–Tryptophan–Leucine (Myr–Trp–Leu) was studied using pig buccal mucosa (Veuillez et al., 1998). Myr–Trp–Leu, being lipophilic, did not readily penetrate across the membrane. Rather, it accumulated in the epithelial and connective tissue of the mucosal barrier. The topological distribution of Myr–Trp–Leu across the mucosa, following its application in ethanol/phosphate buffer (30/70 pH 7.4), was determinated by thin-sectioning of the tissue, extraction of the peptide, and high performance thin layer chromatography (HPTLC). The concentration profile depended, of course, on the duration of the experiment and appeared to be dependent upon the presence of sufficient ethanol in order that the peptide could be solubilized. This important role for ethanol then raised the question of the solvent's effect on tissue integrity. Light microscopic examination of the mucosa was, therefore, undertaken, under identical conditions to those used in the permeation experiments, to evaluate any perturbation induced by the ethanolic vehicle. No obvious effects were observed. © 2002 Published by Elsevier Science B.V.

Keywords: Acylated dipeptide; Myristoylated dipeptide; Tissue accumulation; Buccal mucosa histology; Buccal mucosa permeation; Ethanol

1. Introduction

Buccal transmucosal delivery is an alternative drug administration route which might enable the delivery of high molecular weight substances (including peptides), that must otherwise be injected. The buccal route is of interest since the mucosa is readily accessible and has a rich blood supply. Moreover, it is less prone to irritation by foreign substances (compared, for example, to the nasal mucosa) and allows degradation processes by gastrointestinal and hepatic enzymes to be bypassed (Harris and Robinson, 1992; Vyas et al., 1994). Porcine buccal mucosa has been chosen as an in

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vitro model because its epithelial structure satisfactorily mimics the non-keratinized human buccal epithelium (Squier and Hall, 1985a; Lesch et al., 1989; Senel et al., 1994). The buccal mucosa is significantly thicker than all other regions of the mouth (Squier and Hall, 1985b), although the rate-limiting barrier for the movement of hydrophilic molecules across this barrier appears to reside in the outer one-third of the epithelium (Squier, 1973; Squier and Rooney, 1976). Permeation studies have been performed using a number of electron-dense tracers, such as horseradish peroxidase (Squier, 1973; Squier and Hall, 1985a) and lanthanum nitrate (Squier and Rooney, 1976) which, when applied to the submucosal surface, appear to permeate up to, but not into, the uppermost cell layers (approximately the top 20–25%) of the epithelium. This is completely consistent with the idea that the surface cell layers present the major barrier to permeation. Also, it is in this same region that the extrusion of membrane coating granules (MCG), which are involved in barrier formation, is observed (Knuth et al., 1990).

In our previous studies (Veuillez et al., 1998), the permeation across pig buccal mucosa of Myristoyl–Tryptophan–Leucine (Myr–Trp–Leu), an acylated derivative of a model dipeptide with a very low solubility in water, was studied from an ethanol/buffer vehicle. Ethanol has been widely used as a percutaneous penetration enhancer (Harrigan and Levy, 1975; Sloan et al., 1998) and its action on the cutaneous barrier has been well studied. At low concentration, little irritation is observed following application to the skin (Tanojo et al., 1993). Some topical delivery systems contain ethanol at concentrations of up to 90% (Yum et al., 1994). It was found that 5% ethanol increased the permeation of nitroso-nornicotine across the gingival and sublingual tissues (Squier et al., 1986). Frequent use of ethanolic vehicles during permeation experiments can lead to tissue modification. Nevertheless, the buccal mucosa is known to be more robust (Jacques and Buri, 1998). Microscopic examination has proved useful in showing that the integrity of the tissue is maintained (Du Bois, 1966).

Permeation experiments with Myr–Trp–Leu using pig buccal mucosa showed that the product accumulates within the tissue. Therefore, it was considered important to investigate the precise localization of the peptide within the mucosa as a function of time. The relation between the duration of application of ethanol and the distribution of the drug inside the biological barrier was also evaluated. Finally, since ethanol had to be used to solubilize the acylated peptide, it was necessary to examine the effects of the solvent on the tissue.

2. Materials and methods

2.1. Materials

2.1.1. Tissue preparation

Fresh buccal (cheek) tissue from pigs (age ~ 3 months) was obtained from the slaughterhouse (Annecy, France) and transported in isotonic saline solution. Experiments on the tissue began within 2 h after death of the animal. The buccal mucosal membrane was carefully isolated with surgical scissors. To ensure reproducible samples, nonkeratinized buccal mucosa were always taken from the same region posterior to the angle of the mouth. Slice thickness ranged from 1 to 1.1 mm.

2.1.2. Reagents

Myr–Trp–Leu, a lipophilic derivative of Tryptophan–Leucine (Trp–Leu), was synthesized as previously reported (Veuillez et al., 1999). All reagents were of analytical grade. Chloroform (HPLC), methanol and ethanol (HPLC), disodium hydrogen phosphate, potassium di-hydrogen phosphate and sodium chloride were purchased from Fluka (Buchs, Switzerland). Water was passed through a Milli-Q water purification system before use (Millipore®, Bedford, MA). Tissue-Tek® OCT was used to fix tissue sections. Hematoxylin and eosin were used to stain microscopic sections and were purchased from Fluka (Buchs, Switzerland).

2.2. Methods

2.2.1. Determination of Myr–Trp–Leu solubility

Saturated solutions of Myr–Trp–Leu were obtained by adding an excess of the compound to

the vehicle, i.e. 30:70 (v/v) mixture of ethanol:phosphate buffered saline pH 7.4 (Ph. Helv. VII). The mixture was stirred at 37 ± 1 °C for 24 h. The suspension was centrifuged and the supernatant filtered through a membrane (0.45 μm, polypropylene micro-centrifuge tube filters, Whatman, Maidstone, UK). The concentration of Myr–Trp–Leu was determined by UV spectrophotometry at 280 nm using an absorption coefficient of 12.56 l/g per cm.

2.2.2. Permeation experiments

Permeation measurements of Myr–Trp–Leu across the buccal barrier were performed as described previously using modified Franz diffusion cells (Veuillez et al., 1998, 1999). The receptor medium consisted of 2.2 ml of a 30:70 v/v mixture of ethanol:phosphate buffered saline (PBS), pH 7.4 (Ph. Helv. VIII) and was constantly stirred with a teflon-coated magnetic bar. The diffusion cell was maintained at 37 °C. The tissue was equilibrated for 30 min with the receptor medium, before 0.5 ml of a solution of Myr–Trp–Leu (6 mg/ml in ethanol:PBS pH 7.4, 30:70 v/v) was added to the donor compartment. To prepare the solution of Myr–Trp–Leu, the peptide was solubilized in pure ethanol, before adding the necessary volume of buffer; a clear solution was thus obtained. The transmucosal permeation of the peptide was evaluated by withdrawing (and replacing with fresh ethanolic buffer), 100 μl of the receptor fluid at 8, 16, 24 and 48 h. The peptide contents in the donor compartment and in the tissue (see below) were quantified by HPTLC. In a subsequent experiment, the buccal tissue was pretreated with ethanolic buffer for 8 h prior to the application of Myr–Trp–Leu for a further 16 h.

2.2.3. Determination of the amount of Myr–Trp–Leu in the tissue

At the end of the experiment, the tissue was carefully rinsed on both sides with the ethanol: PBS solution. It was then fixed with tragacantha on a piece of cork, immersed in liquid nitrogen for 20 s, glued with Tissue-Tek® OCT and Cryolab®. About 60 μm cryotat sections (Frigocut N 2800 Reichert-Jung, Merck, Switzerland) were obtained at -40 °C by cutting in the plane of the tissue parallel to the epithelial surface. Four samples in the same anatomical region from four different pigs were evaluated.

Each 60 μm thick cryostat section was finely disrupted and extracted in 2 ml of chloroform for 24 h at room temperature. The resulting suspensions were centrifuged and the supernatant filtered through a membrane filter (0.45 μm, polypropylene micro-centrifuge tube filters, Whatman, Maidstone, England). The clear solutions were assayed by HPTLC as described below (Veuillez et al., 1998).

2.2.4. Analysis by high performance thin-layer chromatography (HPTLC)

The contents of the various compartments were assayed by HPTLC on silica gel 60 (Merck, Darmstadt, Germany) using the Camag Linomat IV (Camag, Muttenz, Switzerland). Standard solutions of increasing concentrations were applied on each plate for calibration. The solvent for HPTLC was chloroform:methanol: water (16:6:1). Plates were dried at 45 °C for 20 min and scanned in the Refl-Abs mode with the CAMAG TLC Scanner II (Switzerland) at 280 nm. Quantification was obtained by integrating the signals (CAMAG SP4290 integrator) with a limit of quantification of 35 ng (Veuillez et al., 1998).

2.2.5. Determination of ethanol in the donor compartment at the end of experiment

The concentration of ethanol in the donor compartment was determined by gas chromatography (Hewlett Packard, HP 6890 Germany) equipped with a HP 7683 column injector and a hydrogen FID detector. Samples from the permeation experiments at 8, 16, 24 and 48 h were assayed using *n*-propanol (300 μl/l) as an internal standard and aqueous solutions of ethanol (1, 2, 3 g/l) as the primary standard. The temperatures at the injector column and the detector were 165 and 250 °C, respectively. Nitrogen was used as the carrier at a flow rate of 30.0 ml/min. The hydrogen FID detector was used at a flow rate of 35.0 ml/min. A Porapak Q 80/100 glass column-internal diameter-2 mm, length 1.8 m (Supelco Cat. No 2.0331) was

employed. The results were integrated with Workstation HPChem.

2.2.6. Statistical analysis

The data were evaluated for statistically significant differences by a one way analysis of variance (ANOVA). The probability level was set at 5%. All data were presented as mean ± S.D.

2.2.7. Light microscopy

In order to evaluate the effect of ethanol on the buccal mucosa, a 30% ethanolic solution in PBS pH 7.4 (v/v) was applied to the tissue for 8, 16, 24 and 48 h. Control experiments with only buffer in absence of ethanol after 0.5, 24 and 48 h were also conducted. At the end of the exposure period, small portions of tissue were fixed in 10% buffered formalin solution and dehydrated. All specimens were then embedded in paraffin wax according to standard procedures (Summerlin et al., 1992) and compared with control cryostat preparations. Sections were cut at 4 μm perpendicular to the epithelial surface. They were then stained with hematoxylin eosin (HE) in the normal way (Du Bois, 1966).The sections were examined by light microscopy (Zeiss Axiophot, West Germany) to evaluate any histological changes, in the epithelium and the adjacent connective tissue.

3. Results and discussion

3.1. Permeation experiments at 24 h

With the objective of improving its absorption, Trp–Leu was acylated with myristic acid at the N-terminal position. The derivative was, of course, a more lipophilic compound (log *K*p (oct/water) = 1.04 ± 0.26; Rf in HPTLC = 0.81) than Trp–Leu (log *K*p (oct/water) = −0.68 ± 0.01; Rf in HPTLC = 0.41). In terms of permeation, the acylation of the peptide resulted in its marked accumulation in the buccal mucosa: about 70% uptake after 24 h permeation (Veuillez et al., 1998). However, this extremely high affinity of Myr–Trp–Leu for the tissue meant that no detectable amount was observed in the receptor chamber using HPTLC; that is, the derivative was retained in the mucosa and not 'released' into the receptor phase. A minimum of 30% ethanol was required to solubilize Myr–Trp–Leu and, therefore, all permeation experiments were performed with a 30/70 ethanol/PBS pH 7.4 solution present on both sides of the mucosa. The distribution of Myr–Trp–Leu as a function of depth in the buccal mucosa after 24 h permeation is shown in Fig. 1. Essentially, all of the product was localized between 120 and 420 μm, with the highest concen-

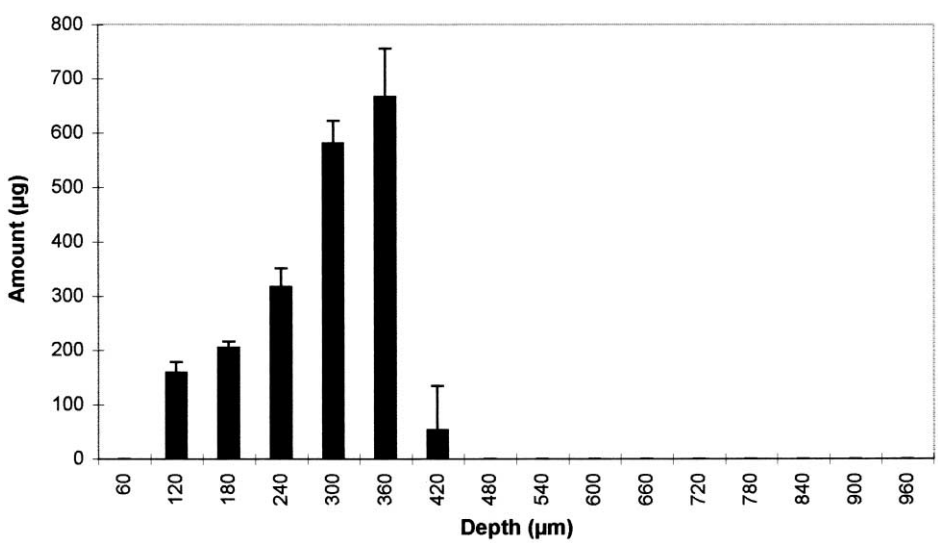

Fig. 1. Distribution of Myr–Trp–Leu across the buccal mucosa after 24 h permeation ($n = 4 \pm$ S.D.).

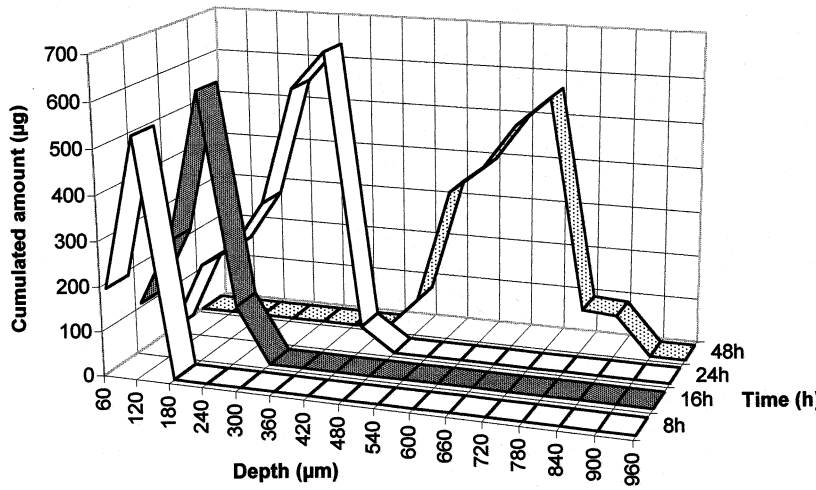

Fig. 2. Concentration profile of Myr–Trp–Leu as a function of time in buccal tissue ($n = 4$).

tration at 360 µm. No peptide was detected in the first 60 µm of tissue, facing the donor compartment; the amount present at 420 µm was very low and was effectively undetectable beyond 480 µm to tissue depth.

3.2. Distribution of Myr–Trp–Leu in the tissue as a function of time

It was important to determine the evolution of peptide transport across the barrier and to determine its distribution in specific tissue layers as a function of time.

Fig. 2 shows that, after 8 h of permeation, the product was found mainly in the outer layers of the barrier. At 16 h, Myr–Trp–Leu had reached the deeper epithelial layers. At 24 h, the permeant was still concentrated in the epithelium (Squier, 1973; Squier and Rooney, 1976) but it had begun to diffuse further into the tissue. The main epithelial barrier has been measured to have a thickness of 282 ± 17 µm (De Vries et al., 1991). At 48 h, Myr–Trp–Leu was found in the submucosa, (i.e. the connective tissue) and was no longer present in the superficial layers of the epithelial layer. It is noteworthy that, after diffusion for 24 h or more, the amount of peptide in the outer membrane layers facing the donor compartment was very small despite the fact that the peptide concentration in the donor compartment was far from being fully depleted (Fig. 3).

A possible explanation for this observation is that the diffusion of ethanol into the membrane was such that an insufficient amount remained in the donor to maintain an appreciable quantity of the acylated peptide in solution. The level of ethanol in the donor did indeed decrease with increasing time of diffusion, with the initial concentration ($30.1 \pm 0.8\%$) falling over the course of 48 h to $24.4 \pm 2.0\%$. The profile of peptide concentration across the buccal mucosa as a function of time somewhat resembles a chromatographic-type process, presumably driven by the migration of ethanol. This conclusion is consistent with the experiment in which the tissue was pretreated with ethanol for 8 h prior to peptide application (Fig. 4). The enhanced amount of solvent present in the membrane clearly permitted the peptide to penetrate into the deeper tissue layers and broadened the concentration profile over a greater distance within the barrier.

Parenthetially, it should also be noted that HPTLC revealed no breakdown of the prodrug in the donor phase or in the tissue, i.e. the compound retained its integrity thoughout the course of the experiments performed.

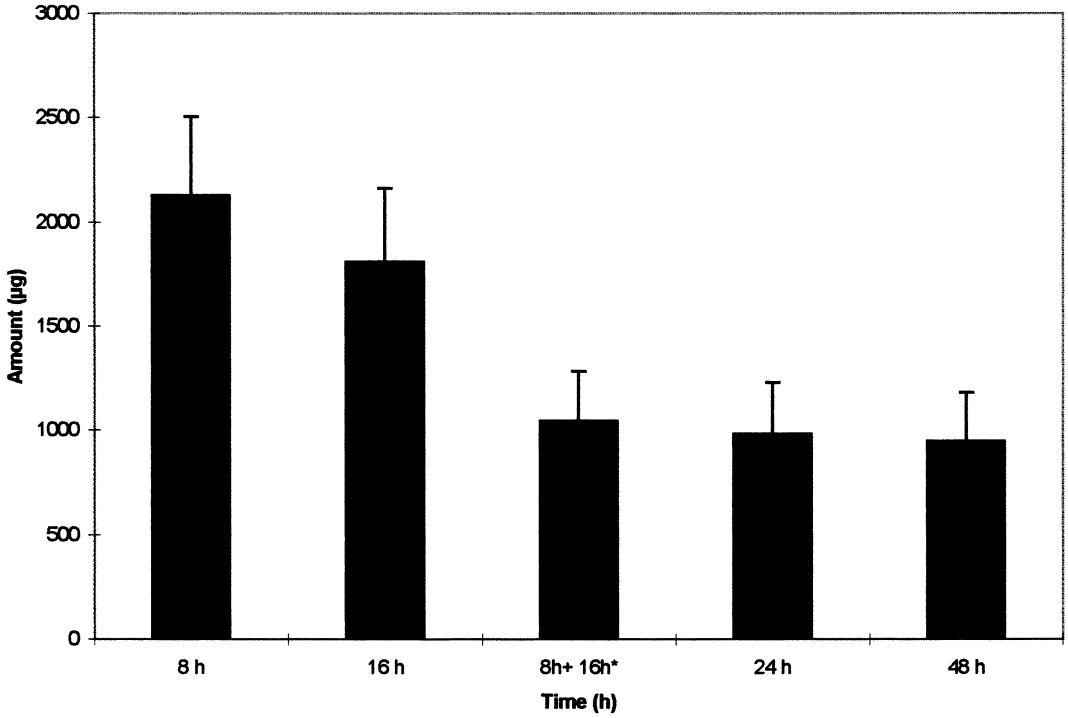

Fig. 3. Amount of peptide recovered in the donor compartment at the end of the permeation experiments.

3.3. Histological studies

Parallel to the permeation experiments, the effect of ethanolic buffer on the buccal mucosa was studied. No modification was observed under direct visual observation. Integrity and appearance (color, smoothness) of the mucosal surfaces, in comparison with the surrounding tissue which was

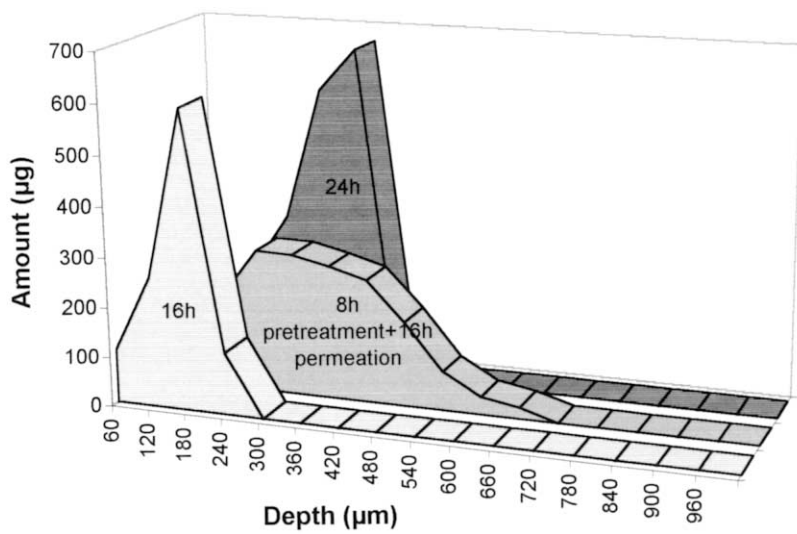

Fig. 4. Effect of ethanol pretreatment for 8 h on the subsequent permeation of Myr–Trp–Leu for 16 h.

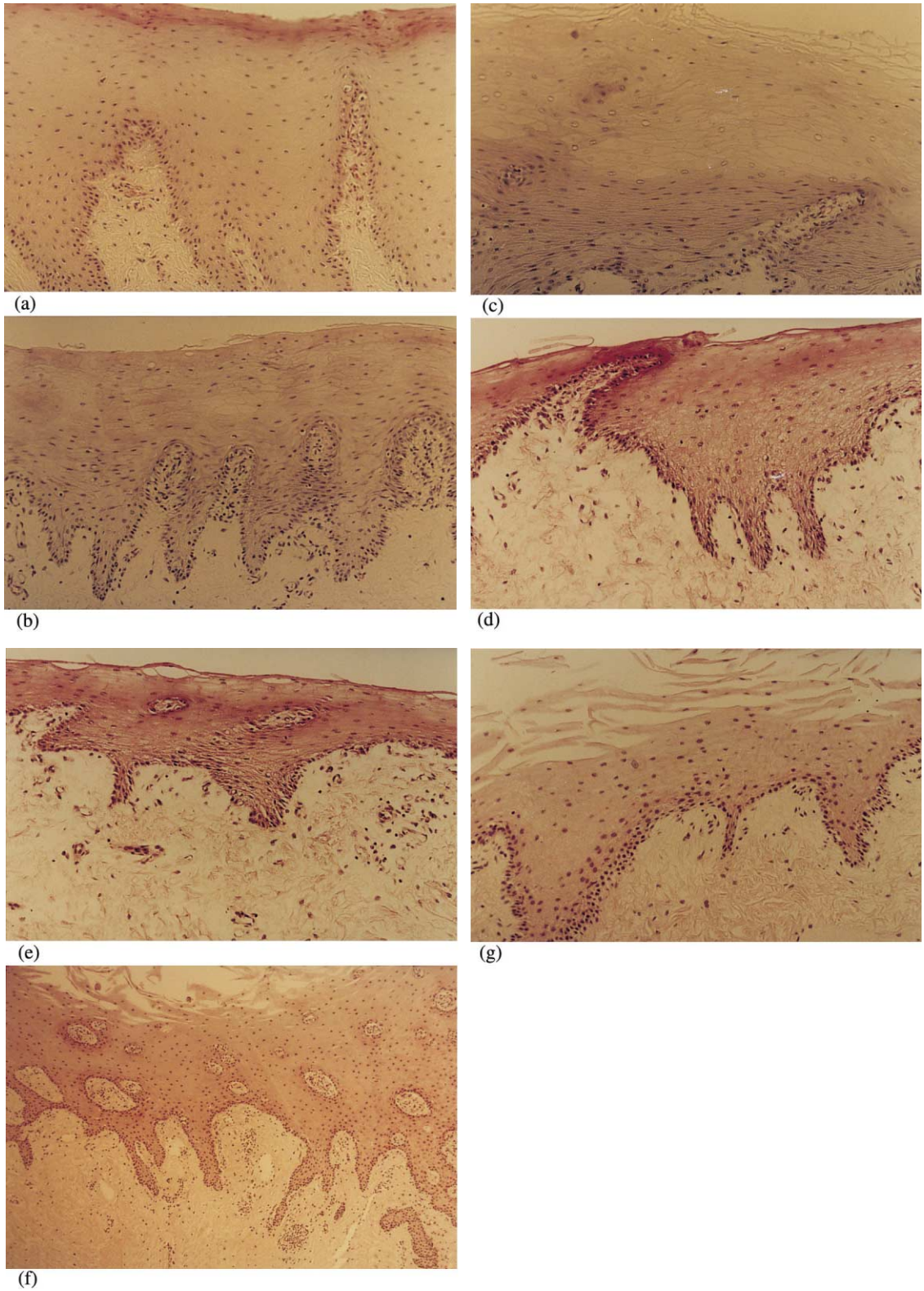

Fig. 5. (*Continued*)

not in direct contact with the solvent, appeared preserved. The tissue specimens examined by light microscopy showed little modification to the epithelial layer, with this superficial barrier always maintaining its integrity. When compared with the controls, the specimens in contact with ethanol were minimally perturbed (Fig. 5a, b). Contact with 30% ethanol for 8, 16 and 24 h, respectively, resulted in only minimal desquamation of the superficial epithelial layer with slight interstitial oedema and swelling of the uppermost epithelial cells. This phenomenon increased somewhat with time (Fig. 5d, e, f). Therefore, buffered ethanolic solution did little or no damage to the structure of the buccal epithelial mucosa. The epithelium and basal membrane, the principal components of the permeation barrier, appeared well-preserved at the end of the different exposure periods. At 48 h exposure to the ethanol medium, desquamation and slight oedema were more pronounced than at 24 h (Fig. 5g). Nevertheless, it is clear from the results of the permeation experiments that no dramatic alterations in the barrier function of the tissue had been provoked by even the longest exposure of the tissue to the ethanolic buffer.

4. Conclusions

The increased lipophilicity of Myr–Trp–Leu resulted in its facile uptake into the buccal membrane. However, complete transport of the prodrug through the tissue was not observed. Although, there was evidence at 24 h that the peptide had begun to diffuse from the outer epithelium (the principal barrier). The concentration profile of the permeant across the membrane as a function of time reveals chromatographic-like behaviour and an essential role for ethanol. Pre treatment of the tissue with ethanol confirmed that the solubilizing capacity of the solvent was critical to the peptide's distribution across the tissue and to its ability to leave the donor compartment and enter the membrane. Histological examination of the mucosa after exposure to the ethanolic buffer vehicle employed revealed either no, or only slight, modification of the tissue.

The strategy used here would appear to have promise, therefore, for controlling the delivery of a drug with a narrow therapeutic index to the systemic compartment, without a 'burst' effect. Clearly, the myristoyl linkage to Trp–Leu would be too lipophilic to be useful clinically (as the compound has such high apparent affinity for the mucosa); nevertheless, this work offers a useful starting point, we believe, for the future optimization and evaluation of this drug delivery strategy.

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Fig. 5. (a) Porcine buccal mucosa emerged in buffer solution pH 7.4 for 30 min, (HE) 160X. Note the thin keratinized layer of about three layers. The remainder of the epithelium is normal and the nuclei are regular, horizontal. (b) Porcine buccal mucosa after 24 h contact with buffer solution pH 7.4, (HE) 160X. There is little or no modification of the epithelium NB: the keratinized layer is not shown. (c) Porcine buccal mucosa after 48 h contact with buffer solution pH 7.4, (HE) 160X. Note interstitial oedema of the lower half of the epithelium associated with the ballonning of the nuclei in that zone. (d) Porcine buccal mucosa after 8 h contact with 30% ethanol, (HE) 160X. The section is slightly oblique, there are no histological modifications from the proceeding images. (e) Porcine buccal mucosa after16 h contact with 30% ethanol, (HE) 160X. Thee are bridges in the keratinized layer suggestive of early desintegration of the epithelial layers, while the remainder of the epithelium is more condensed. (f) Porcine buccal mucosa after 24 h contact with 30% ethanol, (HE) 160X. The section is oblique, however, one notes the swelling of the epithelial cells whose nuclei are of variable sizes. (g) Porcine buccal mucosa after 48 h contact with 30% ethanol, (HE) 160X.The swelling and desquamation of the epithelial cells attain almost the entire thickness of the epithelium. Note the nuclei are of variable sizes.

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