CARBAMAZEPINE TRANSBUCCAL DELIVERY: THE HISTO-MORPHOLOGICAL FEATURES OF RECONSTITUTED HUMAN ORAL EPITHELIUM AND BUCCAL PORCINE MUCOSAE IN THE TRANSMUCOSAL PERMEATION

G. CAMPISI, C. PADERNI, R. SACCONE, M.G. SIRAGUSA[1], L. LO MUZIO[2], C. TRIPODO[3], L.I. GIANNOLA[1] and A.M. FLORENA[3]

Department of Oral Sciences, University of Palermo; [1]Department of Chemistry and Pharmaceutical Technologies, University of Palermo; [2]Department of Surgical Sciences, University of Foggia; [3]Department of Human Pathology, University of Palermo, Palermo, Italy

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Transbuccal drug delivery is an attractive way of administration since several well-known advantages are provided, especially with respect to peroral management. Carbamazepine (CBZ) is an anticonvulsant which is useful in controlling neuropathic pain, and it is currently administered by peroral route, although its absorption and bioavailability is limited due to various factors. The oral cavity could be an interesting site for transbuccal CBZ delivery due to two properties: slow administration of constant low drug doses and less dose-related side effects. However, in transbuccal absorption a major limitation could be the low permeability of the mucosa which results in low drug bioavailability; thus the aptitude of the drug to penetrate the buccal mucosa has to be assessed by using tissue models resembling human normal mucosa. In our experience, CBZ well permeates mucosal membranes. In order to assess the efficacy of CBZ transbuccal delivery and to verify the reliability of these tissues in permeability testing before and after the passage of CBZ, the histo-morphological features of reconstituted human oral (RHO) epithelium (E) and buccal porcine mucosae were investigated. Significant histological changes due to CBZ passage were observed both in RHO-E and porcine mucosa. The main findings detected in RHO samples were cellular swellings with a signet ring-like appearance, nuclear swelling, prominent nucleoli lined against the nuclear membrane and the presence of keratohyalin granules. The most striking finding regarding porcine buccal mucosa was a cytoplasmic vacuolization, mainly involving the basal layer.

Buccal delivery offers a feasible alternative to parenteral delivery and many advantages to *per os* administration, e.g. reduced first pass metabolism, reduced drug decomposition and adverse effects, improved drug bioavailability, and often a more rapid onset of therapeutic effect (1).

Carbamazepine (CBZ) is a drug which could be very attractive if delivered transbuccally. CBZ is really useful in controlling neuropathic pain (2) and it is currently delivered *per os*, using doses ranging from 200-600 mg/die administered in divided doses. Following peroral administration, absorption and

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Mailing address: Dr Giuseppina Campisi,
Dept. of Oral Sciences,
Via del Vespro, 129
90127 Palermo, Italy
Tel: ++39 0916552236 – 0916552221
Fax: ++39 0916552236
e-mail: campisi@odonto.unipa.it

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bioavailability of CBZ is limited due to its physico-chemical and pharmacokinetic parameters. The oral cavity could be an attractive site of delivery also for CBZ due to slow and constant administration of low drug doses with less dose-related side effects.

A comprehensive knowledge of the structure of human buccal mucosa, permeability barriers and transport pathway mechanisms is important for drug development and optimization, and for related transbuccal delivery systems. Studies on drug transport across buccal epithelium have been carried out in the past using several methodologies. Indeed, the reduced availability of human buccal tissue for experimental use has led to the development of cultured tissues and/or application of animal tissue which resembles human buccal mucosa. Two laboratory models have been largely used in recent decades for rapid and efficient examination of permeability, metabolism and toxicity of drugs: *in vitro* tissue-engineered oral mucosal equivalents (3-4), and *ex vivo* buccal mucosa from various animal models (5).

The Reconstituted Human Oral (RHO) epithelium (E) is a cell culture model obtained from TR146 cell line. This type of cells, coming from human neck node metastases of a buccal carcinoma, when cultivated *in vitro* on polycarbonate filters in defined conditions, form an epithelial tissue resembling the mucosa of the oral cavity (6-7). Due to its similarities to human epithelium, RHO-E has recently been utilized as a valuable model for studying drug transport (8).

Many animal models of oral mucosa have been used for *ex vivo* permeability studies: the most commonly used are dogs, rabbits, hamsters, Rhesus monkeys, guinea pigs, rats and pigs (5). When choosing a particular animal model, the main requirement is the resemblance of animal oral mucosae to human oral mucosae regarding ultra-structure and enzyme activities, including physical and metabolic barriers, although no animal tissues can completely resemble human tissue. The porcine buccal mucosa has been used most frequently by various leading research groups as a representative model, resembling human buccal epithelium more closely than any other animal model in terms of lipid content and composition, membrane morphology and permeability barrier functions, structure and composition (5). In particular, the pig seems to be the most attractive animal model for buccal drug delivery since its buccal and mouth floor mucosae contain unkeratinised tissue, as in human beings. Other advantages are their inexpensive handling and maintenance costs (9). Pig excised buccal tissue, mounted in an appropriate diffusion cell system (mainly the vertical Franz-type diffusion cells), has been used in most *ex vivo* studies (10).

The main objectives of this study are to assess the histo-morphological features of RHO epithelium and buccal porcine tissues before and after CBZ passage and to verify the reliability of these tissues in the permeability testing of CBZ and its potential transmucosal delivery.

MATERIALS AND METHODS

Permeation test using RHO

The permeation of CBZ was investigated *in vitro* by measuring drug fluxes through RHO-E, cultured on permeable polycarbonate inserts (11). Briefly, mucosal specimens, non-keratinised type (RHO/S/12), were purchased from Skinethic Laboratories® (Nice, France). Upon arrival, the bags containing the inserts with cultured cell layers were opened under sterile airflow. Each insert (0.5 cm^2) containing the epithelial tissue was removed, and any remaining agarose adhering to its walls was rapidly removed by gently blotting it on sterile filter paper, and it was then placed in culture dishes filled with maintenance medium (Skinethic® Lab. Nice, France). Some inserts, not subjected to the experimental phase, were used as blank. Those samples subjected to the experimental phase in absence of drug were used as permeation controls. The remaining inserts were used for drug permeability tests. Experiments were performed at a constant temperature of 37°C, using the Transwell diffusion cell system (Fig. 1a) as a two-compartment open model (11).

The epithelial tissue was cut out from the plastic insert, together with the polycarbonate filter, using a sharp scalpel for histological analysis. The filter samples were fixed in 10% neutral-buffered formalin for two hours, washed in water for 1 hour, dehydrated in graded ethanol (60%, 80%, 90%, 95%, and 100%) and, after permeation in xylene, embedded in paraffin using standard procedures. Formalin-fixed, paraffin-embedded samples were cut into 4-μm-thick sections on a microtome with a disposable blade and conventionally stained with hematoxylin-and-eosin.

Permeation test using porcine mucosa

The drug permeation through the porcine buccal

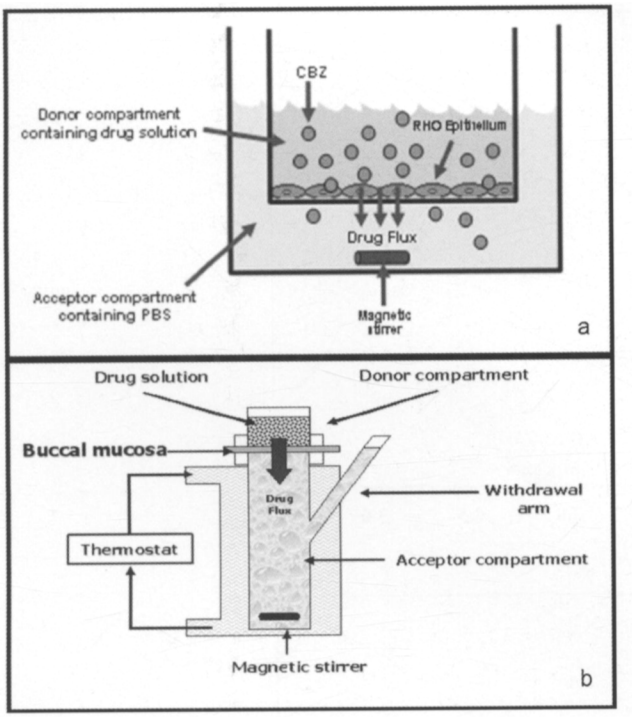

Fig. 1. ***a)*** *Schematic representation of the Transwell system, an open two-compartment model used for the in vitro permeation tests with RHO-E;* ***b)*** *Schematic representation of the Franz-type diffusion cell system: it is an open bi-compartmental model in which the donor compartment was filled with a solution containing CBZ and the acceptor compartment was filled with a solution simulating plasma.*

mucosa was evaluated using Franz type diffusion cells (Fig. 1b) (11). Mucosal specimens were obtained from tissue, which had been removed from freshly slaughtered domestic pigs. After sampling, all specimens were immediately placed in a refrigerated transport box and transferred to the laboratory within 1 h. Excesses of connective and adipose tissue were trimmed away until slides 0.8 ± 0.1 mm thick were obtained. The specimens were stored at -40°C for periods of up to six months. Some specimens, not subjected to the experimental phase, were used as blank. Those specimens subjected to the experimental phase in absence of drug were used as permeation controls. The remaining specimens were used for drug permeability tests. Experiments were performed at a constant temperature of 37°C. Mucosal specimens were fixed in formalin, embedded in paraffin, cut into 4-µm-thick sections and stained with haematoxylin-eosin, using the above-mentioned standard procedures.

Statistical Analysis

Flux and permeability coefficient values, obtained as average value of six replicated experiments, were reported with the standard deviations. All differences were statistically evaluated by the Student's *t*-test with the minimum levels of significance with $P \leq 0.05$.

Histological criteria

Slides stained with hematoxylin-and-eosin were evaluated using optical microscopy in a blind and independent fashion. Digital photomicrographs of relevant fields were taken at 250x, 400x and 1000x magnification. Two different types of negative controls were included in the histomorphological analysis: blank controls and permeation controls. As the aim of the histomorphological analysis was to evaluate the pathological changes occurring in cell morphology and tissue organization, samples treated with 5-fluorouracil (5-Fu) were included in the analysis as markers of cytopathic alterations. The presence of acanthosis, hyper/parakeratosis, koilocytosis, corneal pearls, papillomatosis and phlogosis were evaluated for histological analysis.

RESULTS

The CBZ permeation pattern through buccal mucosae was investigated by using two different bi-compartmental open systems: a Transwell diffusion cells system for RHO-E (*in vitro* experiments) and Franz cells for porcine buccal mucosae (*ex vivo* experiments). The permeability tests were carried out for 3 hours in order to avoid changes in permeability characteristics of the tissues.

In both cases, the amount of CBZ permeated per unit surface area was plotted versus time (Figs. 2 and 3). Permeation through RHO-E was characterized by an initial lag time of about 5 min (Fig. 2b), during which CBZ diffused into the blood stream, although the amount of transferred drug was not easily detectable. The steady state flux was reached in 12 min. For porcine mucosal tissue the steady state flux was reached in about 30 min, as shown in the linear part of the plot in Fig. 3. The linear part of the curves was used for the determination of the steady state flux values (Js) through the membrane and the permeability coefficients (Kp), by means of a standard resolution of the Fick equation for steady state membrane transport. Js and Kp values for CBZ *in vitro* experiments were $7 \cdot 10^{-2}$ mg/cm^{2}h and 0.23 cm/h, respectively, whereas Js and Kp for the *ex vivo*

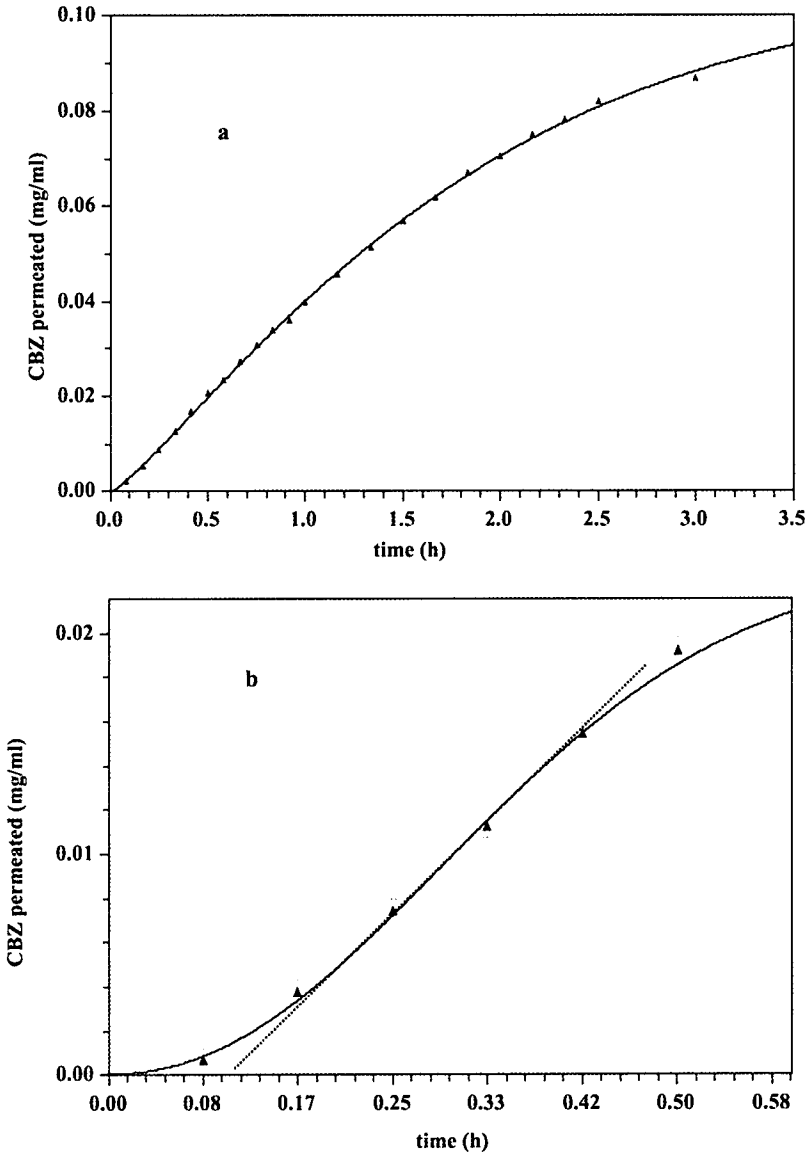

Fig. 2. *In vitro permeation of CBZ throughout reconstituted human oral epithelium:* ***a)*** *cumulative amount–time profile;* ***b)*** *results observed in the first 30 minutes of permeation show an initial lag time of about 5 min. Drug flux was calculated at the steady state from the slope of the linear portion of the plot.*

experiments were $1.81 \cdot 10^{-2}$ mg/cm^{2}h and $4.57 \cdot 10^{-2}$ cm/h, respectively. As expected, the flux through the mucosae was extensively affected by the thickness of the membrane.

After permeability tests, significant histological changes due to permeability of CBZ were observed in the RHO and porcine mucosae. Regarding the former, the two types of negative controls, blank controls and permeation controls revealed an unkeratinized squamous cell epithelium, composed of an average of 5-8 cellular layers. No significant cytological or architectural changes were highlighted (Fig. 4a).

The histological analysis of RHO samples submitted to CBZ diffusion revealed pronounced alterations involving the entire thickness of the membrane; cellular swelling with a signet ring-like appearance, nuclear swellings, prominent nucleoli lined against the nuclear membrane and the presence of keratohyalin granules were the main findings

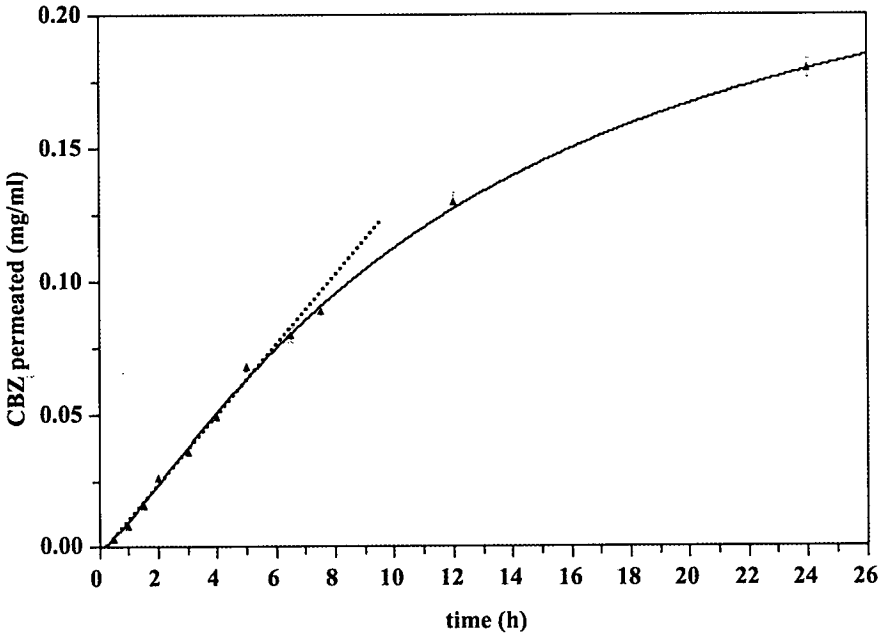

Fig. 3. *Cumulative amount–time profile of CBZ permeated across porcine buccal mucosa during ex vivo experiments.*

(Fig. 4b). Apoptotic figures and signs of abrupt keratinisation were occasionally observed together with cellular disarray with loss of cell polarity (Fig. 4c). In particular, the cells appeared vacuolated and this is likely ascribable to the presence of an intracytoplasmic fatty content due to the presence of CBZ. Other samples revealed less marked alterations with an effacement of the upper 2/3 and a preserved basal layer.

The negative controls of porcine buccal mucosae showed unkeratinized squamous cell epithelia, composed of an average of 20-30 cellular layers. No significant cytological or architectural changes were observed. A slight degree of dysplasia was observed in some samples, as maturational defects of the intermediate layer, and a mild loss of nuclei polarity (Fig. 4d).

The histological evaluation of porcine buccal specimens, submitted to CBZ diffusion, revealed alterations similar to those of the RHO samples. No sign of phlogosis was found in any of the mucosal specimens. The most striking finding was a cytoplasmic vacuolization which mainly involved the basal layer (Fig. 5a). A moderate degree of acanthosis with some piknotic nuclei was present, but neither papillomatosis, nor hyper/parakerathosis, nor horn pearls were detected (Fig. 5b).

The RHO and porcine samples treated with 5-Fu, as marker of cytopathic alterations, showed cytopathic effects more evident than those found in all samples treated with CBZ (Fig. 5c, d).

DISCUSSION

Anticonvulsants are a group of medicines commonly used for treating 'fits' or epilepsy, but which are also effective for treating neuropathic pain. Of these, CBZ is currently the only medication carrying an FDA-approved indication for neuropathic pain which is specified for trigeminal neuralgia and glossopharyngeal neuralgia (12-14). CBZ acts pre-synaptically and post-synaptically by slowing the recovery of sodium ion channels after activation (15). Therapeutic plasma levels range from 4 to 12 μg/mL and the half-life of CBZ varies from 10 to 25 hrs in adults, depending on the patient's age. The most common side effects of CBZ are dizziness, giddiness and dyspepsia. These symptoms are dose-related, and can be minimized by starting with low doses (16).

CBZ is currently administrated at a dosage of 200-600 mg daily, given in divided doses (e.g. every 4 or 8 hours), starting with low initial doses.

Following application to mucosae, the

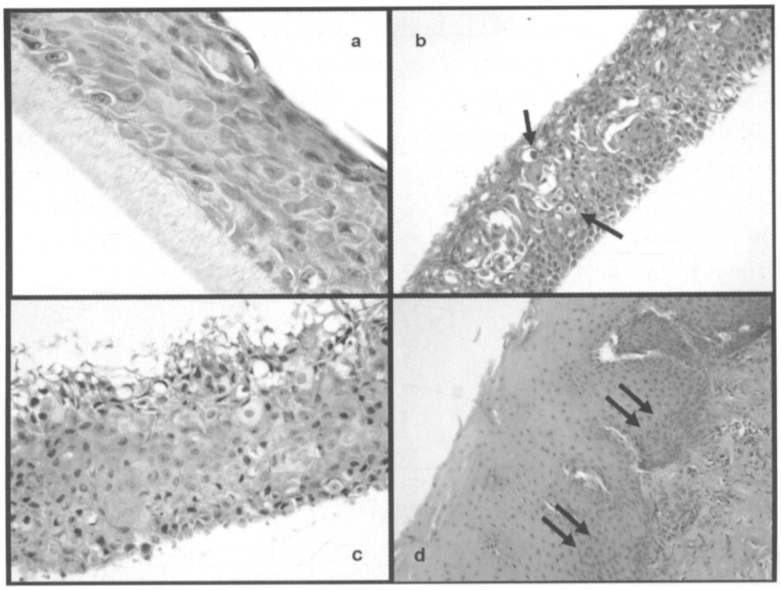

Fig. 4. ***a)*** *RHO sample not subjected to the experimental phase. The picture shows a non-keratinized squamous cell epithelium (five cellular layers) with no significant cytological or architectural changes (H-E x400);* ***b)*** *RHO sample characterized by marked cellular disarray with loss of cell polarity, signs of microvacuolization and sporadic piknotic nuclei (see arrows) (H-E x400);* ***c)*** *RHO sample submitted to CBZ diffusion shows pronounced alterations involving the whole membrane thickness, cytoplasmic and nuclear swelling. Prominent nucleoli lined along nuclear membrane and keratohyalin granules are visible (H-E x250);* ***d)*** *Porcine blank control. The picture shows a non-keratinized squamous cell epithelium composed by an average of 20-30 cellular layers with slight degree of dysplasia in the form of maturational defects (see arrows) (H-E x250).*

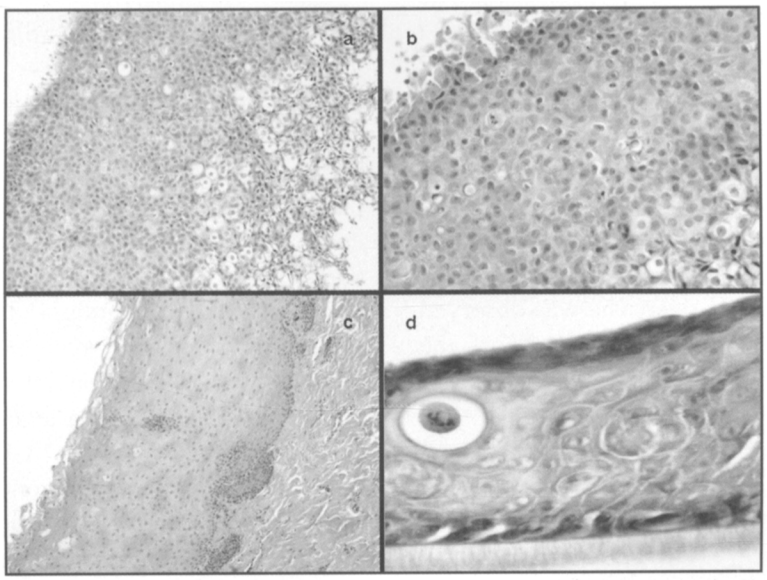

Fig. 5. ***a)*** *This section shows a moderate degree of cytoplasmic vacuolization mainly involving the basal layer along with slight acanthosis (H-E x400);* ***b)*** *At higher magnification nuclear picnosis is clearly evident (H-E x1000);* ***c)*** *Porcine buccal specimen treated with cytotoxic 5-Fu 1% with acanthosis and many pyknotic nuclei; d) RHO sample treated with cytotoxic 5-Fu 1% showing marked nuclear changes with prominent nucleoli and apoptotic cells (H-E x400).*

therapeutic efficacy of a drug for transepithelial delivery mainly depends on its ability to penetrate the tissue fast enough to provide the required plasma concentrations, which result in the desired pharmacological activity. Indeed, the most important limitation in the development of a buccal drug delivery device could be the low permeability of the buccal mucosae. To assess the ability of CBZ to penetrate the barrier and define the permeation pattern, permeation tests were performed using two different bi-compartmental open systems. Firstly, we used the RHO-E model. This tissue, at the 12th day of culture, has about 4-6 cell layers and it is about 120 μm thick (6) whereas the human buccal mucosa comprises approximately 40-50 cells and it is 500-800 μm thick (about 12.5-20.0 μm for each layer). Secondly, to obtain further permeation data, *ex vivo* permeation tests, using 800 µm thick porcine buccal specimens resembling human mucosa, were performed. In both cases the results of permeability tests were expressed using drug flux values (Js) through the membrane and permeability coefficients (K_p). CBZ, when topically administered, effectively permeated the membrane and no drug was entrapped in the cell layers, thus suggesting that the membrane did not constitute a limitation to absorption.

The aim of this study was to perform a histological analysis of epithelial specimens. The main finding was the diffuse clear cytoplasmic appearance, which is probably an effect of intracellular fat, and likely due to intracellular drug diffusion. This finding was abundantly present in RHO samples and specimens of porcine mucosae; the discrepancy regarding the degree of involvement (transmural in RHO membranes, mainly concentrated in the basal layer in the *ex vivo* specimens) has been ascribed to the different thicknesses of the samples. The cytopathic effects observed in RHO and porcine samples treated with CBZ were less intense than those found in samples treated with the potent cytotoxic drug 5-Fu, thereby suggesting that CBZ is not a cytotoxic drug.

On the basis of our results, we have established that CBZ permeates mucosal membranes well, although its permeation across epithelium did cause some cyto-architectural changes. CBZ is not a cytotoxic drug but further investigation is required in order to assess its potential for transmucosal drug delivery.

RHO-E (*in vitro*) and porcine buccal mucosa (*ex vivo*) have been shown to be two reliable and reproducible permeation test models. Even if we observed some minor different histomorphological behaviour, ascribable to the diverse thickness of the tissues used, both models have similar performances after drug passage. The validity of these mucosal models is confirmed by several data, indicating a good correlation of human normal mucosa with porcine and tissue-engineered human oral mucosal equivalents in terms of histology, ultrastructure and organization of the permeability barrier.

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