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New prospective in treatment of Parkinson's disease: Studies on permeation of ropinirole through buccal mucosa

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ABSTRACT

The aptitude of ropinirole to permeate the buccal tissue was tested using porcine mucosa mounted on Franz-type diffusion cells as *ex vivo* model. Drug permeation was also evaluated in presence of various penetration enhancers and in iontophoretic conditions.

Ropinirole, widely used in treatment of motor fluctuations of Parkinson's disease, passes the buccal mucosa. Flux and permeability coefficient values suggested that the membrane does not appear a limiting step to the drug absorption. Nevertheless, an initial lag time is observed but the input rate can be modulated by permeation enhancement using limonene or by application of electric fields. Absorption improvement was accompanied by the important reduction of the lag time; at once the time required to reach the steady state plasma concentration was drastically decreased.

On the basis of these results we could assume that clinical application of ropinirole by buccal delivery is feasible.

1. Introduction

The degeneration of pre-synaptic dopaminergic neurons of the basal ganglia is the main origin of abnormalities in the control of voluntary movement in Parkinson's disease (PD) patients. Currently, L-DOPA is considered as the standard treatment for PD since it initially provides a stable therapeutic response; unfortunately, during long-term treatment, its beneficial effect declines. The main problem with the early use of L-DOPA is its tendency to induce motor complications. This is a particular problem for young onset PD patients who are at a greater risk of developing motor complications and who have to endure this disability over the course of a long and chronic illness. In the management of PD, it is important to minimize the development of motor fluctuations and to postpone them as long as possible. For this purpose, the use of other drugs such as ropinirole (ROP) offers a valid option before initiation of L-DOPA therapy (Ahlskog, 2011; Azeem et al., 2009).

ROP (IUPAC name: 4-[2-(dipropylamino)ethyl]-2-indolinone hydrochloride) is a potent non-ergoline dopamine receptor agonist with high relative specificity and full intrinsic activity at the D_2/D_3 receptors; its affinity for the D_3 receptor is at least 100-fold greater than for the D_2 receptor. ROP is also approved for the treatment of Restless Legs Syndrome (Ahlskog, 2011; Nanaki et al., 2012). ROP stimulates striatal dopamine receptors to produce dopamine. It is being increasingly used as monotherapy in the initial treatment of PD rather than as adjunct to L-DOPA. ROP is also efficacious in the management of more advanced PD in patients experiencing motor complications after long-term L-DOPA use. The usual dose of ROP is 3–9 mg daily to be taken in three divided doses owing to its short half-life (6 h). Multiple daily dosing may lead to decreased medication compliance and unsteady plasma concentrations which can contribute to the occurrence of motor fluctuations (Azeem et al., 2012).

The immediate-release oral formulation of ROP (ROP-IR) is approved for use as monotherapy in the treatment of early-stage PD and as adjunct to L-DOPA therapy in more advanced disease. ROP-IR has already been shown to reduce dyskinesias in long-term studies and is rapidly and almost completely absorbed, with T_{max} generally reached 1–2 h after dosing. Recently, a new oral formulation for 24 h prolonged release (ROP-PR) was introduced in the market. Several benefits of ROP-PR administration were accurately documented, and findings indicate that the formulation may have a relevant impact on the management of PD in early and advanced stages (Onofrj et al., 2009). Nevertheless, ROP-PR allows the achievement of the steady state conditions after 48 h (Thompson and Vearer, 2007; Nashatizadeh et al., 2009).

ROP has a low molecular weight (MW 260), is sufficiently lipophilic (Log P = 3.32), and like L-DOPA suffers from low

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bioavailability of about 50% by oral route due to extensive first-pass metabolism (Ahlskog, 2011; Contin and Martinelli, 2010).

The adverse events that occurred in greater than 5% of patient treated with ROP are nausea, dyspepsia, headache, dizziness, dyskinesia and orthostatic hypotension (Nashatizadeh et al., 2009). Moreover, a common side effect of D_3 agonists, encountered in clinical practice, is pathological behavior. This includes excessive gambling, hypersexuality, shopping, hyperphagia or obsessive hobbying, which may develop in up to 30% of people taking higher drug doses (Ahlskog, 2011).

To overcome the limitations of the conventional oral ROP therapy, recently the delivery through the skin has been proposed. Transepithelial delivery might help in reduction of administered doses and in overcoming dose-dependent side effects. Topical formulations would be ideal for the geriatric Parkinson population who may be suffering from dysphagia (difficulty swallowing). Approx. 85% of PD patients are over the age of 65, and over 45% have difficulty in swallowing (Azeem et al., 2009, 2012; Reichmann, 2009).

The buccal mucosa could be a favorable site of drug absorption as an alternative transepithelial route; it is non-keratinized and strongly supplied with blood by a dense capillary vessel network. The tissue covers a relatively large drug absorption area. Drugs can thus reach the systemic circulation directly through the capillary vessels, bypassing the first-pass metabolism in the intestine and liver or avoiding inactivation in the stomach. Moreover, delivery of drugs through the buccal mucosa should be considered like a slow i.v. infusion. These characteristics contribute to higher bioavailability parameters after administration of a smaller dose of the drug than in conventional oral formulations (Campisi et al., 2010).

Really, continuous stimulation of striatal dopaminergic receptors, as opposed to discontinuous or pulsatile stimulation, could delay or prevent the onset of motor complications. This hypothesis has arisen from studies of the normal basal ganglia demonstrating that nigral dopaminergic neurons normally fire continuously and striatal dopamine levels are relatively constant (Stocchi, 2009).

The most important reason of buccal ROP administration is certainly the expectation that a continuous drug delivery through the mucosal tissue could guarantee a continuous dopamine replacement and, as a consequence, a continuous dopamine receptor stimulation should be achieved.

2. Materials and methods

2.1. Materials

ROP, USP grade, was kindly supplied by Teva (USA). Lysine hydrochloride (LysC) and trisodium citrate dehydrate (TNaC) were purchased from Polichimica s.r.l. (Bologna, Italy). Limonene, sodium dehydrocholate (NaDHC), and all components of buffer solutions were purchased from Sigma–Aldrich (Milano, Italy). Menthol was purchased from Carlo Erba (Milano, Italy). Buffer pH 6.8 solution simulating saliva was prepared using NaCl (0.126 g), KC1 (0.964 g) KSCN (0.189 g), KH_2PO_4 (0.655 g), urea (0.200 g), $Na_2SO_4{\cdot}10H_2O$ (0.763 g), NH_4Cl (0.178 g), $CaCl_2{\cdot}2H_2O$ (0.228 g) and $NHCO_3$ (0.631 g) in 1 L of distilled water (Gal et al., 2001). Phosphate buffered saline (PBS) Ca^{2+} and Mg^{2+} free solution, pH 7.4, was prepared by dissolving KH_2PO_4 (0.144 g), anhydrous Na_2HPO_4 (0.795 g) and NaCl (9.0 g) in 1 L of distilled water and used as simulated plasma. Saline isotonic solution (pH 7.0) was prepared by dissolving NaCl (9 g) in 1 L of distilled water. All chemicals and solvents were of analytical grade and were used without further purification.

2.2. Ex vivo permeation of ROP throughout porcine buccal mucosa

Porcine mucosal specimens (kindly supplied by Mattatoio Comunale, Villabate, Palermo) were obtained from tissue removed from the inner cheek (buccal area) of freshly slaughtered domestic pigs. After sampling, all specimens were immediately placed in a refrigerated transport box and transferred to laboratory within 1 h. Some specimens were surgically treated to remove excesses of connective and adipose tissue until slides 500 ± 100 μm were obtained. Other specimens were treated using the heat shock method until slides 250 ± 25 μm thick were obtained. For heat separation of the epithelium, the mucosal tissues were dipped for approximately 1 min in saline solution warmed to 60 °C. Then the connective tissue was carefully peeled off from the mucosa to obtain the heat-separated epithelium. The connective tissue was completely removed and the epithelium remained along with the intact basal lamina. The thickness of the tissues was measured using a digital micrometer. Slicing of the tissue with a dermatome was not performed to avoid preliminary freezing which may alter the barrier properties of the buccal mucosa (Kulkarni et al., 2010). Appropriate sections of mucosa were mounted in vertical Franz type diffusion cells. Tissue disks (0.13 cm^2) were equilibrated for 10 min at 37 °C [Polimix EH 2 bath equipped with a constant-rate adjustable stirrer RECO S5 (Kinematica, Switzerland)] adding simulated plasma in the acceptor compartment. In the donor compartment was then placed a ROP solution (10, 20, 30, 45 or 60 mg of ROP in 1.0 mL of buffer solution simulating saliva). At regular time intervals (30 min), samples (0.5 mL) were withdrawn from the acceptor compartment and the sample volume taken out was replaced by fresh fluid.

In all experiments the drug transferred from the donor to the acceptor compartment was monitored by UV spectrophotometric analysis (see Section 2.5). Each experiment was carried out for 6.5 h. Results are reported as means ± SD of six different experiments in which fractions of the same portion of tissue were used ($P < 0.05$). The integrity of the mucosal tissue was monitored after each permeability experiment, according to the method reported early (De Caro et al., 2008). No significant differences were observed using specimens treated surgically or by heat-separation. Heat treatment did not adversely affect on permeability and integrity characteristics of the buccal mucosa (Diaz Del Consuelo et al., 2005).

At the end of each experiment, the residual ROP into the mucosal tissue was detected by extraction. Each sample of buccal mucosa used in the permeation experiments was washed with simulated plasma (3 mL) and than was dipped for 5 min in warmed (50 °C) methanol (1 mL). The extraction was repeated three times. The extraction mother liquors were collected, quantitatively transferred in a 5 mL flask and brought to volume. The amount of drug extracted was evaluated by UV spectrophotometric analysis using the appropriate calibration curve and blank. The same extraction treatment was performed also on mucosal specimens subjected to experimental phase in absence of ROP and used as control. No absorption was observed in the λ range of ROP.

2.3. Ex vivo permeation of ROP throughout porcine buccal mucosa in presence of chemical enhancers

The permeation behavior of ROP in presence of chemical enhancers was investigated using the same methods described in Section 2.2.

Permeation tests in presence of water-soluble chemical enhancers (NaDHC, LysC, TNaC) were performed placing in the donor compartment 1.0 mL of buffer solution simulating saliva containing 45 mg of ROP and 0.05 mg of enhancer. Permeation tests in presence of water insoluble chemical enhancers (menthol, limonene) were performed placing in the donor compartment 5 μL of a solutions containing 0.042 g of chemical enhancer in 1 mL of

Table 1
Calculated steady state flux values, permeability coefficients and lag times for ROP permeation through porcine buccal mucosa.

ROP concentration in the donor chamber (mg/mL)	$J_s(\times 10^{-2})$ (mg/cm^2 h)[a]	$K_p(\times 10^{-2})$ (cm/h)[a]	Lag time (min)
10	6.05 ± 0.421	0.605 ± 0.042	150
20	10.05 ± 0.703	0.503 ± 0.035	95
30	17.56 ± 1.582	0.585 ± 0.053	90
45	15.99 ± 0.164	0.355 ± 0.003	75
60	15.43 ± 0.292	0.249 ± 0.005	45

[a] Values are reported as mean ± standard deviation ($n = 6$).

ethanol. This solution was left on the mucosal tissue for about 5 min to permit the tissue imbibition; the membrane was then conditioned again for 2 h in buffer solution simulating saliva (Nicolazzo et al., 2004, 2005). Following this time, 45 mg of ROP in 1.0 mL of buffer solution simulating saliva were added in the donor chamber. At regular time intervals (30 min), samples (0.5 mL) were withdrawn from the acceptor compartment and the sample volume taken out was replaced by fresh fluid. Each experiment was carried out for 6.5 h. Results are reported as means ± SD of six different experiments in which fractions of the same portion of tissue were used ($P < 0.05$). In all experiments the drug transferred from the donor to the acceptor compartment was monitored spectrophotometrically (see Section 2.5).

The integrity of the mucosal tissue was monitored before and after each permeability experiment (De Caro et al., 2008). At the end of each experiment, the extraction of ROP from mucosal tissue was performed as described before (see Section 2.2).

2.4. Iontophoretic permeation

In the donor chamber of the equipment described in Section 2.2, a silver electrode (active electrode) was placed for anode; in the acceptor chamber a silver chloride-coated silver electrode (reference electrode) was applied for cathode. Prior to chloridation, the silver electrode was dipped in distilled water, ethanol, fuming nitric acid and finally washed with distilled water. The electrode was then treated with 0.1 N HCl and a current of 1 mA was applied for 24 h using silver as cathode (Giannola et al., 2007). The distance between the electrode and the tissue was 2 mm. Current density of 1 mA/cm^2 expressed per unit of crossing area of tissue was applied to observe the effects of iontophoresis on permeation rate. In the donor compartment was placed 1.0 mL of buffer solution simulating saliva containing 45 mg of ROP. Withdrawals of samples were timed as described above. Results are reported as means ± SD of six different experiments in which fractions of the same portion of tissue were used ($P < 0.05$). The drug transferred from the donor to the acceptor compartment was monitored by spectrophotometric analysis (see Section 2.5).

The integrity of the mucosal tissue was monitored before and after each permeability experiment (De Caro et al., 2008). At the end of each experiment, the extraction of ROP from mucosal tissue was performed as described before (see Section 2.2).

2.5. Drug assay

The amount of ROP transferred in the acceptor compartment during permeation experiments was measured spectrophotometrically using the appropriate calibration curve and blank (UV/VIS Shimadzu mod. 1700 Pharmaspec instrument). UV method was found simple, accurate and reproducible. Validation parameters in simulated plasma: λ_{max} = 249.60 nm, linearity range 0.005–0.1 mg/mL, E1% = 0.2960, regression equation $Y = 30.81143X - 0.01213$, correlation coefficient 0.9998, standard error 0.00902. At the testing concentrations, no interferences between ROP and buffer components were observed, and no change in the absorbance of the drug at its λ_{max} was experienced when drug solutions were analyzed in presence of chemical enhancers.

In analogy, the amount of ROP entrapped into the membrane was measured after extraction from mucosal tissue by methanol. Validation parameters in methanol: λ_{max} = 249.60 nm, linearity range 0.005–0.1 mg/mL, E1% = 0.3520, regression equation $Y = 36.03882X - 0.00861$, correlation coefficient 0.99703, standard error 0.01965.

Intraday and interday variations, observed during collection of experimental data, were lower than sensibility.

2.6. Data analysis

The flux values (J_s) across the membranes were calculated at the steady state per unit area by linear regression analysis of permeation data following the relationship $J_s = Q/At$ (mg/cm^2 h), where Q is the quantity of ROP which passes through the cell layers into the receptor compartment, A is the active cross-sectional area available for diffusion (0.13 cm^2) and t is the time of exposure (h).

The permeability coefficient (K_p) was then calculated by the relationship: $K_p = J_s/C_d$ (cm/h) where C_d is the drug concentration in the donor compartment (mg/cm^3). Fluxes, obtained as average value of six replicated experiments, are reported with the standard deviations in Table 1.

All differences were statistically evaluated by the Student's t-test with the minimum levels of significance with $P \leq 0.05$.

2.7. Evaluation of ROP chemical stability in simulated saliva solution

Chemical stability of ROP was studied during 60 days at 37 ± 0.5 °C in buffer solution simulating saliva (0.02 mg/mL) by UV measurements. Drug solutions were monitored for potential variation of absorption peak in the λ range 200–800 nm. The UV absorption peak was constant and highly reproducible during the whole period of collection of data. Validation parameters in buffer solution simulating saliva: λ_{max} = 249.60 nm, linearity range 0.005–0.1 mg/mL, E1% = 0.3043, regression equation $Y = 30.92000X - 0.00493$, correlation coefficient 0.9999, standard error 0.00687. Accuracy > 98.5%. At the testing concentrations, no interferences between ROP and buffer components were observed. Intraday and interday variations, observed during collection of experimental data, were lower than sensibility.

3. Results and discussion

Avoidance of pulsatile stimulation of dopamine receptors can prevent or delay the appearance of motor complications in PD. The administration of repeated doses of anti-Parkinson medications by the oral route has as a major inconvenient the large fluctuation of drug levels that in turn determine pulsatile receptor stimulation. To avoid this limitation, recently ROP-PR formulation has been marketed.

Administration of ROP-PR implies peroral supply of one tablet of 8 mg per day. It has been reported that this type of formulation

allows the achievement of the steady state conditions after 48 h (Thompson and Vearer, 2007; Nashatizadeh et al., 2009).

Taking into account the pharmacokinetic parameters of ROP-IR: t1/2 = 6 h, Vd = 7.5 L/kg (526 L) (Nashatizadeh et al., 2009), clearance = 47 L/h, T_{max} = 2 h, first pass effect 55%, plasma concentration at steady-state = 1 ng/mL/mg, (Thompson and Vearer, 2007; Onofrj et al., 2009) we assume that, following peroral administration of 8 mg of ROP (like ROP-PR), at the steady state the drug plasma levels should be 8 ng/mL.

The plasma steady state conditions imply that the amount of drug eliminated from the systemic circulation is balanced by an analogous amount of drug absorbed. In other words:

$$\text{Drug output rate} = \text{Steady state concentration} \times K_e$$
$$= \text{Drug input rate}$$

where K_e is the elimination constant ($0.693/t_{1/2}$).

The rate of ROP elimination was calculated ($V_e = C_p K_e$; where C_p is the plasma concentration related to oral administration of one tablet) as 0.000924 μg mL/h. Based on these considerations, to maintain the steady state levels, the amount of ROP that should reach the systemic circulation is 6.92 μg/h/kg ($V_d V_e$).

Continuous drug infusion might resolve the problems related to pulsatile receptor stimulation, nevertheless, continuous infusion is expensive, impractical for most patients and could have unsafe outcomes.

In this work the possibility of delivery ROP via the buccal mucosa was studied, as an alternative administration route in treatment of PD. Buccal delivery, like a continuous infusion, could be useful to obtain constant receptor stimulation.

Since a major limitation in buccal drug delivery could be the low permeability of the epithelium, the aptitude of ROP to penetrate the buccal mucosa was verified. Modeling and simulation methods that predict the in vivo performance of drug products can greatly improve formulation strategy by aiding scientists in designing a rational approach to formulation development (Jiang et al., 2011).

Accordingly, *ex vivo* permeation studies were performed using vertical Franz type diffusion cells and porcine buccal mucosa which is the most frequently chosen model for mimic human tissue as it is non-keratinized like human buccal mucosa (Giannola et al., 2007). Tests were performed using ROP solutions containing different amounts of drug (10, 20, 30, 45, 60 mg of ROP in 1.0 mL in simulated saliva). Experiments were carried out using porcine mucosal specimens treated surgically (thickness 500 ± 100 μm) or specimens obtained by heat separation of the epithelial layer (thickness 250 ± 25 μm). Following different treatment, no significant disagreement was observed in permeation behavior of ROP through the membrane. Heat separation did not adversely affect the permeability characteristics of the tissue (Diaz Del Consuelo et al., 2005; Kulkarni et al., 2010). We preferred the heath separation method as it is easy, time efficient and gives highly reproducible thickness when compared to surgical trimming. Additionally, complete and uniform removal of connective tissue is not achieved by surgical trimming.

Permeation profiles of ROP through porcine buccal mucosa were obtained by plotting the cumulative amount permeated versus time (Fig. 1). Fluxes were calculated at the steady state from the slopes of the regression line fitted to the linear portion of the corresponding permeation profile. The intercept of the straight line with the abscissa was taken as the lag time (Table 1).

Average flux data showed that the increase in drug concentrations ranging from 10 to 30 mg/mL resulted in a significant increase in drug flux. A further increase in drug concentration, while reducing the lag time, does not enhance the drug flux, probably due to a sort of membrane saturation phenomenon. This suggestion was confirmed by the results obtained from the extraction and

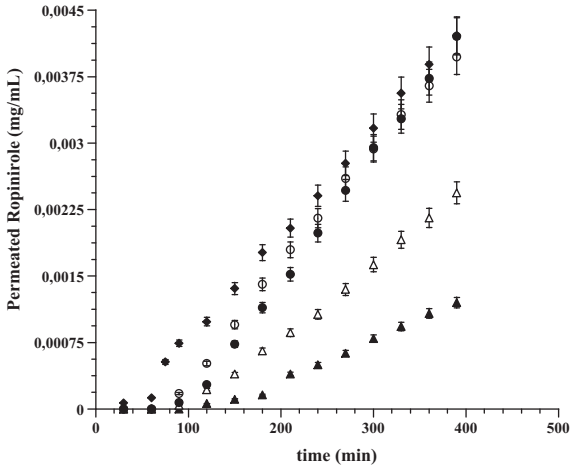

Fig. 1. Permeation profiles of ROP through porcine buccal mucosa, using drug solutions at different concentrations (▲) 10 mg/mL; (△) 20 mg/mL; (●) 30 mg/mL; (○) 45 mg/mL; (♦) 60 mg/mL) in the donor chamber.

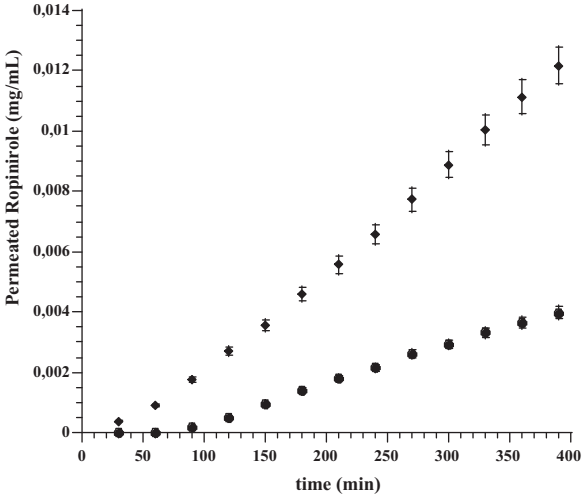

Fig. 2. Permeation profiles of 45 mg/mL ROP solution through porcine buccal mucosa: (●) simple diffusion; (♦) in presence of limonene.

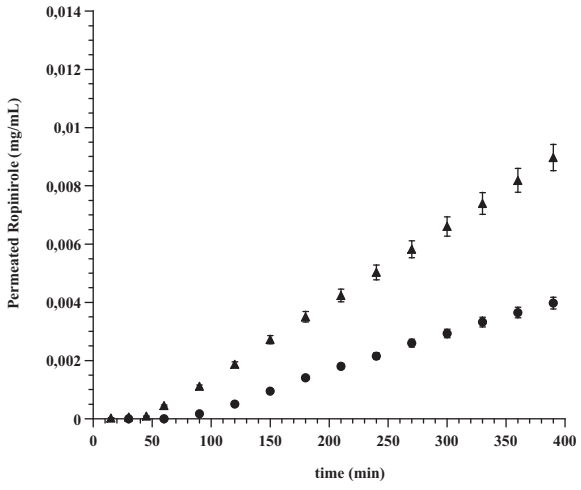

Fig. 3. Permeation profiles through porcine buccal mucosa of ROP 45 mg/mL solution: (●) simple diffusion; (▲) in presence of electric field (1 mA/cm^2).

Table 2
Calculated steady state flux values, permeability coefficients, enhancement ratio, and lag times for a 45 mg/mL ROP solution in studies of permeation through porcine buccal mucosa in enhancement conditions.

	Simple diffusion	In presence of limonene	In presence of electric field (1 mA/cm^2)
J_s($\times 10^{-2}$ mg/cm^{2}h)[a]	15.99 ± 0.164	41.84 ± 0.370	32.52 ± 0.341
K_p($\times 10^{-2}$ cm/h)[a]	0.355 ± 0.003	0.930 ± 0.008	0.715 ± 0.007
ER	/	2.62	2.01
Lag time (min)	75	28	44

[a] Values are reported as mean ± standard deviation (n = 6).

quantification of the drug entrapped into the membrane at the end of each experiment. The average drug amount contained in the membrane was 0.35 ± 0.035 mg in the whole range of concentration used in the donor, thus confirming the proposed saturation phenomenon. On the other hand, the lag time values decreased when the initial drug concentration in the donor compartment increased. When the donor was loaded with a solution containing 60 mg/mL the lowest lag time (45 min) was observed (Table 1).

Flux values suggested that the amounts of ROP that cross the porcine membrane and reach plasma are not adequate to achieve the steady state. The loss of drug that might occur due to dilution by saliva and/or accidental swallowing of portions of the delivered dose, and the value of lag time that we observed could represent limiting factors in those cases requiring a prompt response to the drug.

In buccal drug administration, the barrier properties of the mucosa are dependent on the structural and physicochemical properties of both the oral tissue and the actives. The main resistance to permeation of non-keratinized oral mucosa resides in the outer of the epithelium, in particular the intercellular lipids secreted by membrane coating granules. The arrangement of these lipids is quite amorphous, with only a minor component organized into lamellae, and it appears to be related to the permeability barrier (Diaz Del Consuelo et al., 2005).

To mitigate this barrier and to facilitate the diffusion through the tissue, permeation could be promoted by means of chemical and/or physical enhancement (Giannola et al., 2008). In this study, optimization of the starting drug penetration rate and reduction of lag time was achieved by co-administration of chemical enhancers or by the use of iontophoresis.

On the basis of permeation profile and J_s values, the solution containing 45 mg/mL of ROP was considered the most appropriate to perform the experiments in presence of chemical or physical enhancers. Moreover, ROP at high concentration probably inhibits its electrotransport possibly due to an effect on electroosmosis (Luzardo-Alvarez et al., 2001). The 45 mg/mL solution should be considered as a good compromise between the measured flux value and lag time.

Various substances have been explored as permeation enhancers to increase the flux absorption of drugs through the mucosa, but irritation, membrane damage, and toxicity are often associated with them, and limit their use. A clinically accepted permeation enhancer must increase membrane permeability without causing toxicity and permanent membrane damage (Sohi et al., 2010).

Chemical permeation enhancers could act by different mechanisms. Some act by extraction membrane protein or lipids, membrane fluidization, or production of reverse micellization in the membrane thus creating aqueous channels that affects transport through the paracellular route; others disturb the intracellular lipid packing by interaction with either lipid packing or protein components, thus increasing the fluidity of lipid bilayer membrane, and improving transcellular route (Hassan et al., 2010).

Among penetration enhancers from different classes we selected only substances we experienced that no caused toxic effects on buccal membrane (Giannola et al., 2007). In particular, NaDHC, LysC, and TNaC as hydrophilic molecules, and L-menthol and limonene as lipophilic molecules were chosen. Worth of note, the pleasant taste associated with menthol and limonene may increase patient acceptability to further improve the justification of their use in a buccal drug delivery system.

Permeation tests in presence of water-soluble chemical enhancers were performed by co-administration of ROP and the enhancer in the donor compartment. All hydrophilic promoters were used at the same concentration. Experiments in presence of L-menthol or limonene were performed by pre-treatment of the tissue with the enhancer.

No significant lag time reduction or increase of drug permeation rate through the membrane using NaDHC, LysC, TNaC as promoters were observed. This behavior could be attributable to their enhancement mechanism that primarily affects transport through the hydrophilic paracellular domains, and some molecular properties (i.e. molecular weight, Log P, pK_a) of ROP do not comply with the requirements of paracellular pathway (Deneer et al., 2002; Hassan et al., 2010; Nicolazzo et al., 2005).

Also L-menthol did not cause significant change in transbuccal permeation of ROP, and did not reduce the lag time. It has been reported that L-menthol, due to its —OH group, is able to accept or donate hydrogen bonds and modify the network of the membrane; nevertheless, the real mechanism by which menthol increases transbuccal absorption is not clear. On the other hand, it should be considered that in transmucosal passage the efficacy of penetration enhancers depends on the physicochemical properties of the drug and the enhancement is drug specific (Punitha and Girish, 2010; Prasad et al., 2007; Squier et al., 2010).

On the contrary, using limonene flux and permeability coefficient of ROP through the porcine mucosa were significantly increased (Table 2; Fig. 2) where the lag time significantly dropped down (28 min). This improvement effect could be attributed to the lipid membrane fluidizing activity of terpenes that improves transcellular route and, at least in part, to the effects of limonene on the ROP partitioning in the membrane (Hassan et al., 2010; Ramesh et al., 2007).

The physicochemical characteristics of ROP suggest that also iontophoresis could be used to promote movements through the mucosal membrane. Iontophoresis enhances the transport of charged and neutral molecules across the epithelial tissues by application of mid electric current. The total iontophoretic transport is the result of two mechanisms, the first of which is electromigration, or the direct interaction between the charged molecules and the electric field; the second mechanism is the convective flow (electroosmosis) that results from the permselective properties of the membrane. Being ROP a relatively lipophilic cation, its movement under iontophoretic conditions should be attributable prevalently to electroosmosis (Luzardo-Alvarez et al., 2001; Mudry et al., 2007).

Accordingly, *ex vivo* permeation studies in presence of electric field were carried out. We have previously found that application of current densities of 2 mA/cm^2 or more could cause cytoarchitectural changes consisting in nuclear pycnosis, diffuse signs of

abrupt keratinization and loss of cellular alignment (Giannola et al., 2007), therefore, we investigated ROP permeation in presence of a continuous current density of 1 mA/cm^2.

The application of the electric field determined a good improvement of both transmucosal flux (Fig. 3) and permeability coefficient (Table 2) together with a marked reduction of lag time (44 min).

In experiments carried out with iontophoresis, drug flux through the membrane was lesser and lag time was higher than those experienced with limonene during the same period. These results were attributed to the relative lipophilicity of the ROP cation (Mudry et al., 2007).

The ability of limonene in fluidizing the lipid domain of membrane and its effects on the drug partition into the membrane, coupled with the physicochemical characteristics of ROP, suggest that the transcellular pathway is the preferred mechanism of drug permeation.

Our experimental data suggest that by buccal delivery the time required to reach the steady state plasma concentration of ROP is drastically reduced with respect to ROP-PR.

Since in drug administration one of the limiting factors of using aqueous solutions could be the drug degradation, we evaluated also the chemical stability of ROP in conditions simulating the buccal environment. Degradation was evaluated by monitoring the variation of the drug amount in simulated saliva solutions during 60 days. Data evidenced that the amount of ROP in the tested solutions was constant during the period of storage and no degradation products were formed.

4. Conclusions

Even if no model is right in predicting drug absorption, it can be useful to give good information on *in vivo* drug performance. The results collected during this work, showed that the buccal mucosa does not restrain permeation of ROP. The drug passively crosses the membrane and allows the achievement of therapeutic drug levels in plasma. Nevertheless, an initial lag time is observed but the input rate can be modulated by permeation enhancement using limonene or by application of electric fields. Absorption improvement was accompanied by the important reduction of the lag time; at once the time required to reach the steady state plasma concentration was drastically decreased.

Seeing that buccal delivery is advantageous for those drugs affected by intensive first pass metabolism, we could assume that clinical application of ROP buccal delivery could agree with a rapid achievement of the steady state conditions and a reduction of the ROP doses.

In the *in vivo* environment, several parameters that together cannot be predicted could affect the absorption process e.g. enzymatic metabolism of the drug in the mucosal tissue. Before proceed to the design of an adequate delivery system, additional work would be needed to verify our assumptions *in vivo* on animal models.

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