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Effect of passive and iontophoretic skin pretreatments with terpenes on the in vitro skin transport of piroxicam

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Abstract

The enhancing effect of several terpenes (thymol, menthone and 1,8-cineole) in the percutaneous permeation of piroxicam (Px), either passive or iontophoretically, was investigated. These terpenes were applied, on the skin membrane, as a passive and iontophoretic skin pretreatment. Px was delivered from carbopol gels containing hydroxypropyl-β-cyclodextrin (2% w/w Px). An increase in Px flux values, both passive and iontophoretic after skin pretreatment with 5% terpenes/50% EtOH, was found to be in the following order: thymol > menthone > 1,8-cineole. Iontophoretic skin pretreatment with terpenes produced a slight increase in the passive flux of Px, in comparison with the passive skin pretreatment. This result indicated that iontophoresis could modify the skin morphology and consequently, increase the passive transport of Px. However, when Px was transported iontophoretically, passive skin pretreatment with terpenes, produced higher flux values than iontophoretic skin pretreatment. These results could be explained by the fact that with the iontophoretic pretreatment, terpenes could penetrate into the skin and limitate the movement of the ionized species, across the skin, during the iontophoretic experiments. The amount of Px retained in the skin after all experiments was related to flux values across skin.

Keywords: Hydroxypropyl-β-cyclodextrin:Px complexes; Iontophoresis; Skin pretreatment; Skin retention; Terpenes

1. Introduction

The stratum corneum (SC), the uppermost layer of the skin, is the main barrier to percutaneous permeation of drugs. However, due to the great interest that this route offers, several strategies can be employed to lessen the barrier function, such as penetration enhancers and physical methods (Bhatia and Singh, 1999; Bhatia et al., 1997).

Penetration enhancers are molecules, which reduce the barrier properties, by acting on different skin components such as lipids and/or proteins (Williams and Barry, 1992). These molecules could be applied on the skin membrane in different ways: included in the vehicle or as a skin pretreatment. In the pretreatment experiments, the chemical enhancers were delivered directly on the skin; meanwhile, when these compounds are included in the vehicle they must diffuse out of it before reaching the skin surface. Consequently,

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skin pretreatment with penetration enhancers could produce a higher and quicker drug permeation.

Terpenes, naturally occurring volatile oils, appeared to be promising candidates for clinically acceptable enhancers (Williams and Barry, 1991). They were reported to have good toxicological profiles, high percutaneous enhancement abilities, and low cutaneous irritancy at low concentrations (1–5%) (Okabe et al., 1990). Terpenes are a series of compounds that consist of isoprene (C_5H_8) units and they have been shown to increase the percutaneous absorption of both hydrophilic and lipophilic drugs (Mohimi et al., 1997).

Iontophoresis is a non-invasive technique, which uses a mild electrical current to facilitate the transdermal delivery of a great variety of drugs. Transdermal delivery, based on this principle, offers many attractive advantages, such as rapid onset of action and high versatility in the control of delivery. In general, the total iontophoretic flux is the sum of electrorepulsion and electroosmosis components (the passive flux contribution is negligible). The relative importance of electrorepulsion and electroosmosis depends on the physicochemical and electrical characteristics of the membrane and of the permeant. In this way, the flow in the anode-to-cathode direction (electroosmosis), facilitates cation transport, inhibits that of anions, and enables the enhanced transdermal transport of neutral, polar solutes (Burnette and Ongpipattanakul, 1987; Kim et al., 1993), which does not undergo flux enhancement by electrorepulsion. The electrorepulsive and electroosmotic contribution to iontophoretic transport remains an important goal as optimization of formulations for drug delivery (Merino et al., 1999).

Besides, iontophoresis and chemical enhancers can produce a synergistic effect, providing an additional driving force for enhancing the flux of different drugs. Recently, some studies reported that iontophoresis synergistically enhanced the permeability of molecules through terpenes/ethanol treated skin (Bhatia and Singh, 1998).

Piroxicam (Px) is one of the most important non-steroidal anti-inflammatory drugs which presents the well-known gastrointestinal side effects (Schiantarelli and Cadel, 1981; Schiantarelli et al., 1982). This drug has two pK_a's (1.8 and 5.2) corresponding to the pyridyl and enol structural groups, respectively (Beetge et al., 2000), therefore, depending upon pH the drug can exist in cationic, neutral (i.e. zwitterionic) or anionic forms. At pH 7.4, the conditions under the experiments were performed, the drug carries a net charge of -1, and cathodal iontophoresis was the most appropriate way to transport this anion molecule.

The present study evaluates the enhancing effect of the passive and iontophoretic skin pretreatment with several terpenes, on the in vitro percutaneous permeation of Px, either passive or iontophoretically. Thus, this study was performed to identify the synergistic effect between the skin pretreatments with chemical enhancers and iontophoresis. Iontophoretic pretreatments were developed in order to obtain further enhancement of Px flux than with the passive ones. Local tissue concentrations of Px from carbopol gels containing Px:HPβCD complexes, after all experiments, were also measured.

2. Materials and methods

2.1. Materials

Px was generously provided by Industrial Kern Española S.A. (Barcelona, Spain). Hydroxypropyl-β-cyclodextrin (HPβCD) was purchased from Sigma-Aldrich. Carbopol ETD 2001® (carboxypolymethylene), triethanolamine 85%, and propylene glycol (PG) USP were supplied by Roig Pharma S.A. (Barcelona, Spain). Menthone, 1,8-cineole and thymol were all obtained from Imatra S.A. The structures of the terpenes used in this study are shown in Fig. 1. Ethanol (EtOH) was provided by Prolabo. All other chemicals and reagents used were of analytical grade.

2.2. Preparation of Px gels

Px:HPβCD 1:1 complexes (2% w/w Px), prepared by the coprecipitation method (Doliwa et al., 2001), were incorporated into carbopol gels.

The gels were obtained by dispersion 1% w/w carbopol 2001® in a mixture of water and 40% PG. The pH of the carbopol dispersions was adjusted to 7.4 with triethanolamine.

2.3. Transdermal permeation experiments

2.3.1. Animal model

Porcine ears were obtained from the local slaughterhouse and after cleaning them under cold running water, the outer region of the ear was cut. The whole skin was dermatomed to 800 μm and inmmediately frozen at -20 °C. After a period of time, the skin samples were clamped between the two chambers of the iontophoretic vertical cells.

2.3.2. Skin pretreatments

The enhancer solutions for the skin pretreatments consisted on 5% terpenes (thymol, menthone and 1,8-cineole) in EtOH/buffer (50:45, v/v). Also, skin pretreatments with EtOH/buffer (50:50, v/v) were carried out. 1 ml of the enhancer solutions was placed onto the skin surface exposed in the cathode chamber and 1 ml of fresh *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffered saline in the anode of a vertical glass diffusion cell. These cells have a hollow chamber which is suitable for both solution and gel formulations. The capacity of the receptor was 7.5 ml and the surface area of the skin, exposed to the enhancer solutions, was 0.7 cm^2.

Skin pretreatments were carried out passive and iontophoretically. As terpenes are considered neutral molecules, anodal iontophoretic pretreatment has carried out for 3 h. The constant current applied was 0.4 mA cm^{-2} for 3 h, in all cases. Besides, silver–silver chloride electrodes (Ag/AgCl) were used for the study, as they have the property of not cause electrolysis of water. The electrodes were prepared by coating silver wire with molten AgCl. Current and voltage control with automatic crossover (Model APH 1000 M, Kepco, Inc) was used. This supply has a specified drift of <2 μA per 8 h for its current-controlled output, an important consideration if drug is sensitive to changes in current.

Passive skin pretreatment were carried out without current in the same conditions.

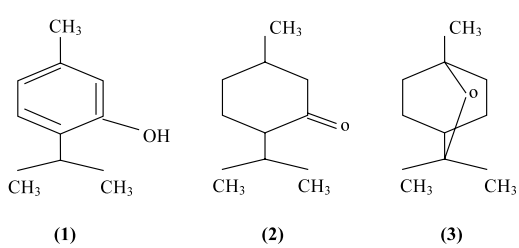

Fig. 1. Structural formula of terpenes used in the skin pretreatment experiments: **(1)**, thymol; **(2)**, menthone; **(3)**, 1,8-cineole.

2.3.3. In vitro skin transport experiments

After pretreating the skin with the enhancer solutions, the skin was washed with distilled water and 1.5 g of Px formulation were placed into the cathode chamber. Constant current was applied at 0.4 mA cm^{-2} for 7 h, in the case of iontophoretic experiments and without current in passive diffusions. As Px was negatively charged, cathodal iontophoresis was carried out. The receptor fluid was continuously perfused using peristaltic pump and 1.3 ml were collected every hour.

The amount of Px in the samples was measured by UV-spectrophotometer (Diode Array 8452 A, Hewlett-Packard) at 354 nm. The absorbance values were read against a linear standard plot to obtain the corresponding concentrations of Px. The results were expressed as the mean $\pm$ S.D. of three experiments.

Statistical comparisons were made between groups by one analysis of variance (ANOVA) and post hoc Tamhane test. Statistical significance level was defined as $P < 0.05$.

2.4. Determination of Px retained in the skin

At the end of the in vitro experiments, the skin was removed from the vertical cell and washed with distilled water. The treated skin area was weighed, placed in 3 ml of phosphate buffer and homogenized using a tissue homogenizer (Euro-Turrax, Ika Labortechink, Germany) for 2 min; 700 mg of potassium carbonate, 1 ml of tetrahydrofuran and 0.5 ml of ethanol were added to 1 ml of the resulting homogenized solution. The

Table 1
Passive and iontophoretic fluxes of piroxicam after passive and iontophoretic pretreatments with 5% terpenes/50% EtOH at 3 h

Pretreatments	Passive pretreatment		Iontophoretic pretreatment	
	Passive flux (nmol cm^{-2} h^{-1})	Iontophoretic flux (nmol cm^{-2} h^{-1})	Passive flux (nmol cm^{-2} h^{-1})	Iontophoretic flux (nmol cm^{-2} h^{-1})
None	12.93 ± 1.26	33.87 ± 0.21	12.93 ± 1.26	33.87 ± 0.21
EtOH	26.37 ± 3.05*	34.61 ± 1.85	28.69 ± 7.39*	29.13 ± 5.88
thymol/EtOH	39.38 ± 3.14*	164.30 ± 5.44*	47.89 ± 4.29*	118.29 ± 5.92*
1,8 cineole/EtOH	25.71 ± 5.98*	80.43 ± 5.87*	20.50 ± 3.63*	56.89 ± 3.21*
menthone/EtOH	36.66 ± 1.15*	126.81 ± 5.61*	40.56 ± 3.51*	110.00 ± 4.29*

Each data point is the mean $\pm$ S.D.; $n = 3$. *$P < 0.05$. ANOVA and post hoc Tamhane test, compared with the non-pretreated skin at 3 h.

tubes were vortex mixed for 1 min and then centrifuged for 10 min at $2500 \times g$. 1.2 ml of the upper phase were placed in a second test tube and evaporated to dryness at 60 °C under vacuum. The residue was then reconstituted in 1 ml of tetrahydrofuran, vortex-mixed for 30 s and filtered with a 0.5 μm filter (Millipore). The amount of Px in the sample was assayed spectrophotometrically at 368 nm. For the calibration procedure, blank samples of skin homogenate were spiked with a known amount of Px and extracted as previously described.

3. Results and discussion

The effect of passive and iontophoretic skin pretreatment with terpenes (thymol, 1,8-cineole and menthone) on the in vitro passive diffusion and iontophoresis transport of Px was studied. Px was delivered from gels containing Px:HPβCD complexes (2% w/w drug).

3.1. Passive skin pretreatment

Passive and iontophoretic Px flux values at 3 h (when the steady-state was reached), after passive skin pretreatment with 5% terpenes/50% EtOH, were presented in Table 1. As terpenes were dissolved in 50% ethanol, skin was also pretreated with this substance, in order to elucidate if ethanol itself acted as a percutaneous enhancer for Px permeation.

In the passive experiments (Fig. 2a), skin pretreatment with 50% EtOH increased 2 fold the Px flux compared with non-pretreated skin. In this way, Srinivasan et al. (1990) have already reported that passive skin pretreatment with 50% ethanol dramatically increased the passive fluxes of several molecules. When 5% terpenes were added to a 50% EtOH solution, the passive flux of Px further increased for thymol, followed by menthone. The less effective was 1,8-cineole, which did not present significative differences in comparison with 50% EtOH pretreatment. Williams and Barry (1991) also reported that terpenes did not present the same effectiveness in promoting the percutaneous permeation of drugs. Also, other authors proposed that this fact might be closely related to the physicochemical nature of drugs and terpenes (El-Kattan et al., 2001).

The literature reveals that hydrophilic terpenes (alcohol, ketone and oxide terpenes) are good candidates in enhancing the permeation of non steroidal anti-inflammatory drugs. In some studies with indomethacin, as a lipophilic molecule, drug absorption was markedly enhanced by the addition of hydrophobic cyclic monoterpenes, while hydrophylic ones showed minor effects (Okabe et al., 1989). In contrast, a study performed, under similar conditions, with diclofenac sodium as a hydrophilic penetrant, suggested an alcohol ter-

pene (menthol) to be the most effective penetration enhancer (Williams and Barry, 1991). Also, Arellano et al. (1996) described the alcohol terpenes as the most effective accelerants for this NSAID followed by ketones and oxides.

Px could be classified as a slightly lipophilic drug, with a balanced lipophilic/hidrophilic character very similar to ketoprofen. In this way, Nakamura et al. (1996) observed that the ketoprofen flux was remarkably enhanced when 2% alcohol terpene (menthol) was applied. These authors explained that menthol might change the dense barrier structure of the stratum corneum of the skin. Our results also presented an enhancement in the flux of Px when an alcohol terpene (thymol) was applied, although, slight differences between the alcohol and the ketona were found. The small enhancement in the flux observed with 1,8-cineole was also reported with a NSAID, the fluflenamic acid, when this oxide terpene was used as a penetration enhancer (Priborsky et al., 1992).

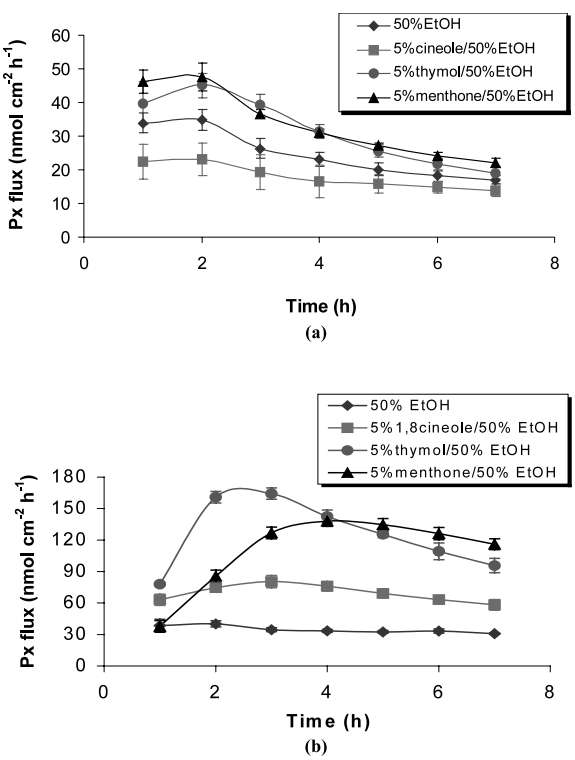

Fig. 2. Piroxicam flux values after passive skin pretreatment with 5% terpenes/50% EtOH: passive transport (a) iontophoretic transport (b). Each data point represented the mean ± S.D.; $n = 3$.

Fig. 2b represented the profiles of the iontophoretic fluxes of Px obtained after passive skin pretreatment with 5% terpenes/50% EtOH. All terpenes in combination with 50% EtOH increased the intophoretic flux of Px, although this enhancement was higher in the case of thymol, followed by menthone and 1,8-cineole. Thymol produced a higher and faster iontophoretic flux than menthone, in the first 3 h. Since then, thymol and menthone did not present significative differences. In the case of 1,8-cineole, the iontophoretic Px flux value was increased 2.3 fold, while this terpene did not provoke any enhancement in the passive flux.

These results suggested that intophoresis synergistically enhanced the permeability of Px through skin pretreated passively with 5% terpenes/50% EtOH. According to this, Bhatia and Singh (1998) obtained a great enhancement in the iontophoretic flux of LHRH after the skin pretreatment with 5% terpenes (limonene, carvone, thymol and cineole)/50% ethanol. Additionally, there were other authors that applying iontophoresis in combination with several enhancers (terpenes, oleic acid, etc.), increased markedly the fluxes of different drugs (Bhatia and Singh, 1999; Bhatia et al., 1997; Srinivasan et al., 1989).

In general, it is possible to interpretate that iontophoresis have a synergistic effect on the enhancing activity of the terpenes and consequently, this technique could boost the terpenes action.

3.2. Iontotophoretic skin pretreatment

The skin permeation enhancement of terpenes (thymol, 1,8-cineole and menthone) is greatly dependent upon their structure, the characteristics of the permeant and the method used to deliver both the permeant and the terpenes on the skin surface. Terpenes were applied as a iontophoretic pretreatment, in order to obtain further enhancement of the Px flux than with a passive pretreatment. As terpenes were considered neutral molecules, anodal iontophoretic pretreatments were carried out. In this way, Burnette and Ongpipattanakul (1987) explained that for neutral

molecules, it cannot experience transport via direct charge repulsion when an electric field is applied across the skin during iontophoresis. However, iontophoresis can increased the flux of these neutral molecules by electroosmosis (the flow in the anode-to-cathode direction when the electrical current passes through the skin). In addition, Delgado-Charro and Guy (1994) supported the idea that with neutral molecules, anodal delivery was significantly higher than cathodal, in a manner expected from the permselective properties of the skin. Hence, the permeation enhancement induced by electroosmosis is predominant during iontophoresis of neutral compounds or compounds with a high molecular weight (Hirvonen and Guy, 1998; Pikal and Shah, 1990; Kirjavainen et al., 2000).

The passive and iontophoretic fluxes of Px at 3 h after iontophoretic skin pretreatment with terpenes were summarised in Table 1. In the passive experiments (Fig. 3a), Px permeation through porcine skin was significantly enhanced by ethanol iontophoretic pretreatment, in comparison with the no pretreated skin. The percutaneous flux for this drug was further increased with iontophoretic skin pretreatment with both 5% thymol and 5% menthone, while 1,8-cineole presented similar effectiveness than ethanol. The Px passive flux values obtained with terpenes, except with 1,8-cineole, were slightly higher after the iontophoretic pretreatments than after the passive ones.

This could indicate that passage of current could increase the original permeability of the skin to an extent that, once it was switched off, passive diffusion continued through a tissue more permeable. In this way, numerous authors have reported a skin alteration after the passage of electrical current during iontophoresis (Manabe et al., 2000; Sims et al., 1991).

Fig. 3b represented the profiles of the iontophoretic fluxes obtained after skin iontophoretic pretreatment with 5% terpenes. All terpenes in combination with EtOH increased the flux of Px further than when the skin was not pretreated. This enhancement was higher for thymol and menthone, but in the case of thymol, the iontophoretic flux of the drug increased faster than

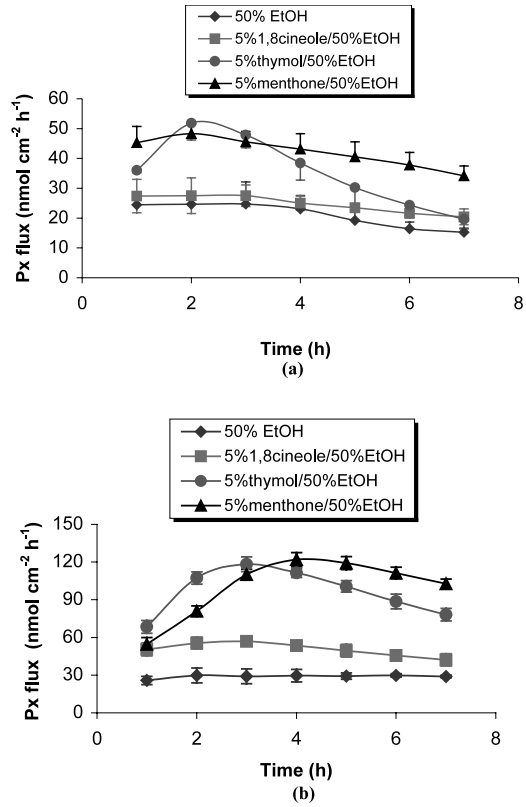

Fig. 3. Piroxicam flux values after iontophoretic skin pretreatment with 5% terpenes/50% EtOH: passive transport (a) iontophoretic transport (b). Each data point represented the mean ± S.D.; $n = 3$.

with menthone, mainly in the first 3 h. 1,8-cineole showed the lowest flux among all the terpenes employed.

When we compare the Px iontophoretic fluxes obtained after passive and iontophoretic pretreatments, it was possible to observe that the passive pretreatment produced a higher flux than the iontophoretic one. In this way, Gay et al. (1992) also reported that after skin pretreatment with oleic acid (OA), the Px flux decreased. These authors indicated a competition in the transport between the OA and the drug, both charged negatively. However, in our case, as terpenes were considered neutral molecules, the decrease in Px iontophoretic flux was not due to a competition in the transport of the charges. It could be explained by the fact that when we applied the ion-

tophoretic pretreatment, terpenes could penetrate into the skin and their presence within the tissue could limit the movement of the ionic species during the iontophoretic transport and consequently, the iontophoretic fluxes of piroxixam decreased.

3.3. Px retained in skin

The amount of Px retained into the skin after passive and iontophoretic pretreatment, was calculated either passive or iontophoretically. Fig. 4 represented the amount of Px retained in skin after passive (Fig. 4a) and iontophoretic (Fig. 4b) pretreatments with 50% EtOH and 5% terpenes in 50% ethanol. The amount of Px in the skin increased in all cases after iontophoretic transport in comparison with the passive transport of the drug. When pretreatment was carried out with 5% thymol, the amount of Px retained either passive or iontophoretically was the highest, following by 5% menthone and 1,8-cineole.

In this way, we could conclude that in most cases, the highest flux of the drug the most quantity of Px retained in the skin. This result was in an agreement with the previous ones of Santoyo and Ygartua (2000) who reported that the skin concentration after topical application of Px could be related to its flux across skin.

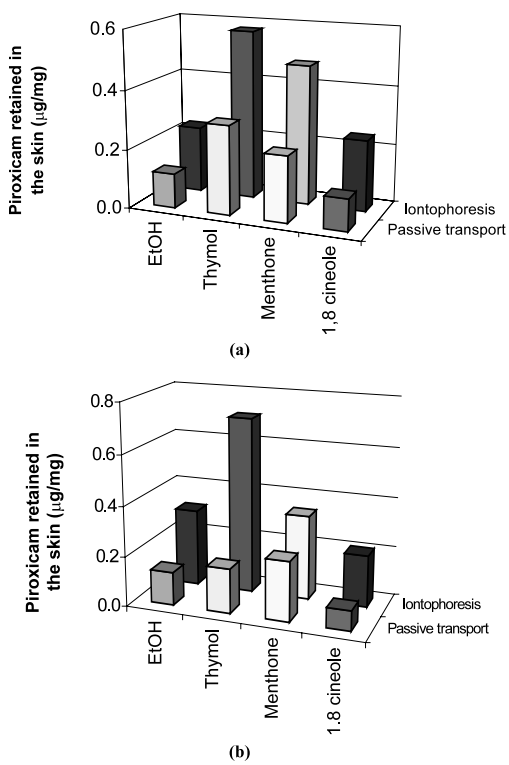

Fig. 4. Piroxicam retained in skin (μg mg^{-1}) after skin pretreatments with 5% terpenes/50% EtOH, followed by either passive or iontophoretic transport: passive skin pretreatment (a) iontophoretic skin pretreatment (b).

4. Conclusions

Skin pretreatment with 5% terpenes in combination with 50% ethanol significantly increased the flux of Px, either passive or iontophoretic, although iontophoresis produced a further increase. In this way, the most effective enhancers for the enhancement of Px flux were in the corresponding order: thymol > menthone > 1,8-cineole.

Iontophoretic skin pretreatment with terpenes produced a slight enhancement in the passive flux of Px, in comparison with the passive pretreatment. However, when the Px was transported iontophoretically, passive skin pretreatment produced higher fluxes than iontophoretic pretreatment. Consequently, the present study showed that the best results for Px transport through the skin were obtained with a passive skin pretreatment with 5% thymol/50% EtOH followed by the iontophoretic transport of the drug.

The amount of Px retained in skin after all experiments, seemed to be in concordance with the fluxes values obtained in each case.

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