European Journal of Pharmaceutics and Biopharmaceutics 80 (2012) 663–673

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

A combination of nonionic surfactants and iontophoresis to enhance the transdermal drug delivery of ondansetron HCl and diltiazem HCl

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ARTICLE INFO

Article history:
Received 26 August 2011
Accepted in revised form 10 November 2011
Available online 25 November 2011

Keywords:
Transdermal drug delivery
Iontophoresis
Chemical permeation enhancers
Surfactants

ABSTRACT

The present work reports the evaluation of three nonionic ether-monohydroxyl surfactants ($C_{12}E_1$, $C_{12}E_5$, and $C_{12}E_8$) as skin permeation enhancers in the transdermal drug delivery of two drugs: ondansetron hydrochloride and diltiazem hydrochloride, formulated as hydrogels. The enhancers are used alone, or in combination with iontophoresis (0.3 mA – 8 h). After 1 h of pre-treatment with 0.16 M enhancer solutions in propylene glycol (PG), passive and iontophoretic 24 h *in vitro* studies across dermatomed porcine skin were performed using vertical Franz diffusion cells. Data obtained showed that the nonionic surfactant $C_{12}E_5$ was the most effective permeation enhancer, both for the passive process as well as for samples subjected to iontophoresis, resulting in cumulative amounts of ondansetron HCl after 24 h of approximately 93 μg/cm^2 and 336 μg/cm^2, respectively. Data obtained using diltiazem HCl showed a similar trend. The use of the nonionic surfactant $C_{12}E_5$ resulted in higher enhancement ratios (ER) in passive studies, but $C_{12}E_8$ yielded slightly higher values of drug permeated (2678 μg/cm^2) than $C_{12}E_5$ (2530 μg/cm^2) when iontophoresis was also employed. Skin integrity studies were performed to assess potential harmful effects on the tissues resulting from the compounds applied and/or from the methodology employed. Skin samples used in permeation studies visualized by light microscopy and Scanning Electron Microscopy (SEM) at different levels of magnification did not show significant morphological and structural changes, when compared to untreated samples. Complementary studies were performed to gain information regarding the relative cytotoxicity of the penetration enhancers on skin cells. MTS assay data using human epidermal keratinocytes (HEK) and human dermal fibroblasts (HDF) indicated that HEK are more sensitive to the presence of the enhancers than HDF and that the toxicity of these compounds is enhancer molecular weight dependent.

1. Introduction

The skin is the human body's largest organ with a primary function to protect the body against external aggression such as microorganisms (bacteria and viruses), radiation and to prevent water loss [1,2]. Its unique highly organized structure comprises four distinct layers: nonviable epidermis (stratum corneum), viable epidermis, dermis and subcutaneous connective tissue (hypodermis) with various fully differentiated cell-types [3]. The stratum corneum (SC), the outermost layer of the skin, is composed of dead, flattened, keratin-rich cells (corneocytes) embedded in a complex intercellular lipid mixture, particularly rich in ceramides, fatty acids, cholesterol and cholesterol sulfate, organized in bilayer arrays [4,5]. Therefore, the barrier function of the mammalian skin is principally due to the SC, which is also responsible for the poor penetration of drugs into the skin [6]. Historically, the biggest challenges in the development of transdermal drug delivery (TDD) systems have focused in overcoming the skin barrier without causing harmful effects, principally local irritation.

Beginning with the introduction of the first nicotine patches for tobacco cessation in the mid-1980s, the transdermal market is estimated to represent today, worldwide, ca. $5.6bn [7]. Some of the advantages associated with TDD are as follows: (a) avoidance of hepatic first-pass metabolism and other gastrointestinal tract issues such as presence of food and pH changes; (b) sustained and controlled release over a long period of time; (c) reduction of

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doi:10.1016/j.ejpb.2011.11.010

adverse effects or therapeutic failures associated with intermittent dosing; (d) improved patient compliance because it is a convenient and painless administration route. However, TDD has also some limitations, including that (a) only drugs with a molecular weight lower than 500 Da and a log*P* (octanol/water) between 1 and 3 are suitable for diffusion and permeation across the SC with the ability to reach the systemic circulation, (b) there is a high variability related to different skin absorption profiles and (c) it may induce local irritation and sensitization [8].

Strategies to increase drug permeation across the skin include passive and active methods or the combination of both. Permeation of drug molecules across the skin can occur also via skin appendages (hair follicles, sebaceous and sweat glands) or directly crossing the cells (transcellular), however, the predominant diffusional path seems to be intercellular [9,10]. The use of permeation modulators/enhancers is particularly useful since they have been reported to increase the diffusivity and/or the solubility of drugs in the skin by reversibly altering lipid organization of the SC, reducing the diffusional resistance [11–13]. Surface active agents (surfactants) comprise a class of compounds with amphiphilic properties and, therefore, can be employed as permeation enhancers. Surfactants are present in many cosmetic and pharmaceutical formulations used as emulsifiers, wetting, suspending, solubilizing, and stabilizing agents. When applied to the skin, surfactants interact with the lipid molecules, intercalating into lipid bilayers [14]. This results in interfacial defects and structure disruption causing the formation of diffusional paths for drugs molecules [10]. Depending on the amphiphilic structure and concentration employed, local irritation of skin may be observed. However, nonionic surfactants are commonly recognized to have the lowest irritating properties among all surfactant classes [15,16].

Iontophoresis is a promising technique capable of extending the number of compounds that can be delivered transdermally and is particularly useful for hydrophilic drugs and/or those with higher molecular weight. Based on the principle that like charges repel, iontophoresis consists of the application of an electrical current of low voltage to the skin, forcing the drug molecules to cross the SC. In the case of positively charged drugs such as those used in this work, drug molecules are repelled by the positive electrode (anode) into the skin, toward the negative electrode (cathode), reaching the systemic circulation. Negatively charged ions migrate in the opposite direction, closing the circuit [17,18]. The use of chemical penetration enhancers with iontophoresis is regarded as one of the best strategies to enhance the permeation of drugs across the skin, combining the advantages of both approaches. It has been shown that in many cases the concentrations of enhancers can be reduced as well as the current levels without the loss of effectiveness of drug delivery. This approach has been extensively reported in the literature for different drugs, and in combination with different permeation enhancers [19–31].

Ondansetron hydrochloride (Fig. 1a) is a serotonin 5-HT3 receptor antagonist with antiemetic activity. It is used in the management of post-operative nausea and vomiting (PONV) and concomitantly with chemotherapy and radiotherapy. Despite that doses are normally expressed in terms of the base form, it is also commonly administered as its hydrochloride form especially in injectable and oral dosage forms. Ondansetron undergoes a significant first-pass hepatic metabolism, which justifies the development of an alternative to the oral and injectable routes, such as the transdermal route, avoiding the gastrointestinal effects and associated pain, and promoting better patient compliance [32].

Diltiazem hydrochloride (Fig. 1b) is a benzothiazepine calcium-channel blocker with peripheral and coronary vasodilator properties, used in the management of angina pectoris, cardiac arrhythmias and hypertension. Like ondansetron, it undergoes an extensive first-pass metabolism (bioavailability ca. 40%) and has been reported to cause GI disturbances, including nausea, vomiting and constipation. Therefore, the transdermal route also appears as a promising route of administration [32].

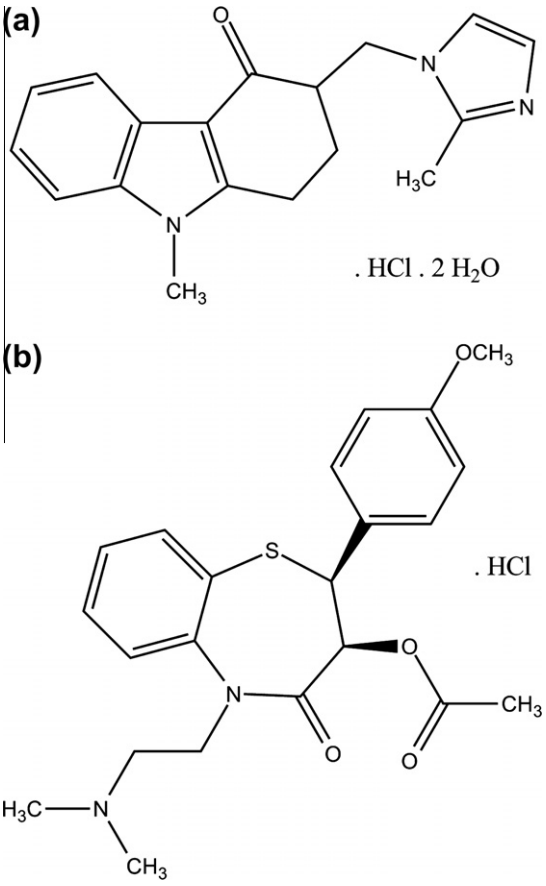

Fig. 1. Molecular structures of ondansetron hydrochloride dihydrate (on the left, Mw: 365.15; log*P*: 2.07; log*D* (pH5.5): 0.19) diltiazem hydrochloride (on the right, Mw: 450.14; log*P*: 3.63; log*D* (pH5.5): 0.76).

In addition, the work we are presenting is of significant value since there are few studies in the literature reporting the transdermal use of ondansetron [33–35]. Diltiazem transdermal delivery has been the center of attention of some studies focusing on film, matrix or patch compositions alone [36–38] or concomitantly with iontophoresis [39]. Nolan and co-workers [24] studied the combined use of oleic acid as permeation enhancer and iontophoresis in the transdermal delivery of diltiazem hydrochloride and some other drugs, across human and murine skin. However, the use of various nonionic ether-monohydroxyl C_{12} surfactants, only differing from each other in the polar headgroups, as chemical permeation enhancers, or combined with iontophoresis has not been investigated to date.

The objective of this work is the development of drug-loaded hydrogels suitable for transdermal drug delivery, and the evaluation of the efficacy of various nonionic ether-monohydroxyl C_{12} surfactants ($C_{12}E_1$, $C_{12}E_5$ and $C_{12}E_8$) as permeation enhancers, in the absence and presence of iontophoresis, for the drug delivery of ondansetron and diltiazem hydrochloride, across porcine skin. Despite the fact that excised human skin is always desirable for *in vitro* permeation experiments, porcine skin has been reported to be the most suitable model when human skin is not available. Porcine skin can be more easily obtained and exhibits anatomical and morphological properties similar to human skin [40,41]. Moreover, previous permeation studies with different compounds reported a smaller variability when porcine skin was employed

and a significant correlation between porcine and human skin [42,43]. In addition, this work presents cytotoxicity studies (MTS assay) in human epidermal keratinocytes (HEK) and in human dermal fibroblasts (HDF), and histology and SEM studies were carried out on cells and skin tissue, respectively, to evaluate potential harmful effects of the enhancers and of the methodology used.

2. Experimental section

2.1. Materials

Ondansetron HCl dihydrate (⩾99%) and diltiazem HCl (⩾99%) were purchased from Polymed Therapeutics, Inc. (Houston, TX, USA). Hydroxypropylmethylcellulose (HPMC K15M) was a kind gift from Dow Chemical Company (USA). Propylene glycol (PG) (Reagent Plus, 99%), nonionic ether-monohydroxyl C_{12} surfactants, ethylene glycol monododecyl ether ($C12E_1$), pentaethylene glycol monododecyl ether ($C_{12}E_5$), octaethylene glycol monododecyl ether ($C_{12}E_8$), silver wire (Ag), silver chloride (AgCl), and citric acid monohydrate were purchased from Sigma Aldrich (Saint Louis, MO, USA). Indicative values of the log*P* and log*D* of the drugs and surfactants were obtained using suitable predicting software (ACD/LabsTM, Toronto, Canada). Azone was synthesized at the New Jersey Center for Biomaterials (Piscataway, NJ, USA). Phosphate buffer saline tablets (PBS) were purchased from TIC Gums (Belcamp, MD, USA). Formalin 10% was purchased from Fisher Scientific, Inc. (Torrance, CA, USA). CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega Corp (Madison, WI, USA). Tissue-Tek® O.C.T™ Compound was purchased from Sakura Finetek USA, Inc. (Agawam, MA, USA). Porcine skin tissue was obtained from young Yorkshire pigs (26.5–28 kg, UMDNJ, Newark, NJ). Human epidermal keratinocytes (HEK) and human dermal fibroblasts (HDF) were purchased from Invitrogen™ (Carlsbad, CA, USA).

2.2. Hydrogel preparation

Ondansetron HCl hydrogel (pH 2.2) was composed of 2.0% (w/w) ondansetron HCl, 1.0% (w/w) HPMC K15M and deionized water. Citric acid 0.01% (w/w) was previously added to dissolve the ondansetron HCl. Diltiazem hydrogel (pH 4.4) contained 3.0% (w/w) diltiazem HCl, 1% (w/w) HPMC K15 M and deionized water.

2.3. Porcine skin preparation

Porcine skin was obtained from young Yorkshire pigs. Prior to dermatoming, subcutaneous fat (hypodermis) was removed with a knife, avoiding contamination caused by contact with the outer layer of the skin. Porcine skin was excised and dermatomed into an approximate size of 1 cm^2, using a Padgett® Model B Electric Dermatome (Integra LifeSciences, Plainsboro, NJ) with 650–750 μm thickness and was stored at −80 °C no more than 3 months prior to use. Immediately before the experiments, skin samples were defrosted, thawed and rinsed with water at room temperature, before immersion in PBS (pH = 7.4) for 1 h to promote equilibration.

2.4. Preparation of Ag and AgCl electrodes

Pure silver (Ag) wire 0.5 mm in diameter was used as the anode. AgCl electrode, the cathode was prepared by placing a pure silver wire and an AgCl powder coated wire in a beaker filled with 0.1 N HCl solution, both connected to a power source at 3.0 mA for 12 h.

2.5. Preparation of enhancer solutions

All enhancer solutions were prepared at 0.16 M in propylene glycol (PG). The enhancers tested were three nonionic ether-monohydroxyl C_{12} surfactants, ethylene glycol monododecyl ether ($C_{12}E_1$), pentaethylene glycol monododecyl ether ($C_{12}E_5$), octaethylene glycol monododecyl ether ($C_{12}E_8$), and Laurocapram/1-dodecylazacycloheptan-2-one (Azone). All enhancers were soluble in PG at the concentration used.

2.6. Drug delivery studies

In vitro permeation studies were carried out over a period of 24 h in vertical Franz diffusion cells (PermeGear, Inc., PA, USA) with a diffusion area of 0.64 cm^2 and a receptor compartment of 5.1 mL filled with PBS solution (pH = 7.4), kept at 37 °C ± 0.1 and stirred at 600 rpm. Drug-loaded hydrogel (0.3 mL) was placed in each donor compartment and occluded with Parafilm® to prevent evaporation. Dermatomed porcine skin pieces (0.76 mm thickness) were placed between the donor and receptor compartments, with the epidermal side facing up. At predetermined time points (0.0, 1.0, 3.0, 5.0, 8.0, 12.0, 20.0 24.0 h), 300 μL samples were collected from the receptor compartment and immediately replaced with 300 μL of PBS solution. Samples were kept in the refrigerator prior to HPLC analysis. All the skin samples, except the control, were pre-treated with 60 μL of the various enhancer solutions, for 1 h, prior to the application of the drug-loaded hydrogel in the donor compartment (t = 0.0), both in passive and iontophoresis experiments. Iontophoresis experiments were carried out at 0.3 mA for 8 h (stage 1) followed by 16 h of passive diffusion (stage 2), using a Phoresor II Auto (Model PM 850) as the power source. The anodal electrode (Ag) was in contact with the hydrogel formulation, whereas the cathode (AgCl) was placed inside the receptor solution. Both electrodes were connected to the power source.

2.7. Quantification of ondansetron HCl and diltiazem HCl

All samples were analyzed using HPLC. The system consisted of an Agilent HP 1100 series with an autosampler, equipped with a quaternary pump and a VMD detector and a Agilent Chemstation. A reverse-phase C_{18} column (150 × 4.6 mm C18 (2) 100 A Luna 5 μm, Phenomenex®) with a guard column was the stationary phase for the Ondansetron HCl assay, at 25 °C. The mobile phase consisted of methanol and PBS (pH = 7.4) at 65:35. The flow rate was set to 1.0 mL/min and the injection volume was 20 μL. Ondansetron HCl was detected at 310 nm and the retention time was ca. 5 min. The method showed a linear response in the 0.1–500 μg range (R^2 = 1.0000) with a daily RSD < ± 3.0%. Diltiazem HCl was detected at 240 nm using a reverse-phase C_{18} column (150 × 4.6 mm C18 Luna 5 μm, phenyl-hexyl, Phenomenex®) with a guard column as the stationary phase at 25 °C. The mobile phase was prepared by mixing 4 volumes of methanol with 1 volume of water. The pH was then adjusted to 3.3 with glacial acetic acid. Final pH was adjusted to 6.6 using TEA. The flow rate was set to 1.2 mL/min and the injection volume was 20 μL. The retention time was ca. 3.3 min. The method showed a linear response in the 0.01–100 μg range (R^2 = 1.0000) with a daily RSD < ± 3.0%.

2.8. Data analysis in the permeation studies

The steady flux at time t (J, μg cm^{-2}) was calculated from the slope of the linear portion of the plot of cumulative drug amount permeated versus time. Q_8 and Q_{24} refer, respectively, to the cumulative drug amount present in the receptor fluid after 8 and 24 h. The enhancement ratio (ER) for flux was calculated using

$$ER = \frac{\text{flux for treated skin with enhancer or iontophoresis or their combination}}{\text{flux for untreated skin}}$$

Results are presented as mean ± standard deviation (SD) (n), where n is the number of replicates. Flux values were examined for significance using unpaired Student's t-test. The p value was set at 0.05, meaning that if $p < 0.05$, there was statistical significance between the control and the enhancers tested.

2.9. Skin integrity evaluation

Morphological changes and integrity evaluation was carried out by analyzing the structure of the skin by means of light microscopy and Scanning Electron Microscopy of the samples used in the permeation studies. After permeation experiments, skin samples were immediately rinsed with deionized water, carefully dried and stored at −80 °C until further use.

2.9.1. Histology studies

Skin samples used in the permeation studies were carefully sectioned and fixed using 10% buffered formalin for 24 h at room temperature. Skin samples were subsequently dehydrated with 50%, 75%, 95% and 2 changes of 100% absolute ethanol for 1 h each. Samples were embedded in Tissue-Tek® O.C.T. compound and frozen using carbon dioxide (dry ice) [44]. Cross-section slices of 7 μm thickness were obtained using a microtome (Leica Model CM 1850, Leica Microsystems, Inc. Bannockburn, IL, USA) and were stained following Ellis Hematoxylin and Eosin (H&E) staining protocol [45].

The stained slices were analyzed using a Nikon Eclipse E 800 light microscope (Micro Optics, Cedar Knolls, NJ, USA) at 10, 20 and 40×. A Nikon Digital Camera (Model DXM 1200) was used to capture images. Images were processed using SPOT TM Imaging Software, Version 5.0 (Diagnostic Instrument, Inc., Sterling Heights, MI, USA).

2.9.2. Scanning Electron Microscopy studies

Skin samples were submerged in Tissue-Tek® O.C.T. Compound and frozen using dry ice. Cross-section slices of the frozen samples were cut with 10 μm thickness using a microtome (Leica Model CM 1850, Leica Microsystems, Inc. Bannockburn, IL, USA). The samples were defrosted and fixed using 4% formaldehyde for 1.5 h. Following to this, skin samples were rinsed and placed in deionized water, at room temperature, for 2 h. Samples were dehydrated using solutions of 30%, 50%, 75%, and 95% ethanol in water for 25 min and finally, skin samples were placed in two changes of absolute ethanol (100%) for 2 h at room temperature. The dehydrated samples were dried with a critical-point drier (model CPD 020) and coated with gold. Surface and cross-sections pictures of the samples were taken using Scanning Electron Microscopy (SEM – model AMARY-18301).

2.9.3. Cytotoxicity studies

MTT/MTS assays are a colorimetric method used to determine the number of viable cells in cytotoxicity assays, widely reported in the literature for similar systems [46–49]. MTS reagent (CellTiter 96® AQueous One Solution Reagent) contains a tetrazolium compound [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] which is converted by mitochondrial dehydrogenase enzymes of living cells into a colored formazan compound. The number of viable cells is directly proportional to the quantity of formazan product formed as measured by the absorbance at 490 nm (Promega Corp. Protocol, 2009). The levels of formazan formed were quantified using a Microplate Power Wave X Scanning Spectrophotometer (Bio-TEK Instruments, Inc. Winooski, VT, USA).

Cytotoxicity studies were conducted both in cultured HEK and in HDF seeded at 8000 cells/well in 96-well plates under sterile conditions. The goal was to assess the toxic effects of the permeation enhancers on these types of cells. HEK were seeded in 100 μL/well of Epilife® (MEPI 500CA) medium supplemented with gentamycin (Gibco®), human keratinocyte growth supplement (Gibco®) and were incubating for 24 h at 37 °C, 5% CO_2 and 90% R.H. HDF were seeded in 100 μL/well of GIBCO®-Dulbecco's Modified Eagle Medium (DMEM) medium with 100 IU/mL penicillin, 100 IU/mL streptomycin and 10% fetal bovine serum. All the media and biological reagents were purchased directly from Invitrogen™, Inc. (Carlsbad, CA, USA).

After seeding, the culture medium was removed and cells were exposed to medium (control), propylene glycol (PG) and various concentrations (160, 16, 1.6, 0.16, 0.016 μM) of azone and nonionic ether-monohydroxyl C_{12}-surfactants ($C_{12}E_1$, $C_{12}E_5$, and $C_{12}E_8$) dissolved in PG and diluted in culture medium and were placed in the incubator for another 24 h in the same environmental conditions. After the exposure, testing solutions were removed and replaced by a pre-mixed solution of MTS reagent (CellTiter 96® AQueous One Solution) and appropriate medium (Promega Corp. Protocol, 2009) and were returned to the incubator for 2 h. After this, the 96-well plates were read at 490 nm using a plate reader (Microplate Power Wave X Scanning Spectrophotometer – Bio-TEK Instruments, Inc. Winooski, VT, USA).

Results are presented as% cell viability (mean ± standard deviation, $n = 6$), CV, after background absorbance (abs), determined from "no cell" control wells, was subtracted and was calculated according to

$$CV = \frac{\text{abs read intreated cells}}{\text{abs read in control (untreated) cells}}$$

3. Results and discussion

3.1. Permeation studies

The permeation studies were conducted essentially in two conditions, either by applying chemical permeation enhancer solutions alone or in combination with iontophoresis.

3.1.1. Effect of the penetration modifiers on the transdermal drug delivery of ondansetron HCl

Three nonionic ether-monohydroxyl C_{12} surfactants ($C_{12}E_1$, $C_{12}E_5$, and $C_{12}E_8$) and azone were dissolved in PG (0.16 M) and applied to the skin (60 μL) 1 h prior to the application to the skin of 0.3 ml of 2% ondansetron HCl loaded hydrogel. The permeation parameters (Flux, Q_{24}, and ER) are presented in Table 1.

All the permeation modifiers tested showed statistically significant enhancement ($p < 0.05$), except azone, and the nonionic surfactant $C_{12}E_5$ (ER = 107) resulted in the highest permeation of ondansetron HCl of all the permeation modifiers. It is worth noting that the pre-treatment with the vehicle (PG) caused an approximately 10-fold increase in the flux and 117-fold increase in the Q_{24}, but this was clearly lower than the effect observed for the surfactants $C_{12}E_1$ (ER = 26), $C_{12}E_5$ (ER = 107), and $C_{12}E_8$ (ER = 47). It should be recalled that these results have shown to be reproducible, with relative standard deviations typically between 25% and 33%.

Surprisingly, azone had little effect and no statistical difference from the control was found. The permeation profile of the cumulative amounts of ondansetron HCl permeated across the skin over a period of 24 h is presented in Fig. 2.

Table 1
Effect of the chemical permeation enhancers on percutaneous permeation of ondansetron HCl across porcine skin. Data are presented as means ± SD ($5 \leqslant N \leqslant 8$).

Penetration modifier	Flux ($\mu g\ cm^{-2}\ h^{-1}$)	Q_{24} ($\mu g\ cm^{-2}$)	ER
Control (drug-loaded hydrogel only)	0.043 ± 0.14	0.8 ± 0.28	–
Propylene glycol (PG)*	0.41 ± 0.27	8.68 ± 5.87	9.5
0.16 M $C_{12}E_1$ in PG*	1.12 ± 0.21	23.32 ± 4.66	26
0.16 M $C_{12}E_5$ in PG*	4.59 ± 0.78	93.41 ± 15.63	107
0.16 M $C_{12}E_8$ in PG*	2.00 ± 0.69	38.53 ± 13.02	47
0.16 M Azone in PG	0.061 ± 0.020	0.98 ± 0.38	1.4

* Statistically significant difference between enhancer and control, at $p < 0.05$ (Student's *t*-test).

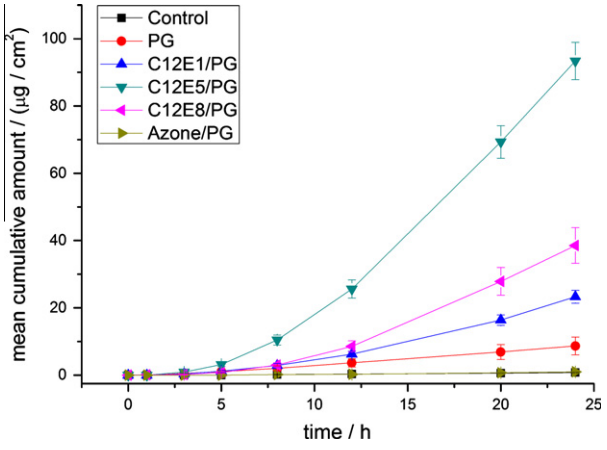

Fig. 2. Cumulative amount of ondansetron HCl permeated across porcine skin as a function of time. Skin was pre-treated with various nonionic ether-monohydroxyl C_{12} surfactants 1 h prior the start of *in vitro* experiments. Data are presented as means ± SEM ($5 \leqslant N \leqslant 8$). (For the interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.1.2. Combined effect of the pre-treatment with penetration modifiers and iontophoresis on the transdermal drug delivery of ondansetron HCl

The combined effects of penetration modifiers and iontophoresis in the transdermal permeation of ondansetron HCl were also investigated. Similarly to the previous experiments, all the enhancers were dissolved in PG and applied to the skin 1 h prior the start of the experiment. Despite *in vitro* permeation experiments were carried out over a period of 24 h, iontophoresis (0.3 mA) was only employed in the first 8 h (stage 1), followed by a post-iontophoresis passive diffusion period of 16 h (stage 2). Results are summarized in Table 2 (stage 1) and in Table 3 (stage 2).

Results showed that iontophoresis (0.3 mA, 8 h) caused a dramatic increase in the flux of ondansetron hydrochloride ranging between 5-fold for skin samples treated with $C_{12}E_5$ and 380-fold for the control samples (formulation only). The effect of the enhancers is barely noticed when iontophoresis is employed, because the contribution of the latter suppressed the contribution of the penetration modifiers, as expected. However, surfactants $C_{12}E_1$ and $C_{12}E_5$ still caused a noteworthy effect on the flux values, respectively, 40% and 30%, which are statistically different from the control in stage 1 (Table 2). The increase is therefore substantial in absolute value, especially if compared with the permeated amounts obtained in the passive process. In stage 2 (Table 3), after iontophoresis was turned off, the flux values decreased as expected. However, skin samples treated with $C_{12}E_5$ and $C_{12}E_8$ showed statistically higher values than those observed for the control samples with an ER of 2. The analysis of Q_{24} values showed that

Table 2
Combined effects of chemical permeation enhancers and iontophoresis (0.3 mA, 8 h – Stage 1) on the percutaneous permeation of ondansetron HCl across porcine skin. Data are presented as means ± SD ($N = 6$).

Penetration modifier	Flux ($\mu g\ cm^{-2}\ h^{-1}$)	Q_8 ($\mu g\ cm^{-2}$)	ER
Control (only formulation)	16.35 ± 3.46	128.30 ± 25.83	–
Propylene glycol (PG)	14.57 ± 2.78	112.31 ± 21.80	–
0.16 M $C_{12}E_1$ in PG*	22.66 ± 3.76	173.48 ± 29.68	1.4
0.16 M $C_{12}E_5$ in PG*	20.79 ± 3.20	159.63 ± 24.13	1.3
0.16 M $C_{12}E_8$ in PG	15.24 ± 3.36	117.47 ± 26.84	–
0.16 M Azone in PG	12.12 ± 0.81	93.19 ± 5.73	––

* Statistically significant difference between enhancer and control at $p < 0.05$ (Student's *t*-test).

Table 3
Combined effects of chemical permeation enhancers and iontophoresis on the percutaneous permeation of ondansetron HCl across porcine skin. Data pertain to post-iontophoresis period (0.0 mA, 16 h – Stage 2), and are presented as means ± SD ($N = 6$).

Penetration modifier	Flux ($\mu g\ cm^{-2}\ h^{-1}$)	Q_{24} ($\mu g\ cm^{-2}$)	ER
Control (only formulation)	5.50 ± 0.46	220.29 ± 24.99	–
Propylene glycol (PG)	5.86 ± 1.12	208.91 ± 35.50	1.1
0.16 M $C_{12}E_1$ in PG	6.99 ± 0.47	289.73 ± 37.22	1.3
0.16 M $C_{12}E_5$ in PG*	10.74 ± 1.87	336.33 ± 48.03	2.0
0.16 M $C_{12}E_8$ in PG*	11.84 ± 0.95	312.62 ± 25.34	2.15
0.16 M Azone in PG	5.29 ± 0.66	175.94 ± 6.88	–

* Statistically significant difference between enhancer and control at $p < 0.05$ (Student's *t*-test).

the combined use of iontophoresis and nonionic surfactants $C_{12}E_5$ and $C_{12}E_8$ caused an approximately 400-fold increase in the cumulative amount of drug permeated in 24 h, in comparison with the passive diffusion in untreated skin (control), and an approximately 40-fold increase comparatively to the passive PG pre-treated skin. The profiles obtained in these permeation experiments are summarized in Fig. 3.

3.1.3. Effect of the penetration modifiers on the transdermal drug delivery of diltiazem HCl

An identical study was carried out using diltiazem hydrochloride as the model drug. Passive diffusion experiments showed that all nonionic surfactants tested increased significantly the permeation of diltiazem HCl. The increase observed when azone was employed was not statistically significant. $C_{12}E_5$ was found to be the most efficient penetration modifier tested, leading to a 9.4-fold increase in the flux values, as compared to control (Table 4). The profiles of the permeation experiments are summarized in Fig. 4.

3.1.4. Effect of combined of the pre-treatment with penetration modifiers and iontophoresis on the transdermal drug delivery of diltiazem HCl

Similarly to what was observed in the studies with ondansetron HCl, values of diltiazem flux, Q_8 and Q_{24} increased significantly following the use of iontophoresis (see the next section). As presented in Table 5, in stage 1 (0–8 h), no statistically significant difference was observed between the three nonionic surfactants tested and azone. Despite the fact that the ER values are similar, $C_{12}E_8$ (1.6) and $C_{12}E_5$ (1.5) had higher activity compared with $C_{12}E_1$ (1.4) and azone (1.3). Interestingly, PG pre-treatment resulted in a significant decrease in the permeation parameters, in comparison with the control.

In stage 2 (8–16 h), the permeation parameters decreased comparatively to those for stage 1, but were clearly higher than those

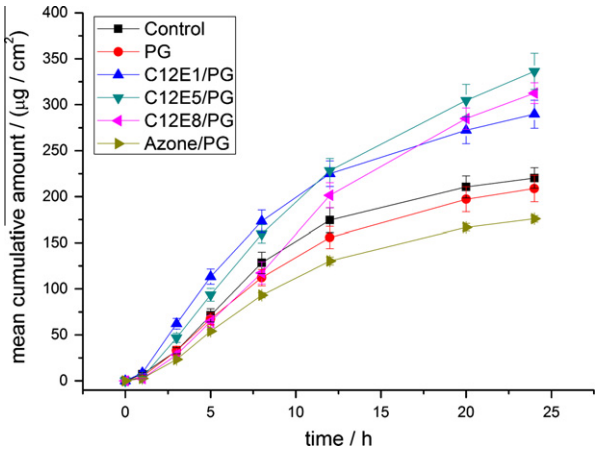

Fig. 3. Cumulative amount of ondansetron HCl permeated across porcine skin as a function of time. Skin was pre-treated with various nonionic ether-monohydroxyl C_{12} surfactants 1 h prior the start of *in vitro* experiments. Iontophoresis (0.3 mA) was applied during the first 8 h (stage 1), followed by a 16 h passive permeation. Data are presented as means ± SEM ($N = 6$). (For the interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 4
Effect of the chemical permeation enhancers on percutaneous permeation of diltiazem HCl across porcine skin. Data are presented as means ± SD ($3 \leqslant N \leqslant 7$).

Penetration modifier	Flux (μg cm^{-2} h^{-1})	Q_{24} (μg cm^{-2})	ER
Control (only formulation)	0.71 ± 0.26	13.53 ± 4.41	–
Propylene glycol (PG)	0.55 ± 0.064	7.59 ± 1.34	–
0.16 M $C_{12}E_1$ in PG*	3.98 ± 1.72	77.83 ± 37.07	5.6
0.16 M $C_{12}E_5$ in PG*	6.68 ± 1.11	137.85 ± 29.40	9.4
0.16 M $C_{12}E_8$ in PG*	3.57 ± 0.90	65.36 ± 19.27	5.0
0.16 M Azone in PG	2.52 ± 1.25	50.95 ± 26.67	3.5

* Statistically significant difference between enhancer and control at $p < 0.05$ (Student's *t*-test).

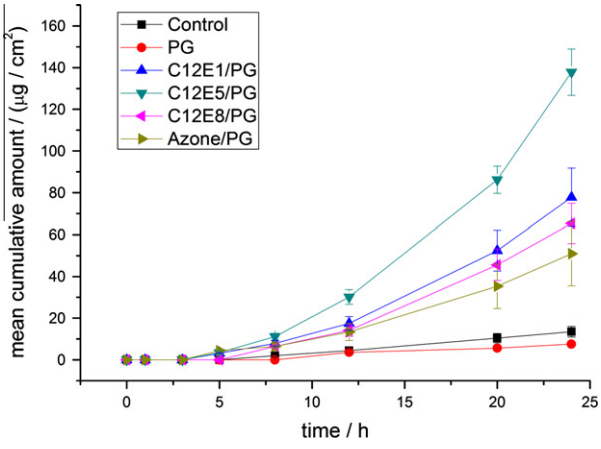

Fig. 4. Cumulative amount of diltiazem HCl permeated across porcine skin as a function of time. Skin was pre-treated with various nonionic ether-monohydroxyl C_{12} surfactants 1 h prior the start of *in vitro* experiments. Data are presented as means ± SEM ($3 \leqslant N \leqslant 7$). (For the interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

observed in the passive permeation study (Table 6). In this period, only enhancers $C_{12}E_5$ and $C_{12}E_8$ showed significantly higher permeation than the control. After 24 h of permeation, samples treated with a combination of iontophoresis (0.3 mA, 8 h) and nonionic surfactants $C_{12}E_5$ and $C_{12}E_8$, reached 2530 and 2678 μg/cm^2, respectively, 1.5 and 1.6 times the amount of diltiazem HCl permeated in the control iontophoresis experiment.

Table 5
Combined effects of chemical permeation enhancers and iontophoresis (0.3 mA, 8 h – Stage 1) on the percutaneous permeation of diltiazem HCl across porcine skin. Data are presented as means ± SD ($N = 6$).

Penetration modifier	Flux (μg cm^{-2} h^{-1})	Q_8 (μg cm^{-2})	ER
Control (only formulation)	123.24 ± 12.78	964.64 ± 98.74	
Propylene glycol (PG)	102.09 ± 12.57	801.50 ± 95.49	-
0.16 M $C_{12}E_1$ in PG*	166.25 ± 33.98	1289.30 ± 264.57	1.4
0.16 M $C_{12}E_5$ in PG*	181.58 ± 24.74	1415.04 ± 190.55	1.5
0.16 M $C_{12}E_8$ in PG*	199.00 ± 25.65	1547.02 ± 192.31	1.6
0.16 M Azone in PG*	163.07 ± 21.81	1267.69 ± 133.68	1.3

* Statistically significant difference between enhancer and control at $p < 0.05$ (Student's *t*-test).

Table 6
Combined effects of chemical permeation enhancers and iontophoresis on the percutaneous permeation of diltiazem HCl across porcine skin. Data pertain to post-iontophoresis period (0.0 mA, 16 h – Stage 2), and are presented as means ± SD ($N = 6$).

Penetration modifier	Flux (μg cm^{-2} h^{-1})	Q_{24} (μg cm^{-2})	ER
Control (only formulation)	43.78 ± 11.59	1689.87 ± 224.28	
Propylene glycol (PG)	36.49 ± 11.07	1409.43 ± 133.91	–
0.16 M $C_{12}E_1$ in PG	47.76 ± 5.47	2092.71 ± 241.23	1.1
0.16 M $C_{12}E_5$ in PG*	66.64 ± 13.38	2530.36 ± 286.97	1.5
0.16 M $C_{12}E_8$ in PG*	66.81 ± 17.45	2678.15 ± 304.20	1.5
0.16 M Azone in PG	42.91 ± 7.48	1998.39 ± 133.68	–

* Statistically significant difference between enhancer and control at $p < 0.05$ (Student's *t*-test).

By comparing Q_{24} values of iontophoresis (Table 6) against those in which this technique is absent (Table 4), it can be seen that the amount of drug permeated increased by ca. 200-fold when a combination of enhancer and iontophoresis was used.

The permeation profiles of diltiazem HCl across porcine skin, using a combination of iontophoresis and various enhancers are presented in Fig. 5.

3.1.5. Overview and comparison

Results show that in both in passive and post-iontophoresis stages, the enhancer with the best overall performance is $C_{12}E_5$. Despite the fact that it is very difficult to know the exact mechanism of permeation enhancement, a possible explanation for the observations will be given in what follows, based on some physico-chemical properties. The $C_{12}E_1$ surfactant is the most hydrophobic compound (log*P* 5.05) and is also the least effective both for passive and post-iontophoresis diffusion for both drugs. In passive diffusion, $C_{12}E_5$ (log*P* 3.62) performs better for both drugs, in what seems a compromise between affinity for the lipid structures on the SC, higher for $C_{12}E_1$ and affinity for the drugs, higher for $C_{12}E_8$ (log*P* 2.54). We note that, in a simple interpretation, affinity for the lipids correlates with disordering of the SC lipids, while affinity for the drug accounts for the facilitated transport of the drugs.

Also reported is the fact that $C_{12}E_5$ is more effective in association with diltiazem than ondansetron. It should be noted that in spite of the fact that the Mw of ondansetron HCl (Mw: 365.15; log*P*: 2.07; log*D* (pH5.5): 0.19) is lower than of the diltiazem HCl (Mw: 450.14; log*P*: 3.63; log*D* (pH 5.5): 0.76), the former is a more hydrophilic drug, thus it permeates less in absolute values. Any increase caused by the enhancers is therefore comparatively more significant than that for a more hydrophobic drug. This more pronounced effect occurs due to a facilitated transport of the hydro-

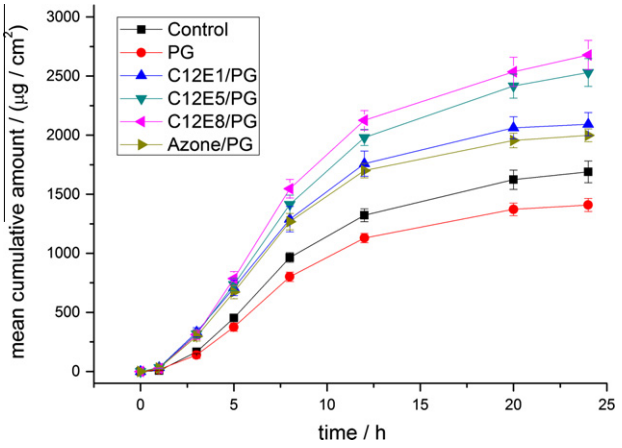

Fig. 5. Cumulative amount of diltiazem HCl permeated across porcine skin as a function of time. Skin was pretreated with various nonionic ether-monohydroxyl C_{12} surfactants 1 h prior the start of *in vitro* experiments. Iontophoresis (0.3 mA) was applied during the first 8 h (stage 1), followed by a 16 h passive permeation. Data are presented as means ± SEM ($N = 6$). (For the interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

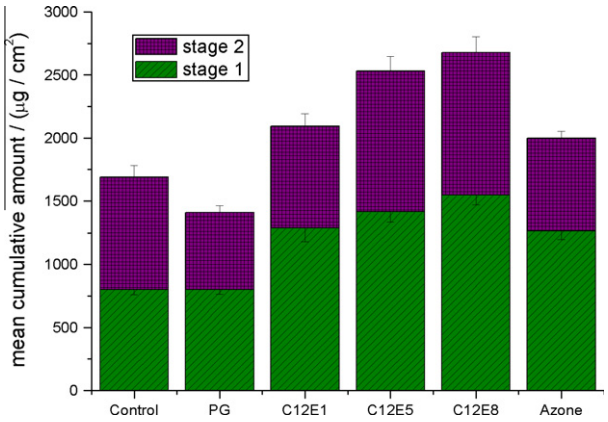

Fig. 7. Cumulative amount of diltiazem HCl permeated after 8 h (stage 1) and 24 h (stages 1 and 2). (For the interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

philic drug through the lipid layers of the skin. This effect has been previously reported by El-Kattan et al. [50], where the ER of the chemical enhancers employed (terpenes) was much higher for the hydrophilic drugs and decreased with an increase in the hydrophobicity.

Figs. 6 and 7 summarize the results obtained in the permeation studies involving iontophoresis. We will first consider the data for ondansetron. Control and PG treated samples produced almost indistinguishable effects, both in stage 1, in which the electrical current was applied for 8 h, and in the subsequent stage of 16 h. Interestingly, azone treated skin displayed a slight reduction in the cumulative amount, compared with the iontophoresis control. The highest drug permeation was achieved with the surfactant treated samples. Relative to the control, the surfactants promoted drug delivery with an increase in flux between 30% and 40% in stage 1. This corresponded to a considerable amount, if we take in consideration the effect of iontophoresis and compare it with the passive process. It is seen that, also for stage 1, the samples treated with $C_{12}E_1$ showed the highest permeated amount of ondansetron, followed by skin samples treated with C12E5 and C12E8, respectively. Interestingly, it was observed that this order

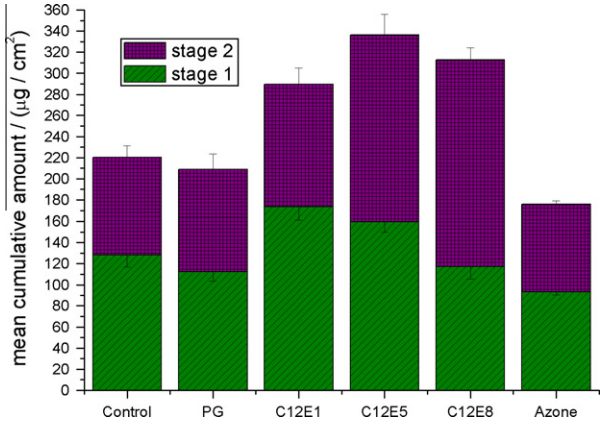

Fig. 6. Cumulative amount of ondansetron HCl permeated across porcine skin after 8 h (stage 1) and 24 h (stage 1 and 2). (For the interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was reversed in stage 2. Overall, during the 24 h, the conjugation of the two opposite trends resulted in a maximum amount of drug permeated, when surfactant $C_{12}E_5$ was employed, with a slightly higher amount of drug permeated in stage 2 than during the iontophoretic stage. The latter effect was even more pronounced in samples treated with surfactant $C_{12}E_8$, while in the remaining samples drug permeation was higher for stage 1. For the most effective enhancer, $C_{12}E_5$, the enhancement ratio calculated on the basis of the total cumulative amount permeated, was 400-fold higher than that for passive control.

Data obtained with diltiazem hydrochloride showed that PG treatment caused a slight decrease in the permeated amount of drug compared with the control. However, all other enhancers tested had the opposite effect. The surfactants were again more effective than azone, and interestingly, the enhancing effect increased with increasing surfactant molecular weight. Generally, this increase was observed both in the iontophoresis and post-iontophoresis stages. It should be noted that, unlike what has been observed in studies with ondansetron, the iontophoresis stage corresponded to a permeated amount of drug which was always higher than that of stage 2. The use of $C_{12}E_8$ in combination with iontophoresis promoted the highest enhancement ratio.

A rough estimate based on the above results obtained with porcine skin, indicated that for the 24 h period, a total dose of ca. 8.4 mg and 67 mg would reach the blood stream for ondansetron and diltiazem, respectively, using a $5 \times 5\ cm^2$ formulation patch. Therefore, these values show that the approaches described in this work may provide a promising strategy to obtain effective therapeutic levels for both drugs, taking into account that slightly higher values drug permeated are expected when using human skin [42,43]. Moreover, higher blood concentrations can be reached with further formulation optimization and iontophoretic parameter adjustments (time and current intensity) applied to the skin.

3.2. Skin integrity evaluation

Skin samples used in permeation experiments were rinsed with water and kept frozen at −80 °C until further use. Skin damage and morphological changes caused by the methodology employed (chemical penetration enhancers and iontophoresis) were assessed (light microscopy and SEM).

3.2.1. Histological studies

Histological procedures followed by light microscope observations at different magnifications (10, 20 and 40×) were carried out in untreated (control) and treated skin samples. All the treated

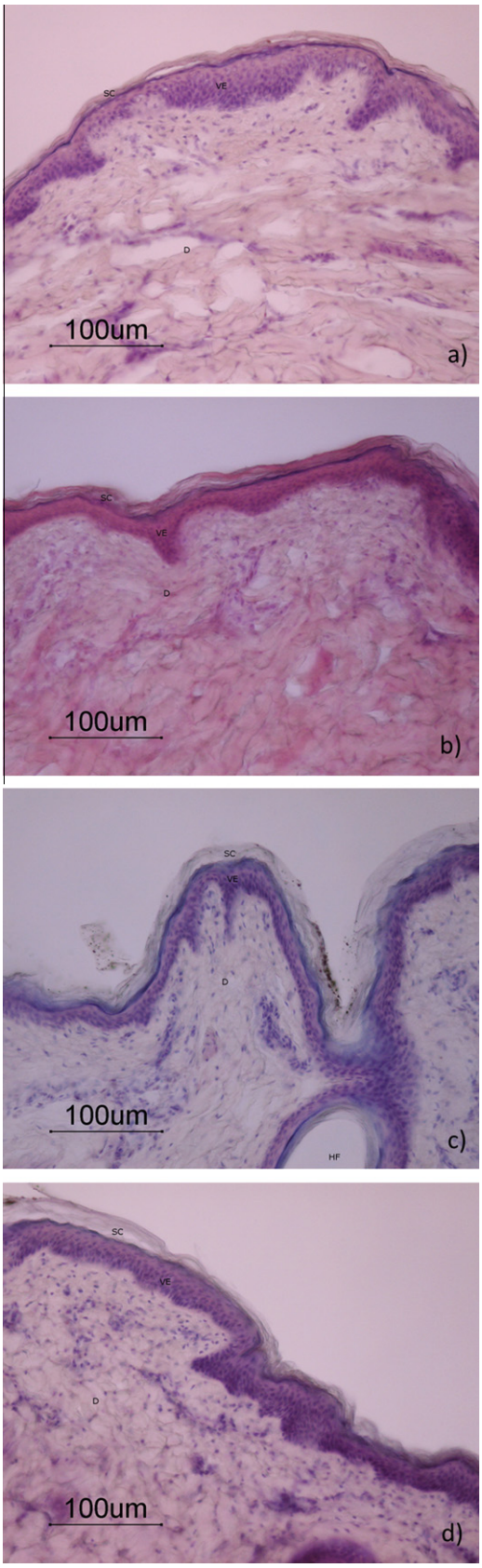

Fig. 8. Porcine skin pictures taken by an optical light microscope at a magnification of 20× after permeation experiments, except for the control (a), using $C_{12}E_5$ (b), $C_{12}E_5$ + iontophoresis (c), $C_{12}E_8$ + iontophoresis (d). The various skin layers and structures can be observed: stratum corneum (SC), viable epidermis (VE), dermis (D) and hair follicles (HF). (For the interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tissues were skin samples previously used as membranes in the *in vitro* permeation experiments, and subject to treatment with various penetration modifiers alone or in combination with iontophoresis.

The histology work was performed on all skin samples treated with enhancers. However, we are presenting only skin samples treated with the enhancers that resulted in the most efficient drug penetration. As can be observed in Fig. 8, no significant morphological or structural changes have been detected when comparing the control samples with those subjected to the combined treatment. No major changes were detected in cell morphology and cohesion, or in the level of organization of the tissues, but some areas of the SC were noted to be detaching or peeling off from the subsequent layer. However, this is also observed in the untreated samples and may be a result of sample handling. The variation observed in the properties related to the skin structure, such as the thickness of the SC, the density and depth of the rete pegs may be ascribed to inter-individual variability and to the body area where the skin was obtained from. It is worth noting that skin samples subjected to iontophoresis exhibited small dark spots, consistent with residues of silver chloride, in the region of the SC.

3.2.2. Scanning Electron Microscopy studies

Scanning Electron Microscopy (SEM) studies were carried out to assess the skin integrity after the drug permeation experiments.

Skin layers such as the SC (the outermost multi-layered structure), viable epidermis (the darker area) and dermis (the thicker structure mostly composed of connective tissue under the viable epidermis) are visible in detail on the following figures (Figs. 9–11).

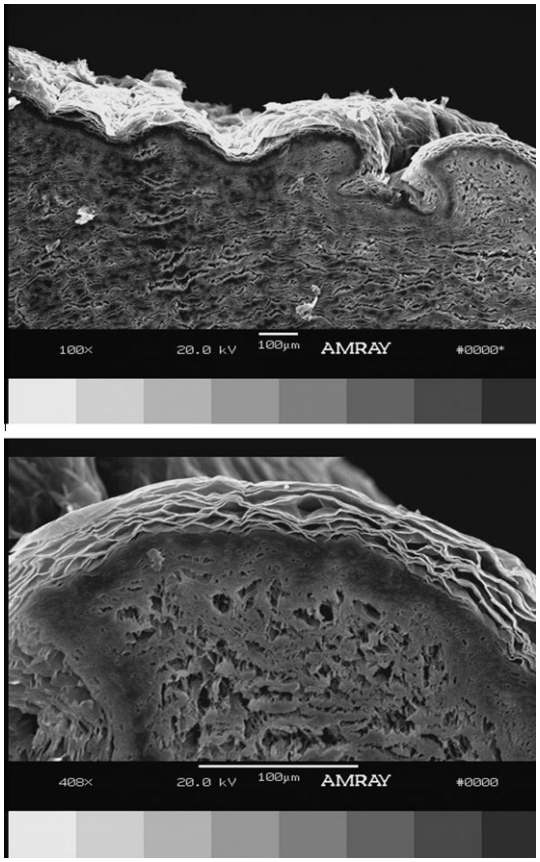

Fig. 9. SEM cross-section of untreated porcine skin (control) at 100× and 400× magnification. This skin sample was not subjected to permeation studies.

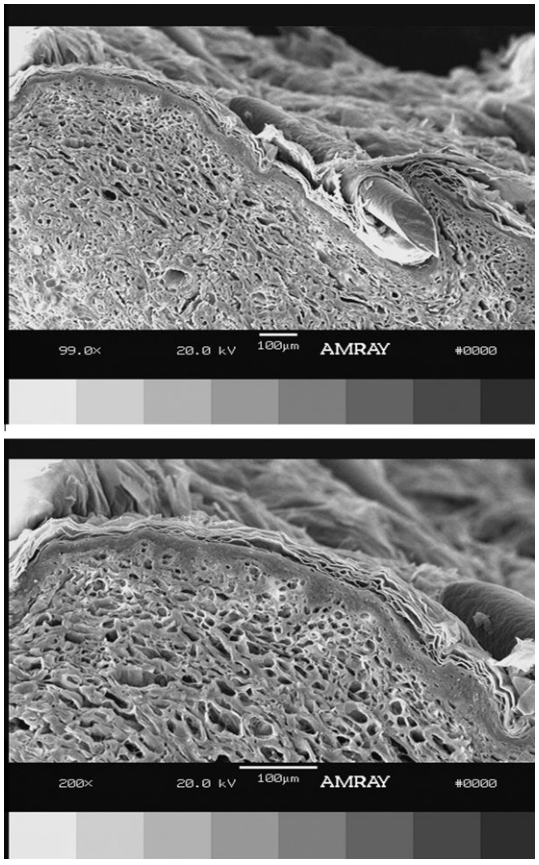

Fig. 10. SEM cross-section pictures of porcine skin treated with nonionic surfactant $C_{12}E_8$ at 100× and 200× magnification taken after permeation studies. On the top picture, a hair filament in the dermis is visible.

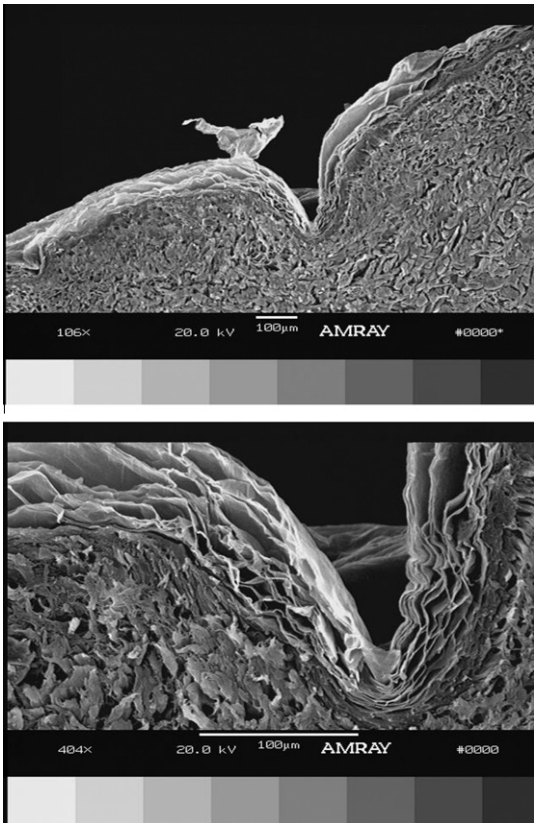

Fig. 11. SEM cross-section pictures of porcine skin treated with nonionic surfactant $C_{12}E_5$ and iontophoresis (0.3 mA, 8 h) at 100× and 400× magnification.

It was observed that all the thirteen skin samples tested retained the cell-to-cell and layer-to-layer cohesion and their macroscopic structure after the cryo-SEM procedure. Despite all the treatments, the tissues remained compact, well stacked and with the same level of organization observed in untreated skin samples. By looking at the skin surface, it can be seen that some corneocytes of the outermost layer of the SC were being shed, a normal process of skin cells renewal known as desquamation (as previously noted in the optical microscopy observations). However, it can also be observed that, despite some swelling of the SC caused by the large amounts of water present in the drug-loaded hydrogels, the corneocytes of the SC are very well packed and in close contact with each other, providing the high organization needed for maintaining its barrier properties. Therefore, it can be concluded that no major differences were found between the control and the treated samples in terms of skin integrity.

3.3. Cytotoxicity studies

The potential cytotoxicity of the chemical penetration modifiers used was assessed by exposing cultured HEK and HDF to different concentrations of these compounds, and by evaluating and comparing the change in the mitochondrial metabolic activity of those cells using the MTS assay.

Standard plots for HEK ($R^2 = 0.986$) and for HDF ($R^2 = 0.978$) were constructed based on the absorbance readings at 490 nm for 0, 2.000, 4.000, 6.000, 8.000 and 10.000 cells/well to ensure the linearity of the MTS assay method used.

The results obtained in the MTS assays are presented in Fig. 12 (HEK) and in Fig. 13 (HDF).

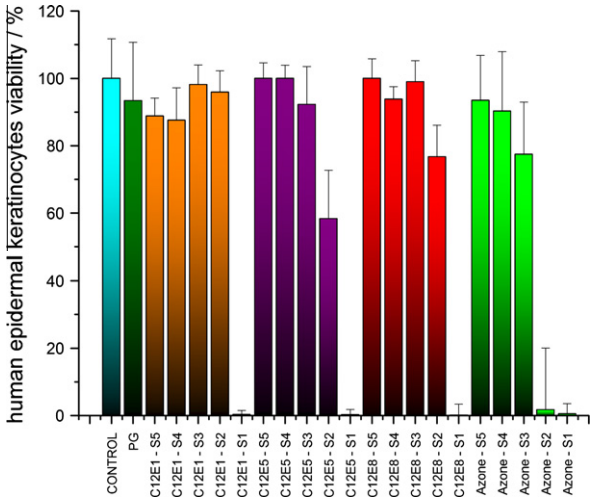

Fig. 12. MTS assay results for cultured HEK. The bars represent the cell viability (%) for each permeation modifier and concentration tested. The error bars stand for the standard deviation ($n = 6$). (For the interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

As can be seen on Fig. 12 PG did not significantly reduce the viability of HEK when compared to the control (untreated cells). However, 160 µM (S1) of all the compounds was lethal for all the HEK. Azone 16 µM (S2) use resulted in cell death at nearly 100%, however, compound $C_{12}E_1$ produced no harm to the cells. The same concentration of $C_{12}E_5$ and $C_{12}E_8$ caused, respectively, a cell viability reduction of 40% and 25%. Concentrations of 1.6 µM (S3) or lower (0.16 µM (S4) and 0.016 µM (S5)) of any of the enhancers did not cause significant cell viability reduction, except in the case of azone.

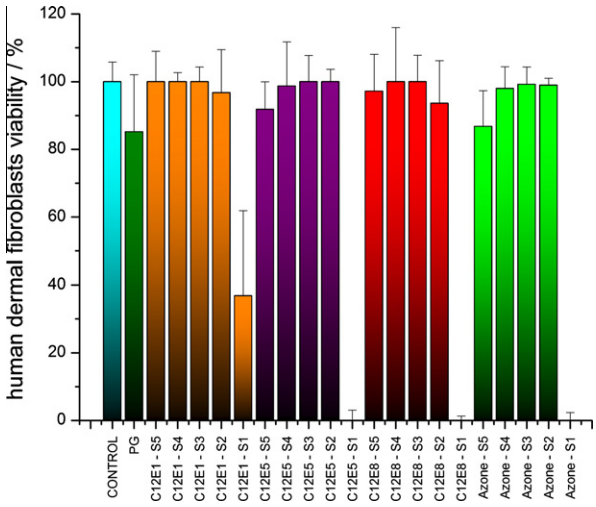

Fig. 13. MTS assay results for cultured HDF. The bars represent the cell viability (%) for each permeation modifier and concentration tested. The error bars stand for the standard deviation ($n = 6$). (For the interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The MTS assay using HDF's showed that the HDF exhibited higher tolerance to the permeation modifiers compared with the HEK. PG caused cell death to a higher extent than in the case of HEK, but this was not statistically significant. Concentrations of 16 μM (S2) or lower of any penetration modifier did not cause a significant decrease in the HDF viability. A concentration of 160 μM (S1) of any of the compounds tested was lethal to all the cells except in the case of the nonionic surfactant $C_{12}E_1$. Results obtained both in HEK and in HDF suggest that the toxicity of the nonionic surfactants tested is molecular weight dependent.

4. Conclusions

The results presented show that the nonionic ether-monohydroxyl surfactants tested were effective skin penetration enhancers for the transdermal drug delivery of ondansetron hydrochloride and diltiazem hydrochloride. The enhancement effects observed were dependent on the penetration modifier and on the drug used. Pentaethylene glycol monododecyl ether ($C_{12}E_5$) produced the highest flux values and cumulative amounts of drug permeated (Q_{24}) observed in the passive transdermal studies of both ondansetron hydrochloride (ER = 107) and diltiazem hydrochloride (ER = 9.4). The combined use of the chemical penetration enhancers and iontophoresis (0.3 mA – 8 h) significantly increased the amount of drugs permeated. Despite the fact that the major contribution in terms of drug permeation was derived from the iontophoresis transport, the various chemical penetration enhancers produced different levels of activity. The best results in terms of drug permeated were obtained with the combination of iontophoresis and the enhancer pentaethylene glycol monododecyl ether ($C_{12}E_5$) (ER = 420, compared to passive permeation without enhancer pre-treatment) for ondansetron hydrochloride, while octaethylene glycol monododecyl ether ($C_{12}E_8$) (ER = 200, compared to passive permeation without enhancer pre-treatment) performed better with diltiazem hydrochloride. Skin integrity evaluation studies did not show significant changes in the tissue morphology when compared to the untreated samples suggesting that these compounds are promising candidates for use in transdermal formulations. The present results suggest that both drugs can be successfully delivered through the skin using a combination of chemical enhancement and iontophoresis, attaining plasma levels comparable to those obtained with oral formulations.

Acknowledgments

S.M.C. Silva acknowledges Fundação para a Ciência e a Tecnologia, Lisboa (Portugal), for Ph.D. Grant reference SFRH/BD/30537/2006. Partial funding provided by the Center for Dermal Research/NJ Center for Biomaterials, Rutgers-The State University of New Jersey www.centerfordermalresearch.org.

The authors acknowledge Mr. Vishwas Rai for the help and assistance provided with the cell culture work.

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