Surfactants from Itaconic Acid: Toxicity to HaCaT keratinocytes in vitro, Micellar Solubilization, and Skin Permeation Enhancement of Hydrocortisone

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Abstract

One of the most widely used approaches for improving drug permeation across the stratum corneum barrier of the skin is the use of chemical penetration enhancers, such as surfactants. In this study, two anionic surfactants, named C12-OPK and C18-OPK, were synthesized via condensation of itaconic acid and fatty amines, with C12 and C18 alkyl chains, respectively. Assessment of impacts on HaCaT keratinocyte cell viability was used as indicator of their potential to cause skin irritation 24 h post exposure (Alamar Blue assay). The LC$_{50}$ values of C12-OPK and C18-OPK (144 and 85 mg/L, respectively) were lower than LC$_{50}$ values of the most used commercial surfactants (e.g. SDS). The effect of different surfactant concentrations (up to ten times the critical micellar concentration, CMC) on hydrocortisone (HC) solubility and permeation through porcine skin was also evaluated. Results showed that drug solubility increased linearly with increasing concentrations of both surfactants, as a consequence of the association between drug and micelles. In vitro permeation results showed that the permeability coefficient increased at surfactant concentrations lower than the CMC. In particular, a higher enhancement effect on drug permeation was obtained with C18-OPK, due to its hydrophobic properties that ensured a more effective HC permeation in comparison to C12-OPK.

Keywords: surfactants, hydrocortisone, cytotoxicity, keratinocyte, micellar solubilization, skin permeation enhancement.
1. Introduction

Transdermal drug delivery offers many advantages over other traditional routes of drug delivery, such as a better control of blood levels, a reduced incidence of systemic toxicity, and the absence of hepatic first-pass metabolism (Nokhodchi et al., 2003). Unfortunately, the transdermal route is associated with a delayed onset of action due to the time required to reach the optimal drug concentration in the skin or blood and the variation of absorption rate based on site of application, skin type and patient age (Barrichello et al., 2006). Moreover, the outermost layer of the skin, the stratum corneum (SC), represents a barrier for most drugs to be delivered into and through it (Barry, 1983), due to the presence of keratin-rich cells embedded in multiple lipid bilayers. One of the most useful strategies to improve transdermal delivery is the use of chemical penetration enhancers (Williams and Barry, 2004), such as surfactants, which enhance the permeability of the SC barrier (Barry, 1983). Surfactants are amphiphilic molecules, consisting of both hydrophilic (head) groups and hydrophobic (tail) groups. Their ability to increase water solubility of drugs and to enhance skin permeability has been widely exploited in the field of transdermal drug delivery (Ghafourian et al., 2015; Pandey et al., 2014; Som et al., 2012). The effect of surfactant molecules on drug availability at the site of application (Rangel-Yagui et al., 2005) and on drug transport across the skin barrier (Pandey et al., 2014) depends on their ability to self-associate in micellar structures as well as to interact with the stratum corneum. The physico-chemical characteristics and the concentration of surfactants have been shown to play an important role in the achievement of these phenomena. In fact, below the critical micellar concentration (CMC), surfactants are present as single molecules, while above the CMC surfactants exist as micelles. Usually, single surfactant molecules do not improve drug solubilization, but they can interact with the dead cornified cells and the matrix of intercellular lipids in the SC to modify barrier permeability and improve drug permeation. In the presence of a micellar solution (i.e. at surfactant concentrations above the CMC), the drug can be efficiently solubilized in the core of the micelles resulting in higher concentration at the site of application with respect to the pure drug. At the same time, the release of the encapsulated drug from the core of the surfactants or the direct transfer of the drug from the micelles towards the membrane, can influence its permeation across the stratum corneum (di Cagno and Luppi, 2013). Therefore, in this situation (surfactant concentration above the CMC) drug permeation is the result of multiple mechanisms. Among the different types of surfactants (anionic, cationic, nonionic, amphoteric), anionic surfactants interact strongly with both keratin and lipids and alter the permeability of the skin by acting on the helical filaments of the stratum corneum; however, the use of anionic surfactants is often accompanied by irritation which limits their applications (Pandey et al., 2014; Karande et al., 2006).
Recently, Malferrari and colleagues synthetized and characterized different surfactants derived from fatty amines and itaconic acid, a dicarboxylic acid industrially obtained from renewable resources (Malferrari et al., 2015). In the present study, two of these surfactants (named C12-OPK and C18-OPK, scheme 1) characterized by a 3-substituted pyrrolidine ring bearing a carboxylate function and a long C12 or C18 alkylic chain respectively, were selected as possible pharmaceutical excipients for topical formulations of therapeutics. C12-OPK and C18-OPK were first tested for their potential to elicit skin irritation via assessment of HaCaT cell viability and then their ability to act as solubility and skin permeation enhancers for hydrocortisone (HC; a naturally occurring corticosteroid that possess strong anti-inflammatory properties) was evaluated.

Scheme 1. Synthesis and structures of surfactants C12-OPK (potassium 1-dodecyl-5-oxopyrrolidine-3-carboxylate) and C18-OPK (potassium 1-octadecyl-5-oxopyrrolidine-3-carboxylate)

2. Materials and methods

2.1 Materials

Hydrocortisone was obtained from Fluka-Chemie (Sigma-Aldrich, Milan, Italy). All chemicals and solvents (HPLC grade) were of analytical grade and were purchased from Sigma-Aldrich (Milan, Italy). Buffer solution at pH 7.4 was composed of 7.4 mM Na₂HPO₄ • 10H₂O, 1.1 mM KH₂PO₄, 136 mM NaCl.

C12-OPK and C18-OPK surfactants were prepared according to a previous reported synthetic procedure (Malferrari et al., 2015): itaconic acid (2-methylidenebutanedioic acid, 1 eq) was added to a stirring solution of the alkylamine (dodecylamine or octadecylamine 1.1 eq) in CH₃CN at RT and then the solution stirred at 70 °C for 24 h. Evaporation of the solvent under reduced pressure left a solid, which was purified by flash-chromatography and treated at RT with an equimolar amount of aqueous KOH, affording after concentration C12-OPK or C18-OPK in 90% overall yield.
2.2 Cytotoxicity evaluation on HaCaT cells

2.2.1 Cell culture

The HaCaT cell line, a nontumorigenic immortalized human keratinocyte cell line, was obtained from Cell Lines Services (Eppelheim, Germany). Cells were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 250 mg/mL fungizone (termed complete cell culture medium) at 37°C in a 5% CO₂ humidified atmosphere. For each experiment, cells were seeded at a concentration 2x10⁵ cells/mL (100 µL) in 96 well plates, and allowed to adhere for 24 h. Stock solutions of C12-OPK and C18-OPK compounds were suspended in complete cell culture medium. The concentration range in which the surfactants were tested was 0.5-1000 mg/L, with 14 intermediate concentrations.

2.2.2 Exposure and viability assay

Cells were treated (100 µL) with the surfactants (suspended in complete cell culture medium) at concentrations ranging from 0.5 to 1000 mg/L, in triplicate, and incubated at 37 °C for 24 h. A positive control (0.2% triton X 100 (v/v)) and a negative control (complete cell culture medium) were also included. For comparative purposes, cells were exposed to SDS at concentrations ranging from 9.77 to 1250 µM (equivalent to 2.81 to 360 mg/L).

Cell viability was assessed using the alamar blue assay (resazurin sodium salt 0.01 mg/mL, in complete cell culture medium). Cells were incubated with the alamar blue reagent for 2 hours and fluorescence measured at 560/590 nm (excitation/emission) using a fluorescence plate reader. Data are expressed as a percentage of the negative control (i.e. % viability).

2.3 HC solubility

A homogeneous suspension of HC was prepared by dispersing an excess amount of drug in water and keeping the suspension under agitation for 48 h at 25°C. Undissolved HC was removed by centrifugation (GS-15R Centrifuge, Beckman Coulter, Milan, Italy) at 10,000 rpm for 15 min and subsequent filtration of the dispersion through a 0.2 µm pore-size cellulose acetate filter. Subsequently, an exact volume was rationally diluted with ethanol and drug concentration was detected by high-performance liquid chromatography (HPLC).

2.4 HPLC analytical assay

Chromatographic separations were performed using a Shimadzu (Milan, Italy) LC-10ATVP chromatographic pump and a Shimadzu SPD-10AVP UV–Vis detector set at 244 nm. Separation
was obtained on a Phenomenex (Torrance, California) Sinergy Fusion-RP 80A (150 × 4.6 mm² i.d., 5 µm) coupled to a Phenomenex Security Guard C18 guard cartridge (4 × 3.0 mm² i.d., 5 µm). The mobile phase was composed of a mixture of acetonitrile/ethanol/phosphate buffer at pH 7.4 50:10:40 (v/v). The flow rate was 0.4 mL/min and manual injections were made using a Rheodyne 7125 injector with a 20 µL sample loop. Data processing was carried out by means of a CromatoPlus computerized integration system (Shimadzu Italia, Milan, Italy). A calibration curve was set up in the 6-121 µg/mL range (ethanol/water 9:1, v/v) and good linearity was found (r² = 0.999). Each analysis was repeated three times (n = 3). For permeations studies, a calibration curve was obtained with a drug concentration range of 0.54-5.4 µg/mL (PBS and ethanol, 80:20 v/v) and good linearity was found (r² = 0.999). LOD and LOQ were 0.15 µg/ml 0.50 µg/ml, respectively. The methodology granted good precision, with RSD values always lower than 5.0%.

2.5 Influence of surfactants on HC solubility

HC solubility was determined in the presence of C12-OPK and C18-OPK surfactants. Table 1 reports the relative molecular weight and CMC of C12-OPK and C18-OPK. For this study, an excess amount of drug was dispersed in water at 25°C, in the presence of different surfactant concentrations until 10-fold the respective CMC. In particular, the tested range of surfactant concentrations was 0.5-10 mM and 0.15-3 mM for C12-OPK and C18-OPK, respectively. After 48 h, undissolved HC was removed by centrifugation and subsequent filtration (conditions as above) and the drug concentration was determined by HPLC. Each analysis was repeated in three replicates (n = 3).

In order to characterize the solubilizing effect of the two surfactants, the molar solubilization capacity (χ) and the molar micelle-water partition coefficient (Kₘ) were determined (Attwood and Florence, 1983; Alvarez-Nunez and Yalkowsky, 2000). χ is defined as the number of moles of the drug that can be solubilized by one mole of micellar surfactant and it characterizes the ability of the surfactant to solubilize the solute. Its value is obtained from the slope of the line that results when drug concentration is plotted against surfactant concentration. The general equation for micellar solubilization is:

\[ χ = \frac{(S_{\text{tot}} - S_w)}{(C_{\text{surf}} - \text{CMC})} \]  (1)

where \( S_{\text{tot}} \) is the total drug solubility, \( S_w \) is the water drug solubility, \( C_{\text{surf}} \) is the molar concentration of surfactant in solution and CMC is the critical micelle concentration.

The micelle-water partition coefficient (K) represents the ratio of the drug concentration in the micelles to the drug concentration in water for a particular concentration of surfactant and it is calculated according to the following equation:
\[ K = \chi \left( C_{\text{surf}} - C_{\text{CMC}} \right)/S_w \]  

(2)

Since \( K \) is restricted by the surfactant concentration, a molar micelle-water partition coefficient \((K_M)\) is generally defined as the micelle-water partition coefficient in a one molar (1M) surfactant solution, i.e.:

\[ K_M = \chi (1 - C_{\text{CMC}})/S_w \]  

(3)

From the thermodynamic point of view, the solubilization can be considered as the partitioning of the drug between micelles and aqueous phase and the standard free energy of solubilization \((\Delta G^0_S)\) can be represented as:

\[ \Delta G^0_S = -RT \ln K_M \]  

(4)

where \( R, T \) and \( K_M \) are the universal gas constant, temperature and molar partition coefficient between micelle and aqueous phase, respectively.

2.6 Preparation of the tissue

Pig ear skin is a widely used model due to its similarity to the human skin in terms of behaviour, composition and permeability (Meyer et al., 1978; Simon and Maibach, 2000). Skin was excised from domestic pig ears, obtained from a local commercial supplier and cleaned with saline solution (0.9 % w/v NaCl). The skin was carefully separated from cartilage using a scalpel, the adipose subcutaneous tissue and the excess of hair were removed. The integrity of the skin was assessed by microscopic observation and any skin that was not completely uniform in appearance was rejected.

The skin was frozen at -20 °C for a maximum period of 2 days (Bigucci et al., 2015).

2.7 Influence of surfactants on HC permeation

Permeation of HC through the skin was investigated using a Franz-type static glass diffusion cell (15 mm jacketed cell with a flatground joint and clear glass with a 12 mL receptor volume; diffusion surface area = 1.77 cm²), equipped with a V6A Stirrer (PermeGearInc., Hellertown, PA, USA). Skin was mounted in the diffusion cell, stratum corneum uppermost, with the dermal side facing the receptor compartment. The receptor compartment was filled with a mixture of PBS and ethanol (80:20 v/v), maintained at 32 ± 1 °C by means of a surrounding jacket and constantly stirred. Samples consist of drug in the presence of different surfactant concentrations. For each surfactant, we selected one concentration below the CMC (0.15 mM) and another one 10-fold higher the CMC (3 mM for C18-OPK and 10 mM for C12-OPK). Moreover, the 3 mM concentration was also tested for C12-OPK in order to study the solubilizing effect on drug permeation at the same surfactant concentration above the CMC. Samples were prepared as described above, including centrifugation and filtration in order to remove undissolved HC; samples
contained different HC amount accordingly to the solubilizing effect of the different surfactants. At
the starting point of the experiment, 200 μL of the of each liquid formulation were applied as donor
phases. The permeation of drug from a control sample, based on HC suspension without surfactants,
was also measured. At predetermined time intervals (1 hour) until 9 hours, 200 μL of the receiver
phase were collected and replaced by fresh receptor medium. Cumulative amounts of drug
permeated per unit area of porcine skin (µg/cm²) was evaluated through HPLC and plotted against
time (s). The concentration of HC in the receptor compartment during the experiment did not
exceed 10% of the saturation solubility (sink condition). Skin components did not interfere with the
reported chromatographic method.

The in vitro permeation rate or flux (J) was generated from the slope of the linear portion of the
curve. Taking into account the starting drug concentration (C₀) within different vehicles,
permeability coefficient (Kp) was calculated according to the following equation:

\[ Kp = \frac{dm}{dt} (A \cdot C₀) \]  

(5)

where \( dm \) is the cumulative amount of drug permeated by the time \( dt \), \( A \) is the area of the skin used
and \( C₀ \) is the initial donor concentration (Abruzzo et al., 2015).

Enhancement ratio (ER) was used to evaluate the effect of surfactant on HC permeation. It is
calculated according to the following equation (Kim et al., 2008):

\[ ER = \frac{\text{Permeation rate in the presence of surfactant}}{\text{Permeation rate without surfactant}} \]

(6)

Each analysis was repeated in four replicates (n = 4).

2.8 Statistical Analysis

All results are shown as mean ± standard deviation (SD) or mean ± standard error of the mean
(SEM). SD and SEM were calculated from the values of at least 3 independent experiments, each
with 3 replicates, except for permeation results which were calculated from the values of at least 4
independent experiments. Data from cytotoxicity assessment were analysed using the Minitab
statistical package using a general linear model with subsequent analysis of variance and post-hoc
Tukey’s test. Data from all the other experiments were analyzed using t-test.

3. Results and discussion

3.1 Cytotoxicity induced by surfactants to HaCaT keratinocytes

The cytotoxic effects of the surfactants to HaCaT cells was evaluated as an indicator of their
potential to elicit skin irritation (Choi et al., 2013; Park et al., 2010; Lawrence et al., 1996). Keratinocytes are a biologically relevant target for skin irritants because they are the first living
cells that contact externally applied compounds. Cell viability of keratinocytes decreased in a concentration dependent manner following exposure to the surfactants in the tested concentration range 0.5-1000 mg/L, 24 hours post exposure (Figures 1 and 2). For both surfactants there was a significant effect of concentration, with toxicity increasing, with increasing surfactant concentration (p < 0.001). For surfactant C12-OPK there was a significant reduction in cell viability observed at concentrations >125 mg/L (p < 0.001). At a concentration of 125 mg/L C12-OPK reduced cell viability to 59.3 % and at concentrations > 250 mg/L cell viability was lower than 20 %. In the case of C18-OPK, cell viability significantly decreased to 22.7 % (p < 0.001) at a concentration of 125 mg/L. At concentrations > 250 mg/L viability was < 20%. A significant difference between both surfactants was observed (p < 0.05), with C18-OPK exhibiting greater toxicity than C12-OPK.

In order to assess the potency of the surfactants, median lethal concentrations (LC50; concentration required to kill 50% of cells) for each surfactant were calculated applying a 4 parameter logistic curve. The surfactant with the shortest chain, C12 chain, had an LC50 value of 144 ± 27 mg/L, eliciting a less toxic effect on the HaCaT cell line than the surfactant with the C18 chain, which gave a LC50 value of 85 ± 14 mg/L. SDS is the most widely used anionic surfactant in gels and shampoos (Bondi et al., 2015). We identified that the LC50 value for SDS to keratinocytes was 50.4 mg/L (equivalent to 175 µM), 24 h post exposure (data not shown). The cytotoxicity of the C18-OPK and C12-OPK surfactants to HaCaT cells was compared to the SDS LC50 value calculated in our study, and those identified in the existing literature. LC50 (24 h) values of 47.6 ± 5.6 mg/L (NRU assay) and 31.4 ± 2.9 mg/L (MTT assay) were reported for SDS (Sanchez et al., 2006). These values are lower than the LC50 value we calculated but this is likely to be due to differences in sensitivity in the cytotoxicity assays employed in the different studies. Taken together these data suggest that the surfactants tested in this study are less toxic to this cell type than other commonly available surfactants. The relatively lower toxicity of our surfactants makes them promising on the field of cosmetics and pharmaceutical applications, however a more comprehensive assessment of skin irritation and sensitization potential (according to OECD protocols) is required in future studies as well as assessment of toxicity at other target sites (e.g. liver).

3.2 HC solubility

HC solubility was detected by HPLC analysis of the clear solution obtained after centrifugation and subsequent filtration of a drug suspension. The solubility of the drug in water at 25 °C was found to be 0.295 ± 0.003 mg/mL (0.814 ± 0.009 mM) following Bergstrom et al. (2002).
3.3 Influence of surfactants on HC solubility

Figure 3 and 4 reported the influence of different surfactant concentrations on drug solubility. The increase of C12-OPK and C18-OPK concentrations above the CMC determined an increase of the drug solubility due to the association between the drug and the micelles. In particular, HC solubility increased from $0.814 \pm 0.009$ mM to $2.203 \pm 0.042$ mM and $1.639 \pm 0.097$ mM in the presence of surfactants C12-OPK and C18-OPK at concentrations 10-fold higher than their CMC ($p < 0.01$). As can be seen in Table 1, the molar solubilization capacity ($\chi$) and the molar micelle-water partition coefficients ($K_M$) of C12-OPK were higher than those of C18-OPK. Moreover, $\Delta G^0_S$ was negative for both surfactants, indicating spontaneous solubilization. The lowest value was observed for C12-OPK, confirming that the solubilization process of HC is energetically more favorable in this micellar systems with respect to C18-OPK. This is likely to derive from the improved hydrophilic-lipophilic balance (HLB) of surfactant C12-OPK (8.6) than the HLB of surfactant C18-OPK (6.7) (Malferrari et al., 2015).

3.4 Influence of surfactants on HC permeation

The cumulative amount of HC permeated through porcine skin ($1.77 \text{ cm}^2$) was plotted against time for the different tested samples (Figure 5). All permeability profiles show good linearity from approximately the first 3 hours until 9 hours. Furthermore, among the different tested samples, C12-OPK at a concentration of 10 mM provided the highest flux of HC through the skin. In fact, the level of HC solubility achieved with C12-OPK (10 mM) allowed to maintain the highest driving force across the skin barrier (concentration gradient). As reported in different works (Raghavan et al., 2001; Raghavan et al., 2000), according to Fick’s first law, the flux of a drug ($J$) is proportional to the concentration of the drug in the vehicle. The flux is related to the chemical potential gradient of the drug and any concentration above its solubility limit will increase its chemical potential leading to an increase in the flux across the membrane. Since each selected surfactant concentration provided the solubilization of different amount of drug, the normalization of fluxes to the initial drug concentration $C_0$ (estimation of permeability coefficients) is useful to compare the different samples. Figure 6 reports the $K_P$ (cm/s) for all the tested samples calculated through equation (5). In the presence of 10 mM and 3 mM concentrations of C12-OPK (above CMC), HC permeability coefficients were lower ($p < 0.01$) than those obtained with 0.15 mM concentration (under CMC). The same trend can be observed for C18-OPK: the $K_P$ in the presence of 3 mM concentration (above CMC) was lower ($p < 0.01$) than the $K_P$ obtained with 0.15 mM surfactant concentration (under CMC). $K_P$ reduction at a surfactant concentration higher than CMC can be attributed to surfactant ability to form micelles which do not penetrate the skin on the account of bulkiness.
Moreover, micellar aggregates appeared to be unable to interact with the skin barrier and modify drug diffusivity, unlike single surfactant molecules. In fact, $K_p$ increase at a surfactant concentration lower than CMC is normally attributed to the ability of the surfactant molecules to penetrate the skin and provide environmental changes capable of improving drug diffusivity. Finally, a higher ($p < 0.05$) enhancing effect on drug permeation with C18 derivative can be observed by comparing the $K_p$ obtained in the presence of the two compounds at the same concentration below their respective CMC (0.15 mM). This behaviour can be attributed to the different physical-chemical properties of the two surfactants. In fact, as reported in Malferrari et al. (2015) C12-OPK is more hydrophilic ($\log P = 4.8$) than C18-OPK ($\log P = 7.0$) and consequently less effective in enhancing skin penetration of a poorly soluble and lipophilic drug such as HC (Pandey et al., 2014). At surfactant concentration of 0.15 mM, the ER was 1.62 and 1.3 for C18-OPK and C12-OPK, respectively. The only study reported in literature with experimental conditions similar to those used in our study is the work of Sarheed (Sarheed, 2013). In this study, the author described the use of different enhancers and determined the ER. Among these permeation enhancers, SDS is the only surfactant employed. ER calculated for two different concentrations of SDS (0.25 and 1%) were higher than the ER measured for C12-OPK and C18-OPK. Despite these results, our study demonstrated that C12-OPK and C18-OPK surfactants are less irritant with respect to SDS.

Godwin and colleagues (Godwin et al., 1998) synthesized twelve urea and thiourea compounds and examined them for their transdermal penetration enhancing properties using hairless mouse skin and hydrocortisone as a model drug. They observed an increase of drug permeation in the presence of these enhancers (ER from 1.1 to 6.6). Although these compounds showed promising properties as penetration enhancers, the authors concluded that the investigation of their irritation potential is needed for a future application.

Considering the same concentration above the CMC of C12-OPK and C18-OPK (3 mM), no significant differences between the $K_p$ values of HC and each surfactant can be observed ($p > 0.05$), thus suggesting that no enhancing effect was reached in the presence of micelles for C12-OPK as well as for C18-OPK.

4. Conclusion

The present work synthesised C12-OPK and C18-OPK surfactants from renewable resources, and investigated the influence of their tail length on the extent of HC solubilization and skin permeation. Accordingly to the HLB and logP values of the two surfactants, that are strictly linked to the tail
length of the molecules, the micellar system based on surfactant C12-OPK presented the best solubilization profile of HC, with respect to the system based on C18-OPK. The toxicity of C12-OPK to HaCaT keratinocytes was less than that of C18-OPK, although a more comprehensive assessment of their safety is required prior to their widespread use. At the same time, the higher hydrophilicity of C12-OPK determines a less effectiveness in enhancing skin penetration of the lipophilic drug HC with respect to C18-OPK. In vitro permeation results showed that, in the presence of surfactant concentrations above CMC, HC permeability coefficients were lower than those obtained with surfactant concentration under CMC. Moreover, comparing the enhancement ratio values with data in literature and considering the low toxicity on HaCaT cell line, we can conclude that these surfactants can be promising enhancers for transdermal delivery of HC. On the base of these considerations, the choice of the suitable surfactant and its concentration should be evaluated depending on the formulation purpose. These results also suggest the possible concomitant use of the two surfactants (C12-OPK at concentrations above its CMC and C18-OPK at concentrations below its CMC) for HC solubilization and the transdermal delivery.
References


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**Figure captions**

Figure 1. Cytotoxicity exhibited by C12-OPK surfactant to HaCaT keratinocytes in vitro. HaCaT cells were exposed to complete cell culture medium (control), or surfactants at concentrations ranging from 0.5-1000 mg/L for 24 h and cell viability assessed using the Alamar Blue assay. Data are expressed as % viability (% of control), (means ± SEM, n= 3). Significance indicated by *** = p < 0.001, compared to the control.

Figure 2. Cytotoxicity exhibited by C18-OPK surfactant to HaCaT keratinocytes in vitro. HaCaT cells were exposed to complete cell culture medium (control), or surfactants at concentrations ranging from 0.5-1000 mg/L for 24 h and cell viability assessed using the Alamar Blue assay. Data are expressed as % viability (% of control), (means ± SEM, n= 3). Significance indicated by *** = p < 0.001, compared to the control.

Figure 3. Solubility curve of HC as a function of C12-OPK concentration in water at 25 °C. Data are expressed as means ± SD, n= 3. Significance indicated by ** = p < 0.01, compared to HC solubility.

Figure 4. Solubility curve of HC as a function of C18-OPK concentration in water at 25 °C. Data are expressed as means ± SD, n= 3. Significance indicated by **= p < 0.01 and †= p > 0.05 compared to HC solubility.

Figure 5. Permeation profiles of HC and fluxes (J) in the absence and presence of different concentrations of C12-OPK and C18-OPK through porcine skin. Data are expressed as means ± SD, n= 4. Significance indicated by *** = p < 0.001, **= p < 0.01 and †= p > 0.05 compared to J<sub>HC</sub>.

Figure 6. HC permeability coefficients (K<sub>p</sub>) in the absence and presence of various surfactant concentrations. Data are expressed as means ± SD, n= 4. Significance indicated by **= p < 0.01 and by *= p < 0.05 and †= p > 0.05 compared to K<sub>p</sub> of HC.
Figure 1

Cell viability (% control)

log [C12-OPK] mg/L

\[ y = 10.37 + \frac{82.4}{1 + 10^{(2.147 - x)}} - 3.585 \]
Figure 2

\[ y = 8.178 + \frac{82.762}{1 + 10^{(1.924 - x)}} * -3.708 \]
Figure 3

C12-OPK concentration (mM) vs. HC concentration (mM)

** Significant differences
Figure 5

**J** C12-OPK (10 mM) = $4.80 \pm 0.47 \, \mu g/cm^2 \, h$

† J C12-OPK (3 mM) = $2.24 \pm 0.41 \, \mu g/cm^2 \, h$

** J C12-OPK (0.15 mM) = $2.24 \pm 0.12 \, \mu g/cm^2 \, h$

** J C18-OPK (3 mM) = $2.72 \pm 0.22 \, \mu g/cm^2 \, h$

** J C18-OPK (0.15 mM) = $2.73 \pm 0.31 \, \mu g/cm^2 \, h$

J HC = $1.68 \pm 0.12 \, \mu g/cm^2 \, h$
Figure 6

![Graph showing the effect of surfactant concentration on $K_p$ (10^(-6) cm/s). The x-axis represents surfactant concentration (mM) ranging from -1 to 11, while the y-axis represents $K_p$ ranging from 0 to 3. The graph includes data points for C12-OPK, C18-OPK, and HC, with statistical symbols indicating significant differences.]