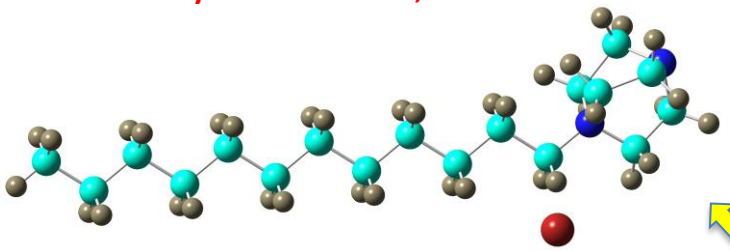
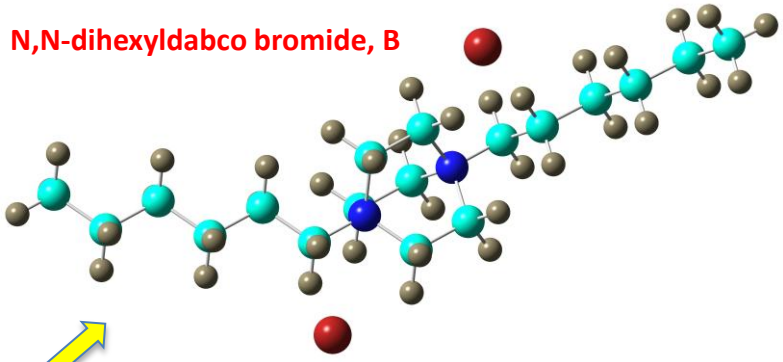
*Graphical Abstract (for review)

N-dodecyldabco bromide, A

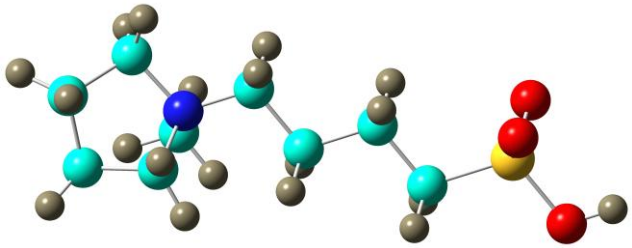


N,N-dihexyldabco bromide, B



IONIC LIQUIDS

(N-methyl-N-(4-butylsulfonic acid) pyrrolidinium hydrogensulfate (E)



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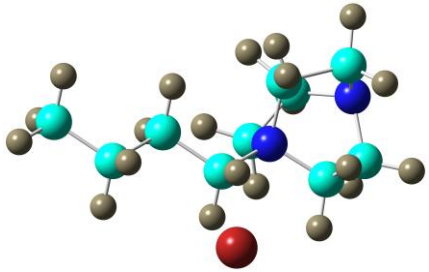
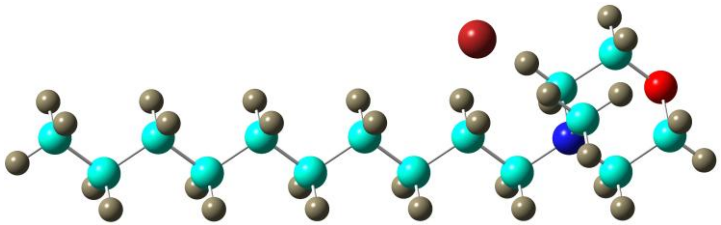
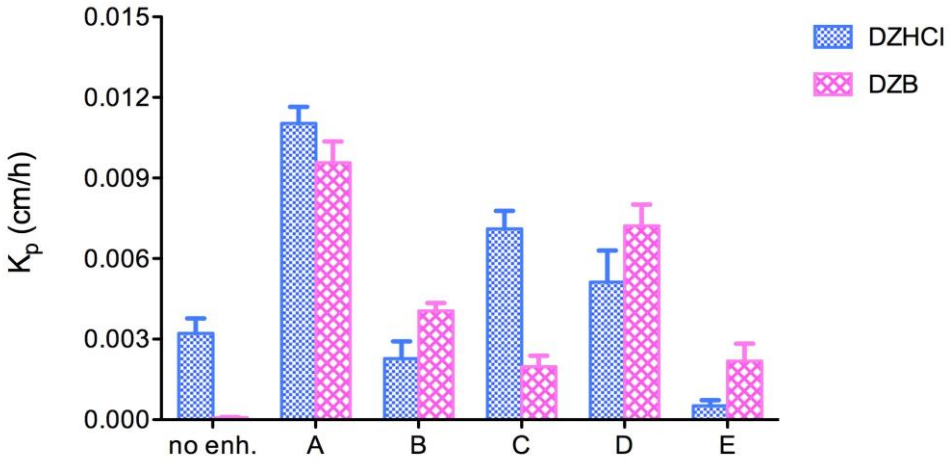


N-methyl-N-decylmorpholinium bromide, D



Effect of different enhancers on the permeability coefficient (Kp) of diltiazem hydrochloride (DZHCl) and of diltiazem free base (DZB)



Ionic liquids as potential enhancers for transdermal drug delivery

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ABSTRACT

The aim of this study was to verify the effect of several cyclic onium based ionic liquids (ILs), including mono- and dicationic derivatives of 1,4-diazabicyclo[2.2.2]octane (DABCO), a dialkyl morpholinium salt and a Brønsted acidic IL, as enhancers of the in vitro transdermal permeation and skin retention of diltiazem through and into hairless rat skin. The drug was used as both the hydrochloride salt (DZHCl) and the free base (DZB) to highlight the relationship between the enhancement effect and the physico-chemical characteristics of the active agent. Permeation tests were carried out using Gummer-type diffusion cells and excised rat skin with a 0.005 M aqueous solution of diltiazem hydrochloride or diltiazem free base with and without the addition of 1% w/w ionic liquids. At the end of the permeation experiments with diltiazem hydrochloride, a suitable extraction procedure allowed for the determination of the drug content retained in the skin. Depending on the ionic liquid structure, a significant enhancement in diltiazem hydrochloride levels in the receiving phase was observed, and the transdermal permeation of the diltiazem free base was markedly increased by treatment with all of the ionic liquids. *N*-dodecyldabco bromide was the best enhancer for both salified and free base drug forms, even though it showed a certain toxicity. On the

other hand, *N*-methyl-*N*-decylmorpholinium bromide showed a good balance between enhancer activity and cytotoxicity.

INTRODUCTION

Transdermal drug delivery systems have been accepted as potential non-invasive routes of drug administration, with the advantages of avoiding first-pass metabolism, sustained therapeutic action and better patient compliance. However, their prevalent use is restricted because of the excellent impervious nature of skin. The outermost layer of the epidermis, the stratum corneum (SC), provides an outstanding barrier to the absorption of chemicals, related to the unique hierarchical structure made up of multiple lipid bilayers and embedded corneocytes. Many approaches have been used to perturb the skin barrier and enhance drug transdermal delivery, including physical disruption (thermal, magnetic, pressure, laser or mechanical modulation, hydration, iontophoresis, phonophoresis, microneedles, skin abrasion and puncture), chemical disruption (permeation or penetration enhancers and prodrug design) and combinations of these methods (Biradar and Sanghavi, 2014; Moghadam et al, 2013). In particular, chemical permeation enhancers act by increasing drug cutaneous permeation through the following: 1) the alteration of SC structure and fluidity; 2) the enhancement of the solubility characteristics of the skin for the drug to be delivered (increase in the partition coefficient of the drug into the skin as well as drug diffusivity in the SC); 3) the creation of disorder among the alkyl chains of SC lipids; and 4) the localized separation of lipid domains to create hydrophilic pores and/or establish a drug reservoir in the SC itself.

Another current trend is the development of structured vehicles acting as carriers through the skin, such as liposomes, niosomes, transfersomes, microemulsions, and solid lipid nanoparticles (Carafa et al, 2009; Bseiso et al, 2015).

In the past decade, to facilitate the passage of molecules through the SC, transdermal permeation enhancers have been extensively studied, and more than 360 chemicals have been demonstrated to enhance skin permeability, including terpenes, sulphoxides, pyrrolidones, fatty acid and alcohol,

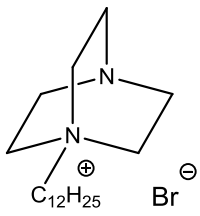
surfactants, urea, etc. (Chen et al, 2014). However, despite these findings, only a few products are currently used in the market because of incompatibilities in the formulation or local irritation. Therefore, the exploration of chemicals to safely improve the skin permeability remains an intensive research area.

Here, we report the use of ionic liquids (ILs) as enhancers of drug delivery into and across the skin. Briefly, ionic liquids are organic salts, generally composed of a large and asymmetric organic cation and an organic or inorganic anion, that are liquid at or near room temperature. They have some peculiar properties, such as a negligible vapour pressure, the ability to dissolve organic, inorganic and polymeric materials and a high thermal stability. Due to these properties, ionic liquids have largely been successfully used as "green" alternatives to volatile organic solvents for a wide range of applications. As designer solvents, they can be synthesized for particular applications or with specific chemical and physical properties by simply changing the anion/cation combination or introducing specific functional groups on the cation or anion.

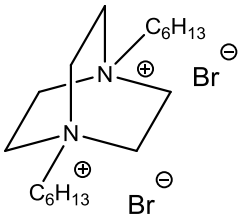
Recently, ionic liquids have gained interest for use in several pharmaceutical applications, although the unknown toxicity profile of many ionic liquids has for a long time hindered their employment in this context, where the tightly regulated active pharmaceutical ingredient manufacturing practices often discourage the use of new solvents, additives and procedures (Smiglak et al, 2014). Ionic liquids have thus been employed for the solubilization of poorly soluble drugs (Jaitely et al, 2008; Mizuuchi et al, 2008; Moniruzzaman et al, 2010a) and to synthesize active pharmaceutical ingredients with modified solubility, increased thermal stability and enhanced efficacy compared to their starting materials (Bica et al, 2010; Hough and Rogers, 2007; Hough et al, 2007). Furthermore, ionic liquids exhibiting antimicrobial activity have been proposed as active pharmaceutical ingredients or formulation preservatives (Pernak et al, 2003). However, because of their physico-chemical and biological properties, they can also be used as additives in formulations for topical drug delivery. Only a few studies have been published regarding the use of ionic liquids in such a specific field, and many of the studies are related to the use of ionic liquids as oil or water

substitutes or, more simply, as additives in microemulsions (Moniruzzaman et al, 2010b). Nonetheless, recently, Zakrewsky et al (2014) have studied the influence of several ionic liquids (including imidazolium, phosphonium, pyrrolidinium and choline based salts, often having long chains on anions) on the transdermal delivery of mannitol and cefadroxil (model drugs), emphasizing some of the beneficial properties, such as enhancer effects, solubilizing abilities, irritating effects and antibacterial activity.

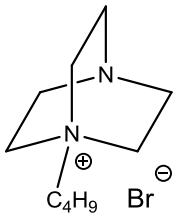
In the present study, we focused our attention on several cyclic onium based ionic liquids, including the mono- and dicationic derivatives of 1,4-diazabicyclo[2.2.2]octane (DABCO), a dialkyl morpholinium salt and a Brønsted acidic ionic liquid. In particular, we have evaluated the influence of the following ionic liquids on drug penetration: *N*-dodecyldabco bromide, A, [C_{12}dabco]Br; *N*,*N*-dihexyldabco bromide, B, [C_6C_6dabco]Br; *N*-butyldabco bromide, C, [C_4dabco]Br; *N*-methyl-*N*-decylmorpholinium bromide, D, [C_1C_{10}mor]Br; (*N*-methyl-*N*-(4-butylsulfonic acid) pyrrolidinium hydrogensulfate, E, [$C_1C_4SO_3$Hpyrr][HSO_4].



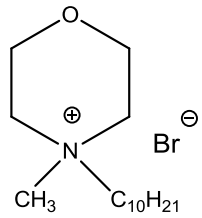
A: [C_{12}dabco]Br



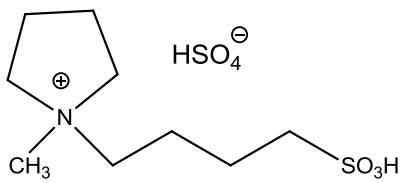
B: [C_6C_6dabco]Br



C: [C_4dabco]Br



D: [$C_{10}C_1$Mor]Br



E: [$C_1C_4SO_3$Hpyrr][HSO_4]

These compounds have been selected based on their surfactant properties derived from the presence of a sufficiently long alkyl chain on the cation, the generally low environmental impact of onium salts and the diffuse use of the DABCO base on pharma applications (Pashirova et al, 2015).

The purpose of this study was, therefore, to verify the effect of these ionic liquids as enhancers of the in vitro transdermal permeation and skin retention of diltiazem, a drug used as a model, through and into hairless rat skin. The drug was used as both the hydrochloride salt and the free base to highlight the relationship between the enhancement effect and the physico-chemical characteristics of the active agent (partition coefficient, water solubility, etc.). The choice of this drug was rational; diltiazem, a calcium channel blocker, is widely used in the management of angina pectoris and hypertension. Because of its short biological half-life (3.5 h), low oral bioavailability (40%), and hepatic metabolism leading to high-frequency drug dosing, the continuous delivery of the drug is required. Therefore, the development of a transdermal drug delivery system containing diltiazem with appropriate enhancers should be of great interest (Limpongsa and Umprayn, 2008).

Materials and Methods

Diltiazem hydrochloride (DZHCl) and sodium dodecyl sulfate were supplied by Sigma-Aldrich, Milan, Italy. Diltiazem free base (DZB) was obtained by treating an aqueous solution of diltiazem hydrochloride with an alkalinized (NaOH) solution, adjusted to a pH 9.5. The precipitate was collected, washed and dried under a vacuum. A white amorphous powder of the diltiazem free base was obtained.

DABCO, *N*-methylmorpholine and *N*-methylpyrrolidine were purchased from Sigma-Aldrich Co. and used without further purification, as well as bromobutane, bromohexane, bromodecane, bromododecane and 1,4-butane sultone. DABCO and morpholinium-based bromides were synthesized by the Menschutkin reaction of 1,4-diazabicyclo[2.2.2]octane with the appropriate alkyl bromide, as previously reported (Chiappe et al, 2009; Pretti et al, 2011). The SO_3H-functional Brønsted acidic IL was prepared by stirring methylpyrrolidine with an equimolar amount of 1,4-butane sultone, under solventless conditions, following a previously reported protocol (Wu et al, 2007).

All other chemicals and solvents were analytical grade.

Cytotoxicity test

A cytotoxicity test was performed on a rabbit corneal epithelial cell line (RCE, European Cell Culture Collection, no. 95081046, ECACC, Salisbury, Great Britain) using a method previously performed by us (Burgalassi et al, 2011). The cell viability test was based on the ready-to-use cell proliferation reagent WST-1.

The results were expressed as the percent optical density of treated vs. untreated control wells. Cell viability was tested after 1 h of exposure at different concentrations (1.0, 0.1, 0.01, 0.001, 0.0001% w/v) of the enhancer under study (Huthala et al, 2003).

***In vitro* permeation studies**

Experiments were carried out on rat skin obtained from 6- to 8-week-old hairless male animals (HsdHanTM/RNU-Foxn1 rnu, Harlan Italy s.r.l., Correzzana, Italy). The study was approved by the Ethical Committee of the University of Pisa, and the protocol was compliant with the European Union Directive 86/609/EEC for the use of experimental animals. The experiments were conducted according to the OECD Test Guideline 428 (in vitro) and the OECD Guidance Document for the Conduct of Skin Absorption Studies (OECD, 2004a; 2004b).

Tests of permeation through excised rat skin were carried out, as previously described, using Gummer-type diffusion cells with an available diffusion area of 1.23 cm^2 and the stratum corneum facing the donor compartment (Monti et al, 2014). The receiving phase (5 ml) was isotonic phosphate buffer saline (PBS, 66.7 mM, pH 7.4) containing 0.003% w/v sodium azide to prevent bacterial growth, maintained at 37°C and stirred at 600 rpm. The skin and receptor phase were left in contact for 30 minutes prior to treatment. Four hundred microliters of a 0.005 M aqueous solution of diltiazem hydrochloride or diltiazem free base containing 1% w/w of the ionic liquids under study was placed on the skin surface. At predetermined time intervals, 5.0 ml samples of the receiving phase were withdrawn for HPLC analysis and replaced with the same volume of fresh fluid. All experiments lasted 24 hours and were repeated four times, and the sink conditions were maintained throughout the entire study. Either diltiazem hydrochloride aqueous solution or diltiazem free base suspension was used as a reference.

Skin distribution studies

At the end of the permeation experiments testing the vehicles containing diltiazem hydrochloride, the skin was removed from the diffusion cells and rinsed with distilled water to eliminate excess vehicle from the skin surface and was gently wiped with cotton wool tampons. The samples were then frozen and sliced horizontally with a cryomicrotome (MEV Cryostat, Slee-Technik GMBH, Mainz, Germany) as previously described (Wagner et al, 2000; Wagner et al, 2002; Monti et al, 2008).

The drug was extracted from the skin slices by treatment with 2.0 ml of 2% sodium dodecyl sulfate for 24 hours. After treatment with methanol (4.0 ml) for 1 hour, the mixture was centrifuged at 4,000 rpm for 15 min. Five hundred microliter aliquots of supernatant were dried *in vacuo* and subsequently dissolved in methanol for HPLC analysis.

The validation of the extraction procedure was performed as previously described in Monti et al 2015. The percentage of recovery was 92.9±2.80 (mean±standard error SE).

HPLC analysis

The concentration of diltiazem in the receiving fluid and in the skin samples was selectively determined by HPLC. The apparatus consisted of a Shimadzu LC-20AD system with an UV SPD-10A detector equipped with an autosampler SIL-10AD VP and a computer integration system. The injection valve was a Rheodyne with a capacity of 20 µl. A Phenomenex Gemini (5 µm; 250x4.6 mm) column was employed. The mobile phase consisted of a mixture of acetonitrile: water (33:67 v/v) containing 20 mM NaH_2PO_4 adjusted to pH 2.5 with H_3PO_4. The detection wavelength was 237 nm, the flux was 1.0 ml/min, and the retention time was 7.0 min.

The amount of diltiazem in the samples was determined by comparison with the appropriate standard curves. In the case of biological materials, the standard curves were obtained by adding

increasing amounts of the drug to a blank biological matrix. The calibration curves were linear in the range of detection, and the assay linearity was good. The limit of detection (LOD) and limit of quantitation (LOQ), calculated on the basis of the response and slope of the regression equation and signal-to-noise ratio, were 0.05 and 0.2 μg/ml, respective, for receiving fluids, and 0.28 and 0.45 μg/ml, respectively, for the biological samples (skin slices).

Data analysis

Linear regression analysis of pseudo steady state diffusion plots allowed calculation of the following parameters: steady-state flux (J), given by Q/At, where Q is the amount of permeant diffusing across area A in time t; lag time (t_L), indicating the time taken by the drug to saturate the membrane and reach the receiving phase, calculated from the X-axis intercept values of the regression lines and the percentage of drug permeated at the end of the experiment ($Q\%_{24h}$).

The apparent diffusion coefficient (D_{app}) through the rat skin was calculated according to the following equation: $D_{app} = h^2/6t_L$, where h represents the thickness of the membrane, and t_L represents the lag time. The thickness of the skin used in this study was approximately 540 μm.

The permeability coefficient K_p was calculated using the following equation: $K_p = J/C_o$, where J is the flux at steady state (μg/cm^2 min), and C_o is the initial drug concentration (μg/cm^3). Drug membrane/vehicle partition coefficient, K_m, was obtained from the following relationship: $K_p = K_m D_{app}/h$. Enhancement factors (EF) were calculated from the Kp ratio of data in the absence and in the presence of an enhancer. Moreover, the extraction procedure allowed the calculation of the drug content (DZ_{skin}, mg/g skin) retained in the skin at the end of the permeation studies.

All data are the average of five determinations ± standard error (SE). Significant differences between permeation parameters were assessed by GraphPad Prism software (GraphPad Software Inc., San Diego, CA). The evaluation included the calculation of the means and standard errors and group comparisons using Student's two-tailed unpaired t-test. Differences were considered statistically significant at $p<0.05$.

Results

In vitro permeation studies

Results of the in vitro permeation experiments related to diltiazem free base and diltiazem hydrochloride, with and without permeation enhancers, are summarized in Table 1, where the relevant permeation parameters (flux, apparent permeability coefficient, lag time, percent drug permeated after 24 h, enhancement factor, D_{app} and K_m) are reported for each vehicle. The marked difference in terms of flux between the salified form of the drug (log $P_{O/W}$ 2.19) (Venkateswara and Satyanandam, 2014) and the free base form (log $P_{O/W}$ 4.197) (Fox, 2014) is immediately evident: the transdermal flux of the diltiazem hydrochloride aqueous solution was approximately 24 times higher than that of the diltiazem free base aqueous suspension (7.3±1.2 vs 0.3±0.01 μg/cm^2·h, respectively). By eliminating the influence of concentration, a change in the permeability coefficient (Kp) of approximately 32 times was found. Kp is composed of factors related to both the drug and the barrier and their interaction. The partition and diffusion properties are affected by both the thermodynamic activity and the vehicle state (solution/suspension). Consequently, even though the drug is more lipophilic as a free base and thus better able to permeate the skin when compared to the salified form, the thermodynamic activity is in favour of the latter. In fact, the resulting J values proportionally increased with an increase in diltiazem activity in the drug solution. The addition of the penetration enhancers under study, ionic liquids that increased the solubility of the diltiazem free base, reduced the effects influenced by the state of the vehicle. The ionic liquids produced differences in the permeation parameters depending on the type of the ionic liquid and the nature of drug used (salt or free base), even though all vehicles exhibited diltiazem permeation through hairless rat skin. In particular, ionic liquids A, C and D resulted in a 3.4-, 2.2- and 1.6-fold enhancement, respectively, in diltiazem hydrochloride levels in the receiving phase with statistically significant differences compared to vehicle without enhancer; B, instead, appeared unable to change diltiazem hydrochloride transdermal flux (5.1±1.4 vs 7.3±1.2 μg/cm^2·h, for B and no enhancer,

respectively), while E seemed to significantly hinder the transfer of the drug through the skin (J= 1.2±0.5 μg/cm^2·h). Moreover, the lag time was significantly reduced by the addition of A and C to the vehicle, decreasing from 6.4 (no enhancer) to 3.7 (A) and 2.2 (C) hours; alternatively, the ionic liquid D did not help the saturation of the skin and produced a high flux value only after a long lag time. Correlated with a high flux value, a high percentage of drug permeated after 24 h was observed in the following order: A (69.7%) > C (47.1%) >D (23.7%). On the basis of all the calculated parameters, A and C appeared to be the best promoters of the cutaneous permeation of diltiazem hydrochloride, as further demonstrated by the Dapp values $13.1 \cdot 10^{-5}$ and $22.3 \cdot 10^{-5}$ cm^2/h found for the A and C vehicles, respectively, taking into account the influence of lag time on the permeation process. Another important parameter is Km, which describes the ability of the drug to escape from the vehicle and move into the outermost layers of the stratum corneum; it is defined as the equilibrium solubility of the drug in the stratum corneum relative to its solubility in the vehicle. In this study, Km values are all above 1, between 1.64 and 5.13, suggesting that the enhancers under study can promote the ripartition in the skin. The sole exception was represented by E (Km = 0.3), which, on the contrary, seemed to promote the affinity of the drug for the vehicle.

Transdermal permeation of diltiazem free base was markedly increased for all ionic liquids under study with EF ranging from 20.4 to 98.7. The most effective are in the order of A (J= 29.7±2.4 μg/cm^2·h), D (J=22.5±2.4 μg/cm^2·h) and B (J=12.6±0.9 μg/cm^2·h). No changes in lag time, and thus in the value of Dapp, were observed. For the diltiazem free base, the enhancers had good solvent properties and, as a result, increased the amount of soluble drug in the vehicle, as demonstrated by Km values in the following order: A>D>B>C>E.

Skin distribution studies

The in vitro penetration data relating to the tested vehicles containing diltiazem hydrochloride are shown in the figures 1a and b as the mg of drug per g of skin retained in each skin layer. The water solution of diltiazem hydrochloride produced a large amount of drug not only in the upper layers but also in the deeper layers of the skin (corresponding to dermis) (Monti et al, 2014), on the order

of approximately 2-3 mg drug/g tissue up to 540 micron. A good balance between hydrophilic (salt form) and lipophilic characteristics was allowed to interact with the components of the tissue by improving the depot effect. By adding ionic liquid A to the drug and water solution, the amount of drug retained in the skin layers was markedly reduced to a value of approximately 0.5 mg drug/g skin after a peak of 2.23 mg drug/g skin at a depth of 15 micrometres (stratum corneum). On the contrary, ionic liquid B produced a depot effect, maintaining concentrations of drug similar to the control in the deeper layers of the skin, after a decrease between 60 and 180 micrometres. In the case of ionic liquids C and D, the amount of drug retained in the skin remained more or less constant throughout the analysed skin depth after the upper layer (beyond 60 micrometres). A lack of activity of ionic liquid E in promoting the retention of diltiazem hydrochloride in the skin at any level is evident.

Cytotoxicity test

Figure 2, based on the toxicity data obtained with the WST-l test, illustrates the effect of the exposure of cells to several concentrations of the different ionic liquids for 1 hour as a percentage of the surviving cells with respect to the controls. The cell viability was above 70% for ionic liquids B, D and E, showing practically no toxicity even at high concentrations, while ionic liquid C showed toxicity (cell viability = 36.4%) only when it reached the highest concentration (1.0% w/v). Only ionic liquid A exhibited a dose dependent response with a sigmoidal curve from which it was possible to calculate an EC_{50} value of 0.006% w/v: the highest tested concentrations (1.0 - 0.1% w/v) produced cell viability values lower than 30%.

Discussion

The present study was designed to demonstrate the activity of several ionic liquids as promoters of drugs skin permeation. Diltiazem was chosen as a model because, on one hand, it can be potentially used for transdermal therapy, and, on the other hand, it is available as both a salt and a base. Therefore, the relationship between the physical and chemical properties of the drug (the ionized and non-ionized forms) and the effect of the proposed ionic liquids as promoters could be verified.

The effects of the different ionic liquids on the apparent permeability coefficient of diltiazem hydrochloride and diltiazem free base are summarized in figure 3.

With the exception of IL E, which is a Brønsted acidic ionic liquid characterized by the presence of a strong acidic group on the cation and an amphoteric anion, all other ionic liquids are neutral (or weakly basic) compounds with a more or less pronounced ability to produce organized systems depending on cation core and alkyl chain length. In particular, ionic liquids A and D, containing a sufficiently long alkyl chain (>C8) on the nitrogen atom of the DABCO-base or morpholinium cation, have surfactant properties, with critical micelle concentrations (CMCs) in water of 14 and 61 mM, respectively (Zhil'tsova et al, 2013; Mirgorodskaya et al, 2014). Analogously, ionic liquid B, with two positive charge centres on the same bicyclic core, can produce multilayer systems (critical aggregation concentration, CAC, 100 mM in water) (XU et al, 2012). On the other hand, ionic liquid C bearing a shorter alkyl chain is unable to produce micelles or multilayers. However, analogous to other ionic liquids, ionic liquid C maintains a degree of structural organization in the presence of relevant amount of water.

The ability of ionic liquids A, B and D to act as enhancers in the case of the diltiazem free base is probably related to their surfactant properties: the hydrophobic core of the self-assembled structures can indeed favour the dissolution of larger amounts of the water-insoluble diltiazem, thus turning these structures into drug delivery vehicles. The enhancer order of A>D>B>C is in agreement with the corresponding CMC values. Conversely, ionic liquid E can affect the water solubility of diltiazem in a completely different way; its acidity can indeed transform the free base into the corresponding water-soluble protonated form. Consequently, the enhancer effect of these ionic liquids can be attributed to an increase in and an optimization of the thermodynamic activity of the drug, particularly when used as a base in the vehicle and in the skin, which is known as the driving force of the passive diffusion process.

However, ionic liquids can also affect transdermal permeation acting on the skin in a more direct way. Cationic surfactants are indeed able to interact with the keratin fibrils of the cornified cells,

favouring cell-lipid matrix disruption (Pandey et al, 2014). Ionic liquids A, D, and B can exert this type of effect. However, the ability of ionic liquid C to increase transdermal permeation suggests that DABCO-based ionic liquids may also operate by other mechanisms independent of their surfactant properties. For example, the positively charged bicyclic core could interact with anionic components of the stratum corneum, thus changing the electric property of this stratum and favouring the transfer of the drug into the skin.

A different role of the promoters, depending on the drug form of a salt or base, has been highlighted by this investigation. When diltiazem hydrochloride was employed, the best promoters were ionic liquids A and C. In particular, ionic liquid A caused a considerable reduction in the lag time and an increase in the drug diffusivity through the skin, ascribable to a decrease of the barrier function of the stratum corneum that is due to lipid-fluidization. This behaviour is shown by an increase in flux, D and Km, consistent with a strong micellization effect. It is noteworthy that in this study, ionic liquid A was used above the CMC (27 mM). On the other hand, ionic liquid C seems to interact with the components of the skin by inserting itself into the lipid bilayer. This observation can be because of the ionic liquid structural features, specifically the short alkyl chains of the cationic moiety and the overall small dimension of the molecule that can determine an increase in drug permeation.

The situation is different in the case of the diltiazem free base, for which A, D and B are the best promoters. In this case, the promoter effect is probably essentially attributable to the increased solubility of the drug in the aqueous vehicle, optimizing the thermodynamic activity. After the addition of the ionic liquid, the aqueous suspension of the diltiazem free base tended towards a solution, although in some case (B and D), the ionic liquid was used below the CMC (31 mM and 22 mM, respectively). The exclusive effect on drug solubility is also demonstrated by the fact that there was no change in the lag time or, consequently, in the diffusion coefficient D.

It is well-known that ionic liquids usually form nanostructures, modelled as a dispersion of spherical aggregates below the CACs or as a system of regularly sized near-spherical charged

micelles at concentrations above their critical aggregation concentration (Wang J and Wang H, 2014). The interaction of these more or less regular aggregates, both with the drug in the vehicle and with biological membranes, is likely based on the enhancement effect. Finally, it has to be noted that although the use of cationic surfactants as transdermal enhancers is generally avoided, DABCO-based ionic liquids, also bearing a long alkyl chain, are generally less toxic than classical surfactants (see below). Usually, the enhancers increase skin permeability by reversibly damaging or altering the physicochemical nature of the stratum corneum. The main problem associated with this activity is the skin irritation arising by the disruption of the organized lipid structures and cell membranes and their components. In this study, the cytotoxicity of ionic liquids was, therefore, evaluated using a rabbit corneal epithelial cell line. The cytotoxicity test clearly demonstrated that ionic liquids B, D and E, were practically non-toxic, while ionic liquid C showed a toxicity only at the highest tested concentration (1.0% w/w). The most toxic salt resulted from ionic liquid A, mostly at the highest concentrations (0.01-0.1-1.0%). In any case, it should be emphasized that the present cytotoxicity test was performed on a particularly sensitive cell line, the ocular line, which represents a significantly worse scenario when compared to structured tissue, leading us to assume that the more toxic ionic liquid in these conditions should also be well tolerated and definitely acceptable for cutaneous use. Further studies will be performed to test the irritation potential on reconstituted skin of new formulations containing the selected ionic liquids.

In conclusion, the experimental approach outlined in this manuscript provides a generalized framework for designing novel ionic liquid-based formulations for transdermal drug delivery. In this regard, we have shown that diltiazem, both as a free base and as the corresponding hydrochloride, can benefit from formulations containing ionic liquids. Ionic liquid A is the best enhancer for both the salified and free base drug forms, even though it shows a certain toxicity; on the other hand, ionic liquid D represents a good balance between enhancer activity and cytotoxicity; therefore, it is considered the best candidate to be used for further studies.

Compared with traditional cationic surfactants (Lane, 2013), ionic liquids offer the important advantage that they can be designed to be less toxic to cells. These findings address a major issue associated with the skin irritation that is typically induced by many skin formulations.

Additionally, the peculiar physicochemical properties of ionic liquids can be exploited to solubilize different classes of drugs and to eventually act as additional penetration enhancers to further increase formulation potency.

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Figure 1- Retention of diltiazem hydrochloride in hairless rat skin after the application of vehicles containing (a) ionic liquids A, B and (b) C, D, E compared to the vehicle without an enhancer (no enhancer) (mean±ES).

Figure 2 – Cell viability after 1 h of RCE exposure to different concentrations of the ionic liquids under study.

Figure 3- Effect of different enhancers on the permeability coefficient (Kp) of diltiazem hydrochloride (DZHCl) and diltiazem free base (DZB). Vertical lines over the bars indicate ±SE.

Table I

Tab. I – Effect of the enhancers on the permeation parameters of diltiazem from liquid vehicles under study (means ± standard error, SE).

ILs	J, μg/cm² · h		$K_p \cdot 10^3$, cm/h		t_L, h		Q%$_{24h}$		EF		D_{app} 10^5, cm²/ h		K_m	
	DZHCl	DZB	DZHCl	DZB	DZHCl	DZB	DZHCl	DZB	DZHCl	DZB	DZHCl	DZB	DZHCl	DZB
No enhancer	7.3 ± 1.2	0.3 ± 0.01	3.2 ± 0.5	0.1 ± 0.01	6.4 ± 0.3	7.8 ± 0.2	17.4 ± 2.8	0.5 ± 0.01	1	1	7.6 ± 0.4	6.2 ± 0.1	2.3 ± 0.4	0.08 ± 0.01
A	24.9 ± 1.4*	29.7 ± 2.4*	11.0 ± 0.7*	9.6 ± 0.8*	3.7 ± 0.2*	6.4 ± 0.7	69.7 ± 4.0*	51.8 ± 6.2*	3.4 ± 0.2	98.7 ± 8.1	13.1 ± 0.7*	7.6 ± 0.8	4.5 ± 0.2	6.8 ± 0.6
B	5.1 ± 1.4	12.6 ± 0.9*	2.3 ± 0.6	4.0 ± 0.3*	6.5 ± 0.1	7.7 ± 0.7	11.9 ±3.4	20.2 ± 0.6*	0.7 ± 0.2	41.8 ± 2.99	7.5 ± 0.2	6.3 ± 0.6	1.6 ± 0.5	3.4 ± 0.2
C	16.0 ± 1.5*	6.1 ± 1.2*	7.1 ± 0.7*	2.0 ± 0.4*	2.2 ± 0.3*	6.9 ± 0.5	47.1 ± 4.1*	10.3 ± 1.8*	2.2 ± 0.2	20.4 ± 4.1	22.3 ± 3.0*	7.1 ± 0.5	1.7 ± 0.2	2.2 ± 1.0
D	11.6 ± 2.6	22.5 ± 2.4*	5.1 ± 1.2	7.2 ± 0.8*	9.1 ± 0.4*	5.1 ± 0.03	23.7 ± 5.9	42.0 ± 4.4*	1.6 ± 0.4	74.5 ± 8.1	5.4 ± 0.3	9.5 ± 0.05	5.1 ± 1.2	4.1 ± 0.4
E	1.2 ± 0.5*	6.8 ± 2.0*	0.5 ± 0.2*	2.2 ± 0.6*	5.0 ± 1.0	6.5 ± 0.3	2.9 ± 1.0*	11.7 ± 3.3*	0.2 ± 0.06	22.6 ± 6.6	8.8 ± 1.0	7.5 ± 0.3	0.3 ± 0.1	1.6 ± 0.5

ILs; ionic liquids; DZHCl= diltiazem hydrochloride; DZB= diltiazem free base; * Significantly different from the vehicle without enhancer, $p < 0.05$.

Figure 1a

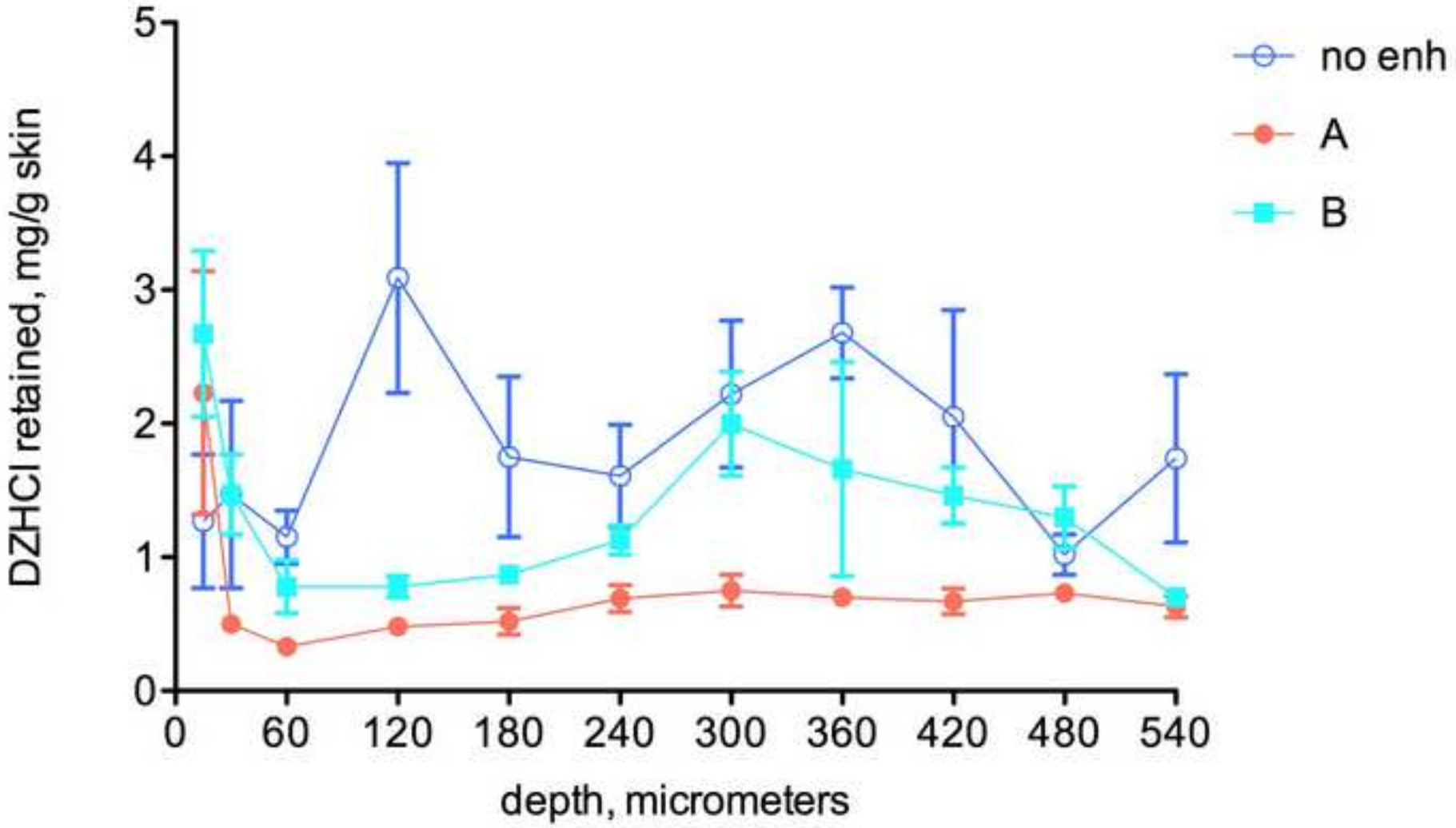
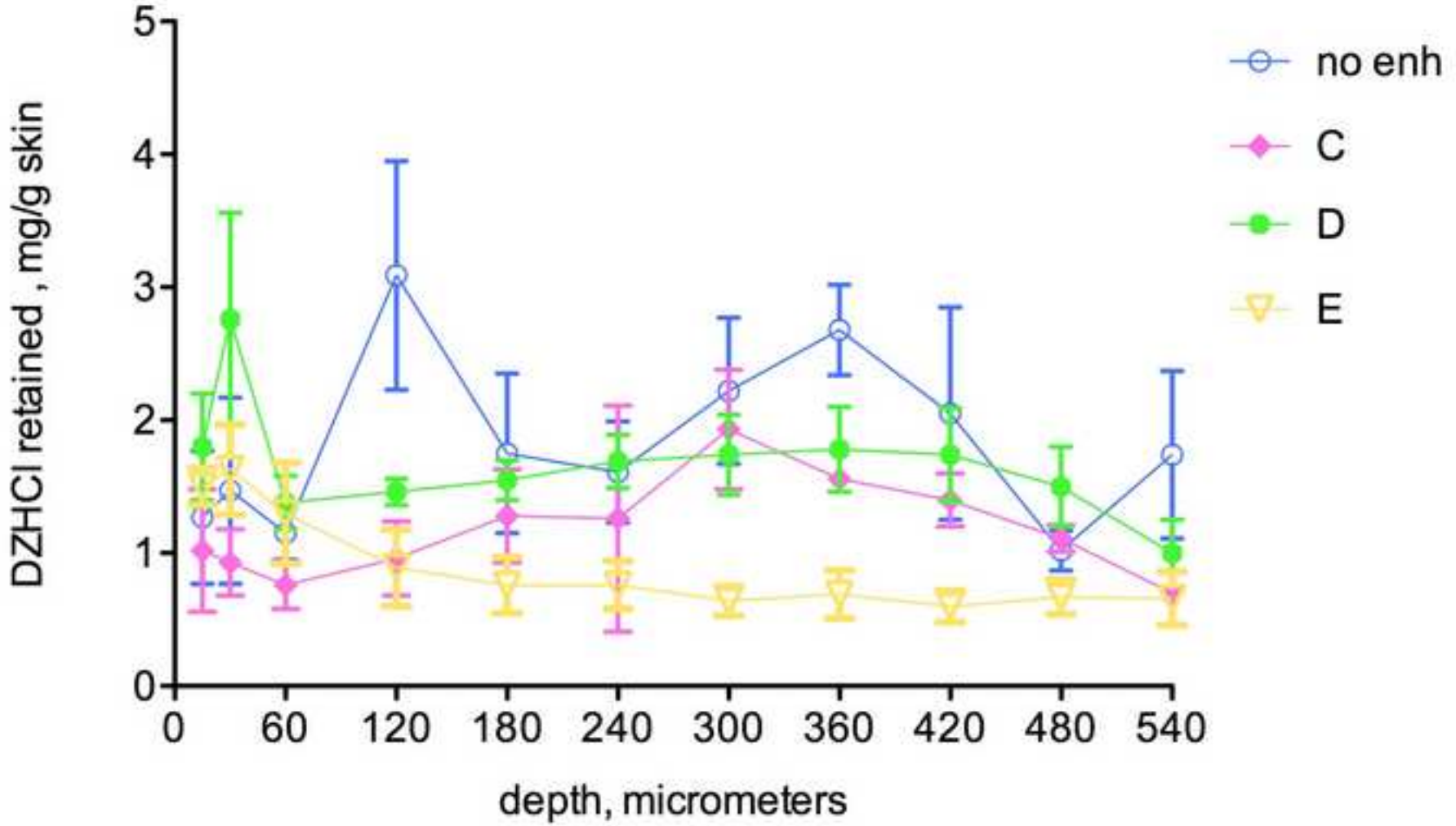

DZHCl retained, mg/g skin
depth, micrometers
no enh
A
B
0
1
2
3
4
5
0
60
120
180
240
300
360
420
480
540

Figure 1b


DZHCl retained , mg/g skin
5
4
3
2
1
0
0
60
120
180
240
300
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420
480
540
depth, micrometers
no enh
C
D
E

Figure 2

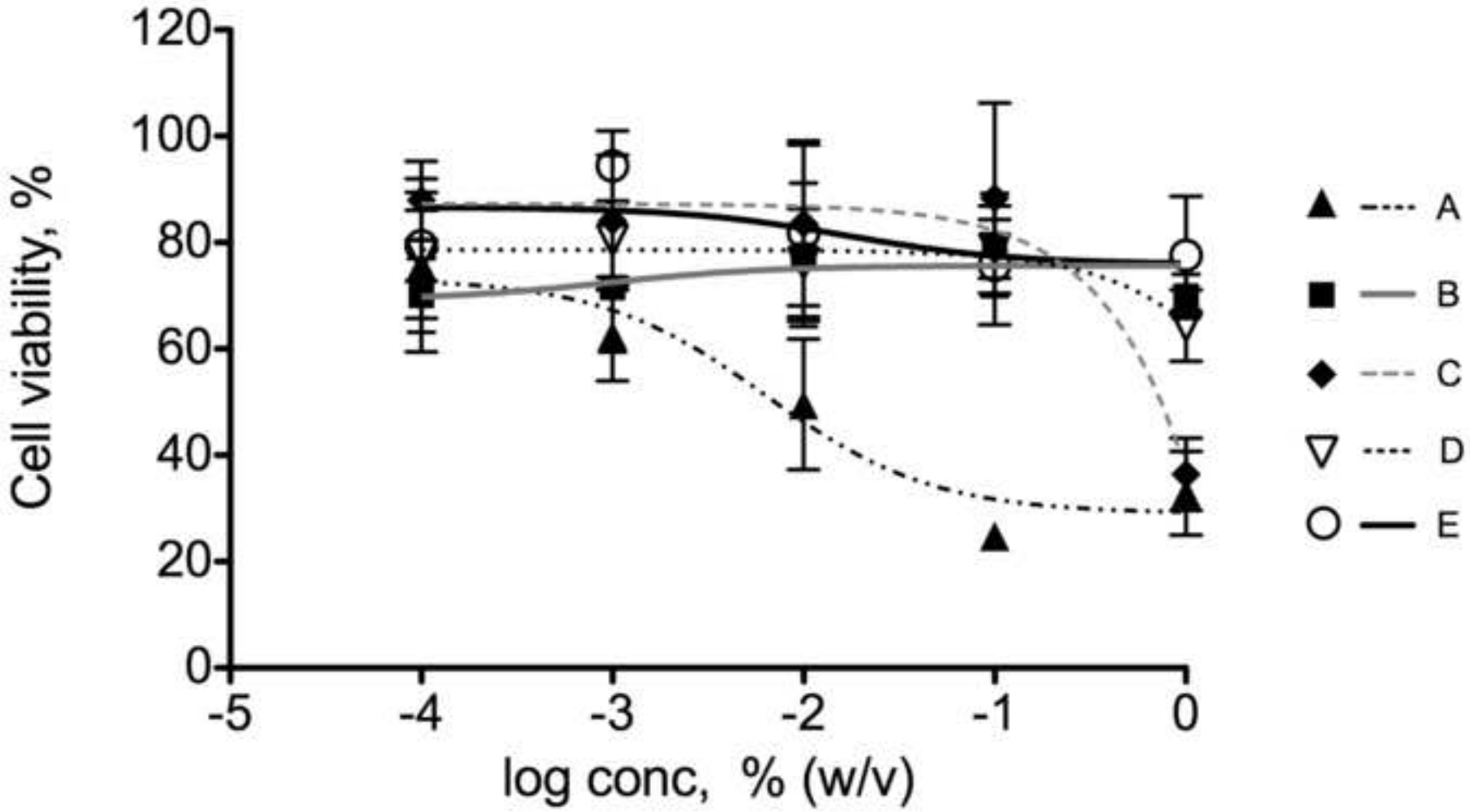

Figure 3

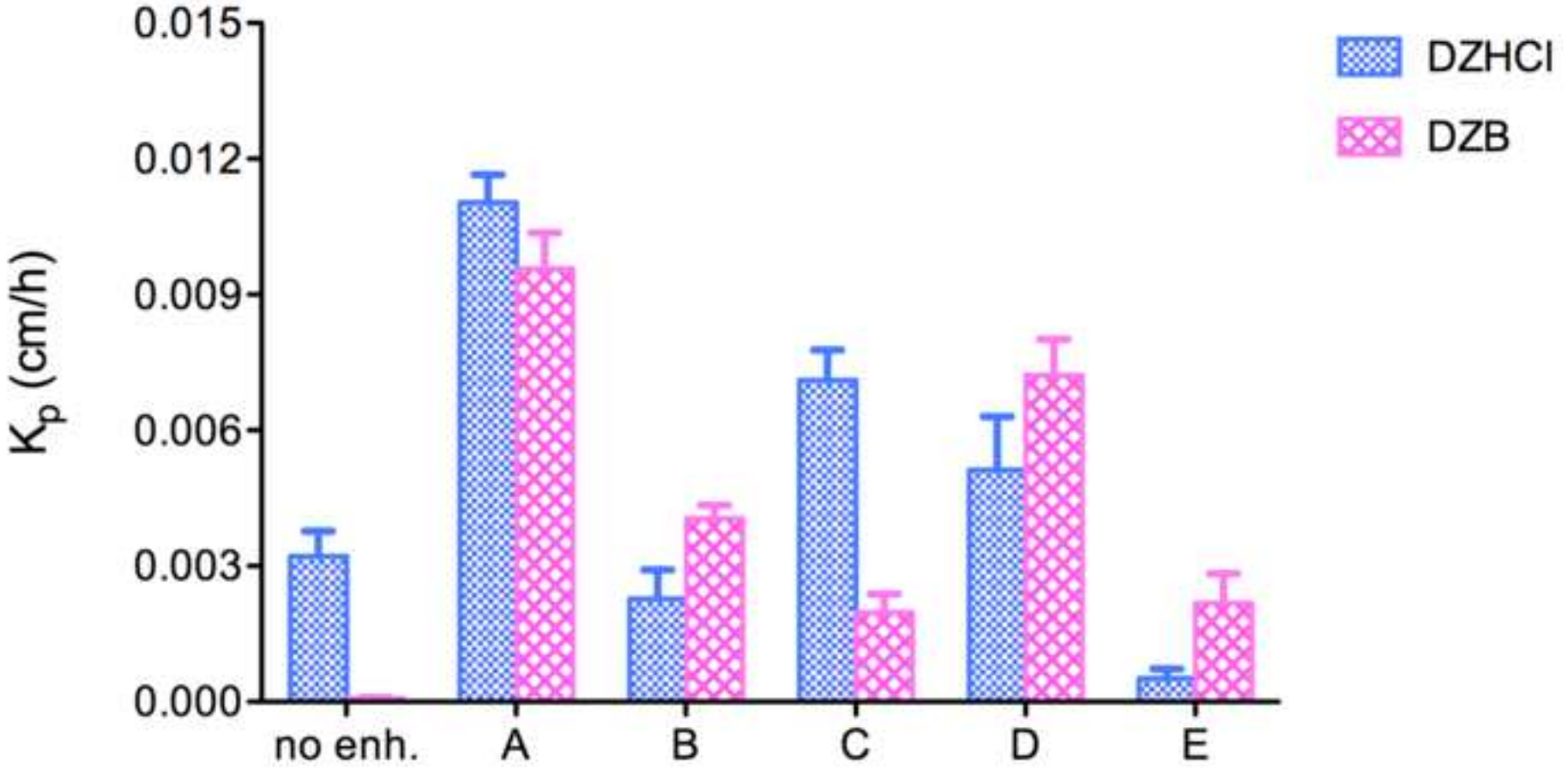

Figure legend

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