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Research Paper

Transdermal Delivery of Ketoprofen: The Influence of Drug–Dioleylphosphatidylcholine Interactions

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Purpose. Considering that most inflammatory diseases occur locally and near the body surface, transdermal delivery of non-steroidal anti-inflammatory drugs (NSAIDs) may be an interesting strategy for delivering these drugs directly to the diseased site. To optimize ketoprofen (KP) transdermal delivery we investigated the influence of dioleylphosphatidylcholine (DOPC) on skin permeation.

Materials and Methods. The formulations studied were: *i*) a physical mixture of KP and DOPC and *ii*) DOPC and KP complex, in a molar ratio of 1:3, obtained by dissolution of the components in chloroform followed by drying under a N_2 atmosphere. Both systems were dispersed in mineral oil and the *in vitro* percutaneous was assayed by absorption using a flow through diffusion cell. Differential Scanning Calorimetry (DSC) and ^{1}H NMR studies were carried out to characterize KP and DOPC interactions. Geometry optimizations using Density Functional Theory and semiempirical methods, as well as a flexible docking procedure were carried out to obtain a binding model for KP with DOPC. KP solubility and partition studies in the formulations, as well as skin irritation and hypersensitivity assays were also carried out.

Results. DSC determinations in the complex showed enthalpy and temperature depressions, indicating KP and DOPC interaction. In addition, dipole–dipole interactions between the KP carboxylic acid and OH groups in phospholipids were shown by ^{1}H NMR studies. Based on the NMR studies, a KP–DOPC binding model is proposed, in which KP is involved by the two long aliphatic chains of the phospholipid. Solubility studies indicated that DOPC improved drug solubility. KP permeation was enhanced by both formulations tested, but the complex also increased its skin uptake. Such behavior could be attributed to the solubilizing, melting and enhancing effects of DOPC. Skin irritation and hypersensitivity were not significantly changed compared to control, suggesting that the formulation may be therapeutically explored for KP transdermal delivery.

KEY WORDS: transdermal delivery; dioleylphosphatidylcholine; drug complex; ketoprofen; skin permeation; skin toxicity.

INTRODUCTION

Drug delivery through the transdermal route is limited by low skin permeability. The stratum corneum, the outermost layer of the skin, acts as a major barrier and is often rate limiting. Considerable research work has been focused on discovering methods to increase stratum corneum permeability. One approach was to employ chemical penetration enhancers, which may increase the permeability of stratum corneum (SC) by increasing drug diffusivity within the membrane and/or by increasing drug partition from the applied formulation into the skin and/or by increasing the effective concentration of drug in the vehicle (1). The effect of these compounds on the drug cutaneous absorption profile has been quantified by permeation parameters, such as drug flux across the skin and skin drug uptake.

Phospholipids have received attention as penetration enhancers (2–9). This class of enhancers has the advantage of containing endogenous components of the human skin, representing 50% of basal layer and 25% of granulosum stratum lipids (10). Phospholipids molecules differ in several features such as polar group contents (choline, serine, glycerol), non-polar chain lengths, characteristic double bonds (in position, number and/or configuration) (11), and these structural variations can influence their effects as skin enhancers (4–9). For example, some phospholipids are capable of insertion among the hydrophobic tails of SC lipids bilayer, decreasing its diffusion resistance to permeants by disturbing packing and increasing fluidity. In addition, some phospholipids are capable to form complexes with drugs, enhancing their thermodynamic activity in the formulation and increasing skin permeation (2,3,12).

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Ketoprofen (KP), a potent non-steroidal anti-inflammatory drug (NSAID) inhibits arachidonic acid metabolism by cyclo-oxigenase and lipoxygenase. Also, it appears to stabilize lysosomal membranes and antagonize the action of bradykinin. The potent inhibitory action on human lipoxigenase distinguishes KP from most other NSAIDs. The compound has been widely used in the treatment of rheumatoid arthritis, osteoarthritis, as well as a mild to moderate painkiller. However, its use by oral administration has been associated with a number of gastrointestinal disorders (13).

Considering that most inflammatory diseases occur locally and near the body surface, transdermal delivery of NSAID may turn out to be an interesting strategy to deliver these drugs directly to the diseased site and to increase local concentration (14,15). However, poor skin penetration limits the efficacy of topically applied KP. Methods trying to improve its cutaneous delivery rely either on chemical penetration enhancers (16,17), novel vehicle systems (18,19), or more complex physical enhancement strategies (20).

The purpose of this study was to investigate the effect of phospholipids, used as penetration enhancers or components of lipid/drug complexes, on *in vitro* percutaneous absorption of KP. The potential of KP transdermal delivery was also assessed by toxicity studies.

MATERIALS AND METHODS

Materials

Dioleylphosphatidylcholine (DOPC) and ketoprofen (KP) were from Sigma (St. Louis, USA). All other chemicals were of analytical or HPLC reagent grade and purchased from Merck (Darmstadt, Germany).

Methods

HPLC Assay for KP

Sample analysis was performed by high pressure liquid chromatography (HPLC) as previously described (21,22). The HPLC system consisted of a model SPD 10A VP Shimadzu Liquid Chromatograph (Kyoto, Japan), fitted with a variable wavelength UV detector operating at 251 nm, a LC-10 ADVP pump, a Rheodyne injector and a Model CR6-A integrator. Separations were performed in a C18 reverse-phase column LichroCART® (Merck, Darmstadt, Germany) (125 × 4 mm i.d.) (5 μm) and a C18 pre-column (4 × 4 mm i.d.) (5 μm) kept at room temperature. Acetonitrile: 0.02 M phosphate buffer (pH 3.0) (45:55) mixture was used as the mobile phase, at a flow rate of 0.8 ml/min. The injected volume was 20 μl and KP retention time 6.103 min. The assay was linear over the range of 0.0125–200.0 μg/ml ($r = 0.999$) with an injection variability of <4.2% for inter-days variation, and <3.2% for intra-day variation.

Formulations

The DOPC and KP complex was obtained by adding the components (molar ratio, 1:3) to chloroform followed by drying under N_2 atmosphere. The dried residue was dispersed in mineral oil. The physical mixture of DOPC and KP (molar ratio, 1:3) was also dispersed in mineral oil. Both dispersion contained KP at 2% (w/w). A formulation containing 2% w/w KP in mineral oil was used as a control.

Solubility Studies

KP solubility, both in the physical mixture and in the complex, was investigated by adding an excess of each to mineral oil and submitting the suspension to constant stirring (300 rpm) at room temperature for 24 h. The dispersion obtained was then centrifuged at 450 × *g* for 10 min. A 1 ml aliquot of the supernatant and 3 ml of methanol were mixed in a vortex for 6 min and centrifuged at 450 *g* for 10 min. KP present in the methanol phase was assayed by HPLC.

Stratum Corneum/Formulation Partition Coefficients ($K_{SC/formulation}$)

Porcine skins were floated (dermal side facing solution) for 12 h in a 0.1% trypsin solution in phosphate buffer (pH 7.2) at 37°C. The transparent SC sheets obtained were briefly washed in water and dried in desiccators containing silica gel for use in partition studies.

The KP partition coefficient from vehicle into powdered SC was determined following the method of Zhao and Sing (22). Briefly, 10 mg of dried SC pulverized in a mortar were mixed with 1 ml of formulation and vortexed for 6 min, ensuring the partition equilibrium. After centrifugation for 10 min at 450 × *g*, 0.5 ml of the supernatant was added to 1 ml of methanol, the mixture vortexed for 6 min and centrifuged at 450 *g* for 10 min. KP present in the methanol phase was determined by HPLC. $K_{SC/formulation}$ was calculated as (Co − C)/C, where Co and C are KP concentrations prior and after partition, respectively.

Characterization of the Complex

DOPC:KP (1:3 molar ratio) complexes were analyzed by Differential Scanning Calorimetry (DSC-200, NETZSCH Gerätebau GmbH, Selb, Germany) and by Nuclear Magnetic Resonance (NMR) Spectroscopy (BRUKER DPX, Billerica, MA, USA). For DSC analysis, the samples (KP, DOPC and DOPC:KP complex) were sealed in an aluminum pan and scanned between −50 and 260°C at a heating rate of 5°C/min under N_2 atmosphere. Temperature calibration was made using indium as standard. For NMR analysis, samples (KP, DOPC, DOPC:KP complex and physical mixture) were dissolved in deuterated chloroform ($CDCl_3$) containing a small amount of tetramethysilane (TMS) as internal standard, and the spectra obtained at 400 MHz. The conditions for Fourier Transform measurements were: acquisition time, 3 s; pulse angle, 30°; delay time, 2 s, number of spectra, 32. In addition, Nuclear Overhouse Enhancement (NOE) experiments were carried out on the complex to get spatial information on KP and DOPC groups and to propose a corresponding molecular model. Thus, some hydrogens of DOPC were selected and irradiated.

Molecular Modeling Studies

We have used GOLD 3.0 (23), Insight II (24) and SPARTAN 4.0.3 (25) softwares to calculate structures and to suggest a binding model for the KP–DOPC complex. GOLD uses genetic algorithm to perform flexible docking in ligands, and each docking result is slightly different from the other. Flexible docking was parameterized for zero ligand bumps, a population size of 100, five islands, 100,000 operations, 95 mutations, and 95 crossovers, adjusted for ten dockings. When the superpositions of the top three solutions (ligand orientations) are within 1.5 Å mean square root deviation (RMSD), the calculation is finished. The fitness function (GoldScore) is evaluated in six stages: (a) a conformation of the ligand inside the receptor binding site is generated; (b) the ligand is placed within the binding site using a least square fitting procedure; (c) a hydrogen bonding energy is obtained for the complex; (d) as well as a steric energy of interaction between the receptor and the ligand; (e) molecular mechanics expressions are used to generate a measure of the ligand internal energy; and (f) the energy terms are summed to give a final fitness.

The docking parameters used have been optimized for single docking calculations, and the program fully validated against 221 diverse and extensively checked protein–ligand complexes with high resolution registered in the PDB (23). In order to consider the DOPC large flexibility, this molecule was used as the ligand in the docking procedure. KP, the receptor had a 20 Å radius sphere centered in the middle carbonyl of the molecule. Docking calculations were performed inside the sphere. Natural Bond Order (NBO) partial atomic charges were obtained for KP from the density functional geometry optimization (at B3LYP/6–31G* level of calculation) and used in the docking procedure. Atomic charges for DOPC were obtained using the AM1 Hamiltonian. Previous to this last calculation, a conformational search on DOPC was performed using the MONTE CARLO method with the Merck Pharmaceuticals force field (25). NMR experiments with the DOPC–KP complex suggested the hydrogen bonding and distance restraints employed in the docking procedure. The hydrogen bond between the phosphate group hydroxyl in choline and the KP carboxyl group as well as the distance between the following atom pairs of KP and DOPC, i.e. C1 and N3, C2 and N3, C4 and N3 have been considered. After docking calculations, the top-ranked GOLD solution for the complex was optimized for implicit solvation conditions, using chloroform as solvent (dielectric constant of 4.8). Initial restrained optimizations were performed for the complex using 1,000 steps of Steepest Descent followed by 5,000 steps of Conjugate Gradient as minimization algorithms. Restraints were released step by step until the convergence of the calculation. An interval of 3–6 Å has been used for distance restraints, in order to satisfy the NOEs observed.

In Vitro Permeation and Retention Studies

Porcine ears obtained from a local slaughterhouse had the dorsal skin removed from the underlying cartilage with a scalpel. Skins were dermatomized to a thickness of approximately 500 μm and stored wrapped in aluminum foil at −20°C for a maximum of 4 weeks before use, as already described (26).

Flow-through diffusion cells with a diffusion area of 1 cm^2 were used in the *in vitro* percutaneous absorption studies. The skin was mounted between the donor and receptor compartments the SC facing the donor compartment and dermis the receptor compartment. The receiving solution (phosphate buffer, pH 7.2) was pumped through the diffusion cell at a flow rate of 3.0 ml/h; a magnetic bar at 300 rpm stirred the contents of the receiver compartment. The whole cell system was maintained at 37°C with a circulating water bath. Samples were collected with a fraction collector at 1 h intervals and the amount of KP permeated was assayed by HPLC. At the end of permeation studies, the skin was removed from the diffusion cell and the surface gently cleaned to eliminate formulation residue. The imprints of the diffusion cell flanges clearly marked the exposed diffusion area. The skin was dabbed dry with cotton, and submitted to the tape stripping method in order to remove SC from the remaining skin, i.e. epidermis (E) without SC + dermis (D). The skins were fixed on a flat surface and the diffusion area of SC was removed by ten pieces of adhesive tape (Scotch Book Tape, n. 845, 3M, St. Paul, MN, USA). KP contained in the strips was extracted with 3 ml of methanol, left overnight and shaken for 1 min before filtering. After the tape stripping procedure, the remaining tissue (E without SC + D) was cut with scissors and placed in a tube. The KP was, then, extracted with 5 ml of methanol under agitation for 6 min in a vortex, sonicated in an ultra-sound bath (40 KHz, continuous mode) for 20 min at 25°C and filtered through a 0.45 μm polytetrafluoroethylene (PTFE) membrane (Corning Incorporated, Corning, NY). The filtrates were then analyzed by HPLC.

The cumulative amount of KP permeated through the skin was plotted as a function of time. The flux was calculated as the slope of the linear portion of the plot (μg/cm^2/h). The KP skin retention (μg/cm^2) was plotted as a function of formulation.

Animals

The skin toxicity studies were carried out in female hairless mice, 5–6 weeks old (strain HRS/J Jackson Laboratories, Bar Harbor, ME) and female Balb/c mice (5–6 weeks old). The animals were housed at 24–26°C, exposed to a daily 12:12 h light: dark cycle (lights on at 6 A.M.), and had free access to standard mice chow and tap water. To reduce the stress associated with the experimental procedure, mice were handled daily for 1 week before experimentation. The protocols were in accordance with guidelines of the University of Sao Paulo Animal Care and Use Committee.

Skin Irritation Test

The skin irritation test was based on a methodology described by Sintov *et al*. (27). The animals were divided into four groups containing three animals each. About 100 μl of each formulation (saline, KP and DOPC:KP complex in mineral oil) were applied non-occlusively over the dorsum of each animal for two consecutive days. On the third day, the animals were euthanized by carbon dioxide vapor and

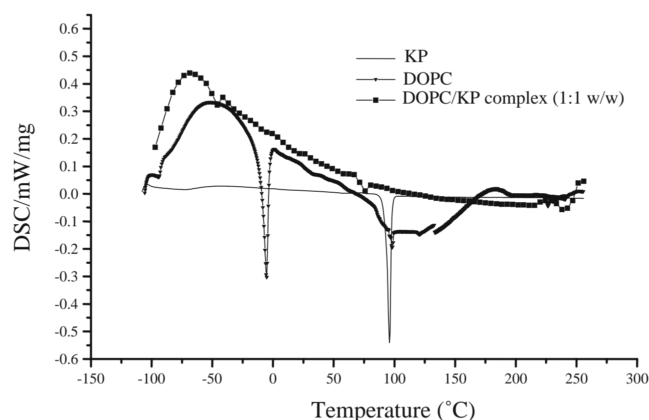

Fig. 1. DSC thermal curves for KP, DOCP and DOPC/KP complex. *KP* = ketoprofen; *DOPC* = dioleylphosphatidylcholine.

samples of the applied skin area were excised and fixed for histological studies by immersion in Bouin solution for 24 h.

After removal of picric acid with alcohol 70°GL, the samples were processed according to the histological technique of inclusion in paraffin and observed by light microscopy. Each tissue sample was cut in 6 μm sections in a microtome. Sections were stained with Masson tricromic and hematoxylin and eosin for histological examination using a 20 fold augmentation objective.

Hypersensitivity Response Test

KP hypersensitivity responses were determined in Balb/c mice using the ear swelling test (MEST) (28), with slight modifications. Mice were divided into three groups containing six animals each. A selected area on the back of the ear was treated with 100 μl of test formulation for three consecutive days. 2,4-dinitrochlorobenzene (DNCB) was used as positive control and saline as negative control. On the eighth day, the ear thicknesses were measured using an engineering micrometer. The animals were then challenged on both sides of each ear with 50 μl of the respective formulation or control substance. The post challenge ear thicknesses were measured 24 h after treatment to determine the percent swelling at 24 h. The percentage of ear swelling was calculated as the ([mean 24 h post treatment ear thickness/mean pretreatment ear thickness] × 100)−100.

Statistical Analysis

The results are reported as means ± S.E.M. Data were subjected to one-way analysis of variance (ANOVA) followed by the Kruskal-Wallis test or to *t*-test analysis followed by the Mann Whitney test. The level of significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

DSC studies were carried out to verify the effect of DOPC on the thermal profile of KP. Figure 1 illustrates thermograms of KP, DOPC and the complex of both (molar ratio, 1:3). The KP thermal curve shows a sharp endothermic peak at 96.1°C and an enthalpy of −24.38 J/g, representing its melting point. A broad endothermic peak at −5.3°C and an enthalpy of −37.62 J/g is shown in the thermal curve of DOPC and a transition temperature at 76.5°C and an enthalpy of −2.932 J/g in the complex (KP–DOPC) thermal curve. However, the thermogram of the physical mixture of KP and DOPC did not show shapes similar to those of drug and phospholipid separately (data not shown). According to the literature, appearance, shift or disappearance of endothermic or exothermic peaks and/or variations in the correspondent enthalpy is indicative of possible interaction between substances (3). Thus, it is possible to infer that there are interactions between KP and DOPC, which were characterized by ^{1}H NMR studies.

Tables I and II show shifts of some signals in DOPC–KP complexes in different proportions (molar ratios, 1:1 and 1:3) compared to the pure samples, (KP or DOPC). Analyzing the 1:3 molar ratio complex, it is seen that the multiplet (H-G3) showed a deshielding (+0.03), while the multiplet (H-Ch2) and the singlet (H-Ch3) showed shielding (−0.18 and −0.22, respectively). On the hand, the 1:1 molar ratio complex showed a different shielding and deshielding profile, resulting from intermolecular interactions dependent on the system concentration and the lipid spatial structure. Although different in value the 1:1 molar ratio results also indicate that the multiplet (H-G3) showed deshielding (+0.06), while the multiplet (H-Ch2) and the singlet (H-Ch3) showed shielding (−0.10 and −0.11, respectively), such as observed in the 1:3 molar ratio complex studies. These results suggest electronic interaction (dipole–dipole) between

Table I. Chemical Shift (δ) Assignments in ^{1}H NMR Spectra in $CDCl_3$/TMS for KP and KP–DOPC Complexes in Different Molar Ratios and the Physical Mixture

Groups	Signal	δ_{KP}	$\delta'_{Complex\ 1:3\ molar\ ratio}$	$\delta'_{Complex\ 1:1\ molar\ ratio}$	$\Delta(\delta_K-\delta'_{Complex\ 1:3\ molar\ ratio})$*	$\Delta(\delta_{KP}-\delta'_{Complex\ 1:1\ molar\ ratio})$*	$\delta''_{Physical\ mixture\ (PM)—1:3\ molar\ ratio}$	$\Delta(\delta_{KP}-\delta''_{PM—1:3\ molar\ ratio})$**
H-7,3′,7′	m	7.80	7.78	7.79	−0.02	−0.01	7.72	−0.08
H-5	m	7.69	7.63	7.66	−0.06	−0.03	7.59	−0.10
H-5′, 9	m	7.58	7.57	7.58	−0.01	–	7.51	−0.07
H-4′, 6′, 8	m	7.46	7.45	7.46	−0.01	–	7.39	−0.07
H-C2	q	3.83	3.78	3.81	−0.05	−0.02	3.74	−0.09
H-C3	d	1.56	1.52	1.54	−0.04	−0.02	1.47	−0.09

δ chemical shift, *m* multiplet, *q* quartet, *d* doublet, *$CDCl_3$* deutered chloroform, *TMS* tetramethysilane, *KP* ketoprofen, *DOPC* dioleylphosphatidylcholine.
*Chemical shifts difference between DOPC/KP complex and KP.
**Chemical shifts difference between KP and physical mixture.

Table II. Chemical Shift (δ) Assignments in ^{1}H NMR Spectra in $CDCl_3$/TMS for DOPC and KP–DOPC Complexes in Different Molar Ratios and the Physical Mixture

Groups	Signal	δ_{DOPC}	$\delta'_{Complex\ 1:3\ molar\ ratio}$	$\delta'_{Complex\ 1:1\ molar\ ratio}$	$\Delta(\delta_{DOPC}-\delta'_{Complex\ 1:3\ molar\ ratio})$*	$\Delta(\delta_{DOPC}-\delta'_{Complex\ 1:1\ molar\ ratio})$*	$\delta''_{Physical\ mixture\ (PM)—1:3\ molar\ ratio}$	$\Delta(\delta_{DOPC}-\delta''_{PM—1:3\ molar\ ratio})$**
H-AG 9 e 10	m	5.33	5.34	5.33	+0.01	–	5.26	−0.07
H-G2	m	5.19	5.18	5.20	−0.01	+0.01	5.12	−0.07
H-G1a	dd	4.38	4.33	4.36	−0.05	−0.02	4.28	−0.10
H-Ch1	m	4.29	4.24	4.30	−0.05	+0.01	4.22	−0.07
H-G1b	dd	4.11	4.08	4.12	−0.04	+0.01	4.03	−0.08
H-G3	m	3.92	3.95	3.98	+0.03	+0.06	3.90	−0.02
H-Ch2	m	3.77	3.59	3.67	−0.18	−0.10	3.57	−0.20
H-Ch3	s	3.33	3.12	3.22	−0.22	−0.11	3.11	−0.22
H-AG2	m	2.27	2.26	2.28	−0.10	+0.01	2.20	−0.07
H-AG8	m	2.00	2.00	2.00	–	–	1.93	−0.08
H-AG3	m	1.57	1.56	1.57	−0.01	–	1.49	−0.08
H-AG (CH2)	wl	1.29	1.27	1.27	−0.02	−0.02	1.19	−0.07
	wl	1.26	1.26	1.26	–	–	1.20	−0.09
H-AG18	t	0.87	0.87	0.87	–	–	0.80	−0.07

δ chemical shift, *m* multiplet, *dd* double doublet, *s* singlet, *wl* width singlet, *t* triplet, *$CDCl_3$* deutered chloroform, *TMS* tetramethysilane, *KP* ketoprofen, *DOPC* dioleylphosphatidylcholine.
*Chemical shifts difference between DOPC/KP complex and DOPC.
**Chemical shifts difference between DOPC and physical mixture

DOPC and KP in both systems (1:1 and 1:3, molar ratio), where choline group hydrogens (H-Ch2 e H-Ch3) are shielding because the group is located near the center of the aromatic ring, and the glycerol group hydrogens (H-G3) are deshielding due to hydrogen bonding between the hydroxyl and carboxylic groups of DOPC and KP, respectively. In addition, the spectra of both complex and physical mixture (1:3 molar ratio of DOPC to KP) are different

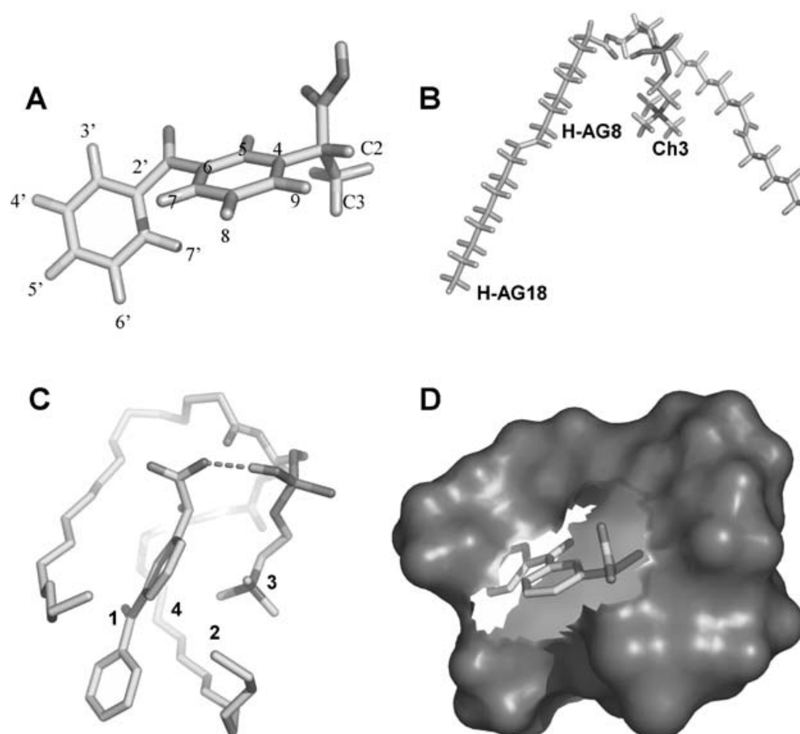

Fig. 2. (A) KP structure fully optimized at B3LYP/6–31G* level of calculation. (B) DOPC structure optimized at AM1 level of calculation. No restraints were applied. Labeled atoms are the hydrogens investigated by NOE. (C) The DOPC and KP complex structure, after docking procedure and subsequent optimization. Hydrogen bond restraint was applied between the hydroxyl of the choline phosphate group and the carboxyl group of KP, whereas distance restraints were applied to the following atom pairs: C1 and N3, C2 and N3, C4 and N3. Hydrogens were omitted for clarity. (D) The complex structure with a density surface built for DOPC and showing the KP short area accessible to the solvent. Hydrogens were omitted for clarity. *KP* = ketoprofen; *DOPC* = dioleylphosphatidylcholine.

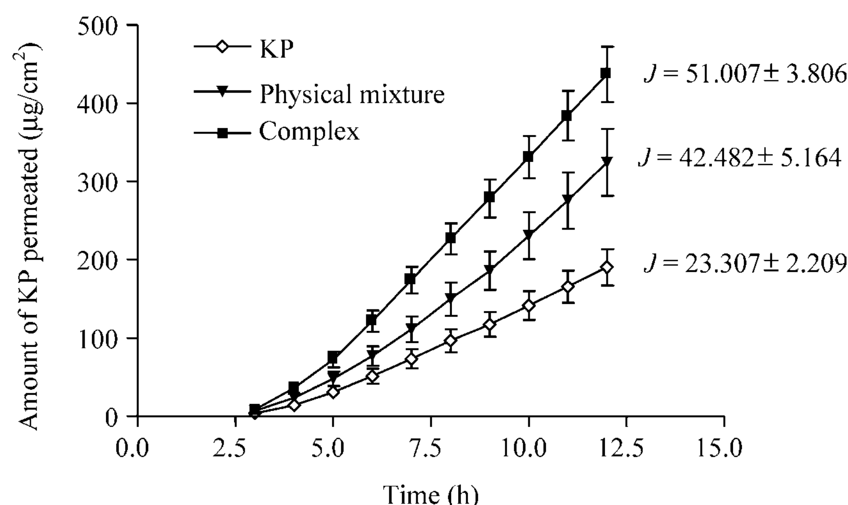

Fig. 3. Cumulative amount of KP permeated *in vitro* through excised porcine skin from different formulations in mineral oil. Each value is the mean of five different experiments ±SE. *KP* = ketoprofen; *DOPC* = dioleylphosphatidylcholine; *J* = drug flux through excised porcine skin, obtained from the slope of the linear portion of the curve.

(Tables I and II). By comparing the spectra of the physical mixture and of its components separately it is shown that the multiplets (H-G3 and H-Ch2) and the singlet (H-Ch3) are shielding (−0.02, −0.20 and −0.22, respectively), suggesting that there is no hydrogen bonding between the DOPC–OH group and the carboxylic acid of KP in this system.

The nature of KP and DOPC interactions was elucidated by molecular modeling studies. ^{1}H NMR and NOE studies of the isolated components and the complex were used for this approach. Due to the large flexibility of DOPC and the number of possibilities for the complex model, these experimental biases were important to propose the more reliable model shown in Fig. 2. It suggests a lipophilic envelope partially shielding KP from the apolar solvent, as observed by other authors (29) for phospholipid–polyphenol complexes in low polarity solvents.

In vitro permeation studies carried out in flow-through diffusion cells using porcine skin as a model evaluated formulation KP delivery. The results are shown in Fig. 3. The permeation profiles (flux values) of complexed or physically mixed KP with DOPC were higher than the control formulation (KP in mineral oil). Fluxes for complexed KP and control formulation were 51.007 and 23.307 μg/cm^2/h, respectively, representing an increase of 2.19 fold. The physical mixture showed an intermediate flux value (42.482 μg/cm^2/h) and an increase of 1.82 fold compared to control. Both results indicate that the phospholipid (DOPC) improves KP permeation. Previous reports by Paolino *et al.* (19) indicated that soybean lecithin microemulsion significantly increased KP permeation compared to a conventional formulation. They attributed this result to the mean size of the internal phase microemulsion droplets, to the drug solubilizing effect of the microemulsified lecithin matrix and to the penetration enhancer effect mediated by the lecithin component.

In vitro KP skin uptake (Fig. 4) was also facilitated by the complex in comparison to the control formulation. In either SC or E (without SC + D) the retention was significantly increased ($p < 0.05$ and $p < 0.01$, respectively) while the physical mixture only produced a significant higher retention ($p < 0.05$) in the SC.

Furthermore, the accumulation of drug in the skin was related to the flux values since the amounts of KP present in the tissue increased as KP percutaneous absorption increased. Our findings are in good agreement with those previously reported (30,31), in which the skin concentration of several NSAIDs was correlated to their flux.

According to the literature, the phospholipid penetration enhancer effect may be mediated by its high affinity to

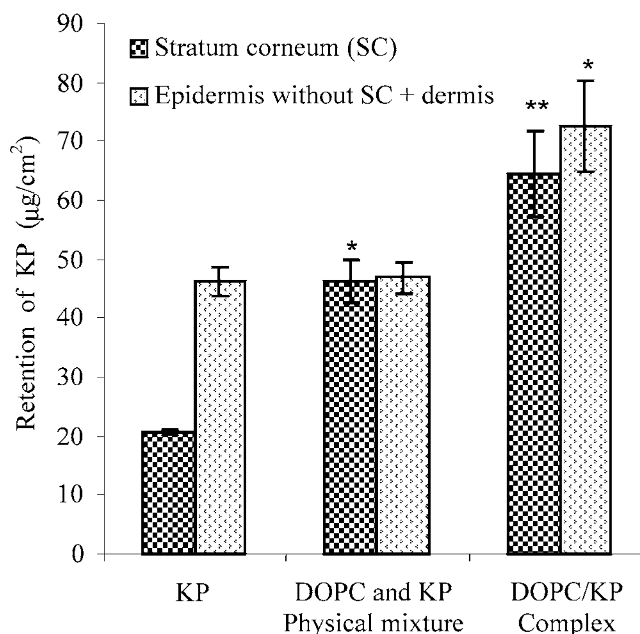

Fig. 4. *In vitro* KP skin uptake from different formulations in mineral oil. Each value is the mean of five different experiments ± SE. *$p < 0.05$; **$p < 0.01$ *vs* control—Kruskal-Wallis test. *KP* = ketoprofen; *DOPC* = dioleylphosphatidylcholine.

epidermal tissue and by being able to mix with the skin lipid components (1). Skin lipid fluidity may be changed by this behavior, thus leading to an enhanced percutaneous absorption of drugs (4–9). Fourier Transform Infrared Spectroscopy and DSC studies indicated that phospholipids affect the stratum corneum lipid organization (4,5,7,32).

On the other hand, the solubility of the drug may be altered when a penetration enhancer is added to a vehicle, thereby increasing or decreasing the degree of the drug saturation. The penetration enhancer can increase the drug concentrations in the vehicle or decrease the vehicle drug solubility. Both approaches lead to an enhanced drug thermodynamic activity in the formulation (33). Previous reports show that addition of phospholipids to liquid paraffin enhances indomethacin and miconazole solubility in vehicle and their skin permeation. According to the authors, this profile may result from drug interactions with phospholipids and their presence in a supersaturated condition (2,3). KP solubility as a complex or in a physical mixture was investigated (Table III). The physical mixture of KP and DOPC in mineral oil showed the highest KP solubility compared to the control formulation. The complex (DOPC/KP, 1:1 w/w) showed an intermediate solubility value. The results showing that KP and DOPC interactions improve KP solubility in mineral oil are in accordance with studies by Battachar *et al.* (12), in which the solubility of indomethacin in squalene was improved by complex formation, as well as by physical mixing.

However, the results also indicate different KP solubility when in complex or physical mixture with DOPC in mineral oil. The solubility of complexed KP was about four fold compared to control, while in physical mixture the solubility was up to 11 fold. Since the amount of KP in both systems was the same (2% w/w) the difference between the solubility values must be related to the process of obtainment.

^{1}H NMR studies of the KP and DOPC complex in two molar ratios and the physical mixture (Tables I and II), as previously described, showed that the interactions were more effective in the complex than in the physical mixture. In other words, Van der Walls interactions and hydrogen bonding were present in the complex while the physical mixture showed only Van der Walls interactions. Since different processes originated the complex or the physical mixture, it is possible to infer that interactions between KP and DOPC are influenced by the preparation method, resulting in different degrees of KP solubility.

According to Fick's law, the flux values should be proportional to the chemical potential gradient, or in other words, the degree of saturation (34,35). As already mentioned, the degree of saturation can be increased by vehicle drug concentration or solubility (32). Raghavan *et al.* (35) showed that hydrocortisone acetate flux from a supersaturated system increased with increasing polymer concentration, reached a maximum and decreased at higher polymer percentages. The decrease in flux at high polymer concentration was attributed to changes in microviscosity and a marginal increase in solubility, since it decreases the thermodynamic activity.

By analyzing the permeation profiles (fluxes) shown in Fig. 3 and the KP solubility results (Table III), it is concluded that the physical mixture provides the highest KP solubility in mineral oil, but the permeation profile remains between the control and complex formulation. Although the KP solubility proportioned by the complex was intermediate between control and physical mixture, its permeation properties were better. It is possible that the expressive increase of KP solubility in the physical mixture caused a reduction in the thermodynamic activity, which in turn interfered with KP permeation.

Another explanation is that drug permeation depends not only on its partition between SC and vehicle (formulation), but also on its diffusion through the membrane. The ability of a drug to partition into the skin, a critical requisite for topical delivery, depends on a number of physicochemical properties including its solubility in the vehicle and its partition coefficient between vehicle and skin (36). In addition, it has been reported that absorption enhancers can affect the chemical environment throughout the lipid domain and thus, theoretically, modify the solute partition coefficient (37). Otherwise, it has been shown that interactions between drug and other substances in vehicle may result in changes of drug physicochemical properties, improving or delaying drug permeation.

A marked change in drug properties was obtained by the interaction of anionic drugs with phospholipids forming fairly stable complexes, which had greater chloroform/water partition coefficients than ionized forms of the drugs themselves (36). The authors also showed that complexes transport through the skin was improved. Buyuktimkin *et al.* (38) suggest that interaction of indomethacin with an enhancer (dodecyl 2-(*n*,*n*-dymethylamino)propionate) may lead to the formation of a new structure, more easily permeated through the SC. In this study KP partition between SC and vehicle was determined to verify the formulation effect on the partition process (Table III), since DSC and ^{1}H NMR results showed an interaction between KP with DOPC, resulting in a new species. The $K_{SC/formulation}$ of KP complexed with DOPC was similar to the control formulation (only KP dispersed in mineral oil). In contrast, the $K_{SC/formulation}$ of KP in physical mixture was lower than the drug alone or complexed with DOPC. Associating partition coefficient and solubility studies, it was observed that the complex increased KP solubility and maintained its $K_{SC/formulation}$, as compared to control, while physical mixture although increasing KP solubility by a greater extent

Table III. Solubility and Partition Results for KP in Different Formulations

Formulation[a]	Solubility in Mineral Oil (μg/ml)	$K_{sc/formulation}$[b]
KP in mineral oil (control)	118.36 (±16.04)	−0.48 (±0.11)
Physical mixture KP + DOPC (1:1) in mineral oil	5,318.37 (±723.48)	−1.14 (±0.10)
Complex DOPC/KP (1:1) in mineral oil	482.43 (±10.05)	−0.44 (±0.12)

Each value is the mean of three different experiments ± SE.
[a] All formulations contained KP 2% (w/w).
[b] Partition coefficient of KP between stratum corneum and formulation.

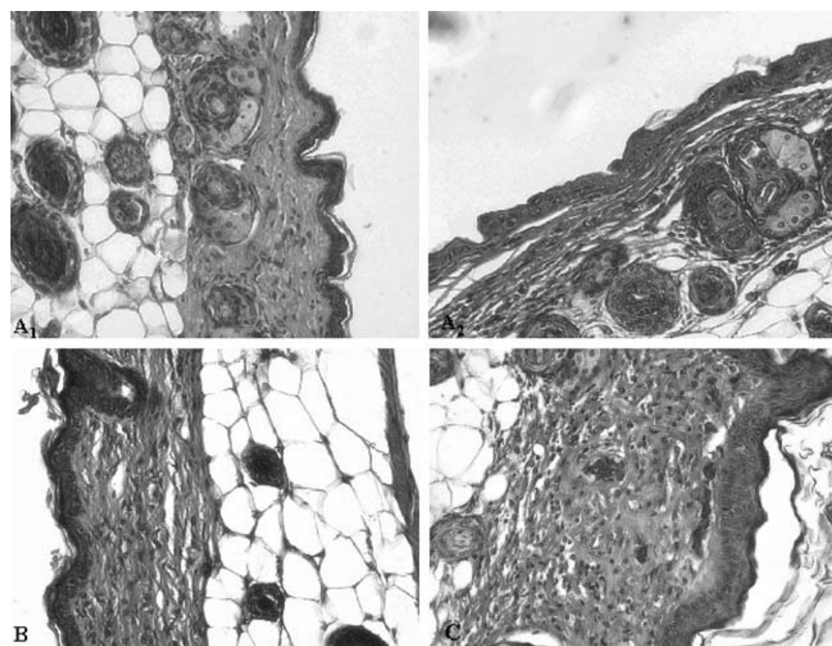

Fig. 5. Histological photomicrographs of skin biopsies taken from non-treated mice or treated with various formulations. H&E stain and MT stain. Objective augment: 20×. (A_1) Untreated mouse skin, (A_2) saline treated mouse skin. Both show thin stratum corneum and distinct boundaries between epidermis and dermis; (B) mouse skin treated with complex, showing no significant change in the skin; (C) mouse skin treated with 5% w/v sodium lauryl sulfate solution, showing considerable damage to the epidermis such as thickness of epidermis with hyperkeratosis and infiltration of the dermis.

decreased its partition coefficient. Again, it suggests that the process of obtainment interferes in these parameters, resulting in different degrees of KP solubilization and partition, which in turn influence drug permeation and skin uptake.

It has been shown that a reduction in the melting point of a permeant has a direct effect on its solubility in skin lipids. It follows that, if the melting point of a drug can be reduced without causing unfavorable changes to other physicochemical parameters, its transdermal flux should be enhanced (39). Eutectic systems have been suggested to reduce the melting point of delivery systems (40). Analyzing the present DSC studies (Fig. 1), a temperature and enthalpy depression is observed in the complex formulation, suggesting that complexation of KP and DOPC has changed (increased) the solubility of the drug in skin lipids and interfered in its permeation profile.

It may be concluded, that the optimization degree in KP permeation may be due to changes in drug thermodynamic activity by interaction with DOPC, modifying KP solubility and partition coefficient. These interactions may also contribute to KP melting point reduction, resulting in a favorable solubility of drug in stratum corneum skin lipids in addition to the DOPC mediated penetration enhancer effect.

Since pharmaceutical preparations containing the proposed complex DOPC:KP (molar ratio, 1:3) will be applied topically, skin tolerance is a crucial condition for its therapeutical use. The potential skin irritating activity of formulations was analyzed by histological assessment in treated mice skins. Treated skin biopsies were examined microscopically and histological changes reported. Figure 5 shows the microscopic appearance of hairless non-treated (Fig. 5A_1) and saline treated mouse skin (Fig. 5A_2). The skin is a multilayered organ and anatomically, it has many histological defined layers: the stratified, avascular, cellular epidermis, the underlying dermis connective tissue and a subcutaneous fat layer. Moreover, the highly vascularized dermis and the epidermis support several skin appendages (41). Chronic skin treatment with sodium lauryl sulfate causes extensive damage (Fig. 5C), including hyperplasia of stratum granulosum and spinhosum, epidermal thickening,

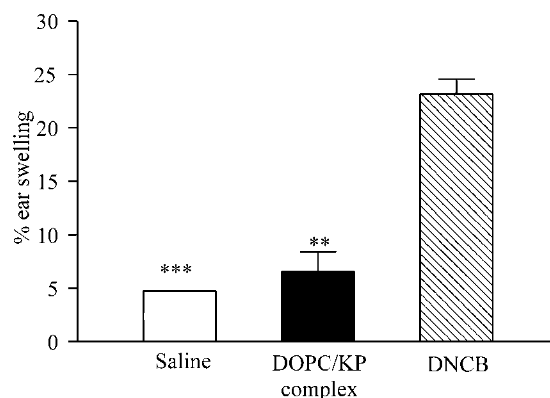

Fig. 6. Percent ear swelling 24 h after Balb/c mice were sensitized and challenged with saline, complex, and DNCB, according to protocols for the mouse ear test. Each value is the mean of six different experiments ± SE. $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* positive control—Kruskal-Wallis test. *KP* = ketoprofen; *DOPC* = dioleylphosphatidylcholine; *DNCB* = 2,4-dinitrochlorobenzene.

hyperkeratosis, increase in blood vessel and inflammatory cell infiltration. Our results are in accordance with Sintov *et al*. (26), who submitted the animals to acute treatment with 5.0% sodium lauryl sulfate.

In comparison, Fig. 5B shows skin submitted to acute treatment with the drug–phospholipid complex in mineral oil. The system caused a slight increase in the stratum spinhosum, resulting in a little epidermal thickening, hyperkeratosis and slight inflammatory cell infiltration.

Hence, the mild skin irritation caused by the DOPC/KP complex system in the present study may be regarded as acceptable in order to achieve some degree of drug permeation through the skin barrier. According to the literature, penetration enhancing effects are caused by structural alteration of the SC, the main skin barrier (1). However, the stronger the effect, the greater the changes in the deeper cutaneous layers. Although it is difficult to quantify the effect of accelerating agents on drug penetration by histological observations, histopathology is still an effective tool for evaluating optimal enhancer concentrations to produce desired permeation defects in the SC with minimal damage to the epidermal and dermal layers (27).

The delayed hypersensitivity response to the KP system was evaluated using the mouse ear swelling test (MEST). MEST requires the use of induction and elicitation phases for evaluation but relies on the measurement of ear swelling as an endpoint (28). Ear swelling responses at 24 h after challenge are shown in Fig. 6. DNCB (1%), the positive control, led to an increase in ear swelling of 23% after 24 h, while saline and DOPC/KP complex increased ear swelling by 5 and 7%, respectively, in the same conditions. Both systems (saline and complex) were significantly different from DNCB, but there was no difference between saline and complex.

Therefore, the complex containing formulation seems to be adequate for topical application of KP, although it is important to mention that the MEST is less reliable for detecting weak to moderate sensitizers (41). It has been reported that in controlled clinical studies, local skin reactions and systemic side effects are not more common than in placebo. However, several case reports have identified KP as being responsible for photosensitive reactions, including both phototoxic and photoallergic adverse effects (42). Thus, further investigation is necessary to characterize the phototoxic and photoallergic potentials of the proposed formulation.

CONCLUSION

The present study elicits the importance of a well devised drug and lipid association by a method resulting in stable complexes, which can optimize delivery in biological membranes such as skin.

DSC and ^{1}H NMR studies showed interactions between KP and DOPC that improved the solubility and decreased the melting temperature and enthalpy of the drug. In addition, DOPC may increase KP diffusion through the SC. Together these considerations may signal enhancement of KP skin permeation and skin uptake when complexed with DOPC. Skin irritation and ear swelling studies did not show significant different effects when comparing complex and saline use. The results suggest that the formulation can be therapeutically used for KP transdermal delivery, although its phototoxic and photoallergic effects must be assessed in a further investigation.

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