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Influence of lipophilic counter-ions in combination with phloretin and 6-ketocholestanol on the skin permeation of 5-aminolevulinic acid

B.G. Auner [a], C. Valenta [a,*], J. Hadgraft [b]

[a] *Institute of Pharmaceutical Technology and Biopharmaceutics, Centre of Pharmacy, University of Vienna, Althanstr. 14, A-1090 Vienna, Austria*
[b] *Skin and Membrane Transfer Research Centre, Medway Sciences NRI University of Greenwich, Central Avenue, Chatham Maritime, Kent ME4 4TB, UK*

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Abstract

In this study, the effect of lipophilic counter-ions on the permeation of 5-aminolevulinic acid (ALA) in combination with skin impregnation by phloretin and 6-ketocholestanol was evaluated. Standard in vitro permeation experiments with porcine skin were performed analysing the ALA content by HPLC and fluorescent detection after ALA derivatisation. The shake flask method in combination with a trinitrobenzensulfonic acid test for ALA analysis was performed to calculate the apparent partition coefficient ($\log P_{Oct}$). The permeation of ALA was enhanced by cetylpyridinium chloride and benzalkonium chloride at pH 7.0 and by sodium-1-octanesulfonic acid, sodium-1-heptanesulfonic acid and sodium-1-pentanesulfonic acid monohydrate at pH 4.0. Corresponding effects of these additives were observed on the partitioning of ALA. Pre-impregnation of porcine skin with phloretin and 6-ketocholestanol increased the ALA diffusion about 1.7-fold at pH 7.0. Moreover, this transport enhancement by 6-ketocholestanol was 3.5-fold higher when a combination of ALA and cetylpyridinium chloride was used as donor.

Keywords: Phloretin; 6-Ketocholestanol; 5-Aminolevulinic acid; Cetylpyridinium chloride; Porcine skin

1. Introduction

Transdermal and topical delivery of drugs provide advantages over conventional oral administration. The advantages of transdermal systems include convenience, improved patient compliance and elimination of hepatic first pass effect. Although transdermal systems have many advantages, most drugs are not applicable to this mode of administration due to the excellent barrier properties of the skin. Molecules must first penetrate the stratum corneum, the outer horny layer of the skin. The molecule then penetrates the viable epidermis before passing into the papillary dermis and through the capillary walls into systemic circulation. It is the stratum corneum, a complex structure of compact keratinized cell layers that presents the greatest barrier to absorption of topical or transdermal administered drugs. The flux of a drug across the skin can be increased by increasing the diffusion coefficient or the driving force by improving the solubility of the drug in the stratum corneum. There are now a wide array of chemicals that have been shown to increase the flux across the skin and a number of reviews of the types of enhancers that are available

* Corresponding author. Tel.: +43-1-4277-55410; fax: +43-1-4277-9554.
E-mail address: claudia.valenta@univie.ac.at (C. Valenta).

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(Barry, 2001; Hadgraft and Guy, 1989; Smith and Maibach, 1995). Since the nature of these compounds is so diverse, it seems unlikely that they all share a common mechanism of action although they have a common aim, to increase the flux across the stratum corneum. Conventionally, penetration enhancers have been categorised into those that disrupt lipid packing in the stratum corneum and those that act as solvents for the penetrating molecule.

Phloretin and 6-ketocholestanol cannot easily be assigned to one of these two categories. In model membrane studies, it has been shown that the membrane dipole potential of bilayers is decreased by phloretin and increased by 6-ketocholestanol (Rokitskaya et al., 2002).

Since the stratum corneum contains a complex bilayer system, which consists mainly of six different types of ceramides that occur freely or attached to proteins of the corneocytes (Franklin and Cafiso, 1993; Swartzendruber et al., 1987), phloretin as well as 6-ketocholestanol may have also an effect on the permeability of skin.

As a model drug, we chose 5-aminolevulinic acid (ALA), which is poorly absorbed into the skin as a result of its physicochemical properties. ALA is topically applied in photodynamic therapy (PDT) of selected percutaneous diseases, including skin cancer. The endogenous conversion of ALA to protoporphyrin IX (PpIX) has broadened the use of PDT. Due to the hydrophilic properties of ALA, ALA-PDT may clinically be limited by the rate of ALA uptake into the neoplastic cells and its penetration through the tissue (Cairnduff et al., 1994; Wolf et al., 1993). The ALA molecule is hydrophilic and does not penetrate through intact skin in sufficiently high doses. Several attempts have been undertaken to increase the flux, e.g. iontophoresis (Barry, 2001; Lopez et al., 2001) or addition of DMSO (Casas et al., 2001) as penetration enhancer. Ester prodrugs have also been considered (Casas et al., 2001; Gaullier et al., 1997; Moan et al., 2001).

Recently, it has been shown that impregnation of porcine skin with phloretin-loaded PC-liposomes and 6-ketocholestanol-loaded PC-liposomes, respectively, can have a small positive effect on the ALA flux at pH 4.0 (Valenta et al., 2002).

The aim of the present study was to investigate whether a combination of lipophilic counter-ions and phloretin or 6-ketocholestanol are able to increase the cumulative amount of ALA permeation through porcine skin.

2. Materials and methods

2.1. Materials

ALA, phloretin (3-[4-hydroxyphenyl]-1-[2,4,6-trihydroxyphenyl]-1-propanone), phosphatidylcholine (L-α-phosphatidylcholine solution, 100 mg/ml in chloroform) (PC), 6-ketocholestanol, 2,4,6-trinitrobenzenesulfonic acid (TNBS), acetylacetone, cetylpyridinium chloride (CP) and sodium-1-octanesulfonic acid (OS) were purchased from Sigma (St. Louis, MO). Sodium-1-pentanesulfonic acid monohydrate (PS) was purchased from Fluka (St. Louis, MO). Cetyltrimethylammonium bromide (CT) was purchased from Merck (Munich, Germany). All other chemicals used in the study were of analytical reagent grade and were used as received without any further purification.

2.2. Skin preparation

Porcine abdominal skin was shaved and then prepared with a dermatome (GB 228R, Aesculap) set at 1.2 mm. The skin was stored in a freezer at −20 °C until use. The samples were thawed 2 h prior to the experiments.

2.3. Unilamellar liposomes

Liposomes with phloretin or 6-ketocholestanol were prepared by a modified method, which has been previously reported by (Cladera and O'Shea, 1998). PC was obtained in a chloroform solution (100 mg/ml). Phloretin and 6-ketocholestanol were dissolved in chloroform/methanol solution (3:1). Either 200 μl PC solution, 200 μl PC–phloretin solution or 200 μl PC–6-ketocholestanol solution were, respectively, mixed.

Mixtures were carefully dehydrated, using a gentle stream of nitrogen, until a thin homogenous lipid film was formed. The films were put under vacuum for 12 h in a desiccator to ensure that the solvent has evaporated. The lipid films were rehydrated in 2 ml of 10 mM phosphate buffer (pH 7.2). The resulting multilamellar liposomes were frozen with liquid nitrogen and re-thawed five times. They were then extruded

(LiposoFast®, Avestin Inc., Canada) 10 times through a polycarbonate Nuclepore® membrane and support (Nuclepore, CA) with a diameter of 100 nm. Nitrogen was used to extrude the mixture using a pressure of 200–500 psi.

2.4. Diffusion cell preparation

Permeation of ALA was investigated using Franz-type diffusion cells with a permeation area of 1 cm^2. The receptor compartment was filled with 2 ml phosphate buffer (0.05 M, pH 6.0), thermostated at 32 °C. The excised skin was mounted on the cell, stratum corneum uppermost. The diffusion cells were placed in a water bath at 32 °C. The cells were allowed to equilibrate for 30 min before the liposomes were added. In all experiments, 150 μl of liposomes were applied to the top of the skin. Three sets of experiments were performed.

(I) All cells were impregnated with PC-liposomes. The donor phase consisted of ALA solutions at pH 4.0 and 7.0 plus additional counter-ions (Table 1) were used.
(II) Three different impregnation formulations are:
  (1) PC-liposomes (control),
  (2) phloretin-loaded PC-liposomes,
  (3) 6-ketocholestanol-loaded PC-liposomes.
  The donor phase was an ALA solution at pH 4.0 or 7.0.
(III) The same types of impregnation as described in (II) were used. The donor phase was either an ALA solution or an ALA solution plus CP at pH 7.0.

Table 1
Different donor solutions containing ALA (0.4 mg/ml) and lipophilic counter-ions (molar ratio 1:1 with ALA) at different pH values in 0.05 M phosphate buffer adjusted to the indicated pH values

Donor solution	pH value	Code
ALA	4.0	ALA 4
ALA + sodium-1-octanesulfonic acid	4.0	ALA 4-OS
ALA + sodium-1-heptanesulfonic acid	4.0	ALA 4-HS
ALA + sodium-1-pentanesulfonic acid monohydrate	4.0	ALA 4-PS
ALA	7.0	ALA 7
ALA + cetylpyridinium chloride	7.0	ALA 7-CP
ALA + benzalkonium chloride	7.0	ALA 7-BZ
ALA + cetyltrimethylammonium bromide	7.0	ALA 7-CT

As described in other studies (Valenta et al., 2001a, 2002), 15 h of incubation time was chosen for all experiments in order to give phloretin and 6-ketocholestanol the chance to diffuse to the possible site of action within the lipid bilayer. After this time, 1.0 ml of the donor solution was applied to each cell. The time of this application was quoted as starting point. The receptor phase was totally removed and replaced with fresh solution at 30-min interval and samples analysed. The experiments were performed over a 4-h period.

2.5. Analysis of ALA by HPLC

The amount of permeated ALA was analysed by HPLC using a slightly modified method described initially by (Oishi et al., 1996). The samples were centrifuged at a speed of 13,500 rpm for 5 min before use. The fluorescence derivatisation method for ALA involved the following: 1.4 ml of acetylacetone reagent (15 ml acetylacetone with 10 ml ethanol 96% (v/v) and 75 ml distilled water) were mixed with 0.18 ml formaldehyde 10% and 0.02 ml of the sample. Then the samples were vigorously shaken for about 5 s and were then heated to 95 °C with slow shaking, followed by a cooling period of 10 min.

A high performance liquid chromatograph (Series 200LC, Perkin Elmer) equipped with an automatic sample injector (ISS-200, Perkin Elmer) was used. A total of 20 μl of the prepared ALA-derivative was injected onto a Nucleosil 100-C18 reversed-phase column (150 mm × 3 mm). Elution was performed at 40 °C with a mobile phase consisting of methanol–water–acetic acid (50:50:1, v/v/v) at a flow rate of 0.7 ml/min. The fluorescence intensity of the eluate was monitored at a wavelength of 370/460 nm excitation/emission with a fluorescence monitor (LS40, Perkin Elmer). The detection limit of this method was about 50 ng/ml.

2.6. Analysis of ALA by determination of the primary amino group

A colour reaction between the primary amino group of ALA and TNBS was used. Standard solutions of

ALA (Table 1) were prepared. The calibration curve achieved a correlation coefficient of 0.999. The following procedure was performed: 100 μl of each sample was pipetted into a microtiter plate. After adding 100 μl 4% $NaHCO_3$, 1 μl 5% TNBS was applied. The plate was incubated for 2 h at 27 °C. Afterwards, the change of colour was determined with a microtiter plate-reader (Anthos™ 2001, Anthos Labtec Instruments) at a wavelength of 450 nm. The detection limit of this method was about 50 μg/ml.

2.7. Apparent partition coefficient

The apparent partition coefficient of ALA was investigated between *n*-octanol and phosphate buffer (0.05 M) at pH values of 4.0 and of 7.0, respectively. In case of additional counter-ions (Table 1), the pH was adjusted again after adding the counter-ion, respectively. *n*-Octanol pre-saturated with phosphate buffer (0.05 M) was used. ALA and an equal molar amount of counter-ion were dissolved, the pH adjusted, *n*-octanol added and stirred continuously for 24 h at a temperature of 4 °C excluding light. After phase separation, the ALA content was analysed in buffer by determination of the primary amino groups with TNBS. Since the amount of ALA initially used in buffer was known, the amount in the organic phase could be determined by difference.

2.8. Statistical data analysis

Results are expressed as the means of at least three parallel experiments ± S.D. Statistical data analysis was performed using a non-parametric Kruskal–Wallis test. All tests have $P < 0.05$ as a minimal level of significance.

3. Results

3.1. Apparent partition coefficient

ALA has two pK_a values of 4.0 for the carbonyl group and 7.9 for the amino group. Therefore, an anionic charge dominates at a pH of 7.0 and a cationic charge dominates at a pH of 4.0. Due to the physical properties, ion pairing with anionic as well as with cationic lipophilic counter-ions would be possible.

Table 2
The apparent partition coefficient ($\log P_{Oct}$) of ALA in phosphate buffer (0.05 M) at pH values of 7.0 and 4.0, respectively, was determined using various lipophilic counter-ions

Lipophilic counter-ion	pH value	$\log P_{Oct}$
Control[a]	7.0	−0.82 ± 0.09
Cetylpyridinium chloride (CP)	7.0	−0.04 ± 0.07
Cetyltrimethylammonium bromide (CT)	7.0	−0.09 ± 0.03
Benzalkonium chloride (BZ)	7.0	−0.22 ± 0.14
Control[a]	4.0	−0.92 ± 0.12
Sodium-1-octanesulfonic acid (OS)	4.0	−0.67 ± 0.28
Sodium-1-heptanesulfonic acid (HS)	4.0	−0.33 ± 0.19
Sodium-1-pentansulfonic acid monohydrate (PS)	4.0	−0.57 ± 0.24

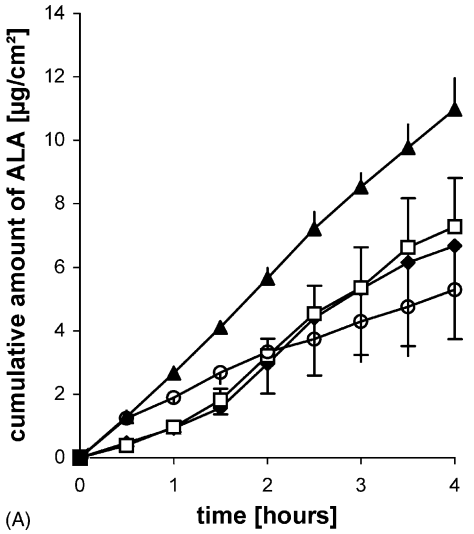
[a] ALA without counter-ions served as control.

At pH 7.0, CP, CT and benzalkonium chloride (BZ) were used as lipophilic counter-ions, respectively. At pH 4.0, sodium-1-octanesulfonic acid (OS), sodium-1-heptanesulfonic acid (HS) and PS, were added to the ALA solution, respectively (Table 1). Firstly, we determined the apparent partition coefficient of pure ALA at pH 7.0 and 4.0. Afterwards, the apparent partition coefficients of ALA together with the lipophilic counter-ions were determined (Table 2). As can be seen, the lipophilicity of the ALA/counter-ion species in general is increased compared to the single ALA molecule. This indicates a possible ion pairing. In case of pH 7.0, the lipophilic shift was higher than in case of pH 4.0. The highest lipophilic shift was caused by CP from −0.82 to −0.04. The question was whether this higher lipophilicity has also a positive effect on the permeation.

3.2. Diffusion of ALA

A comparison of ALA diffusion at pH 7.0 and 4.0 showed a slightly higher cumulative amount of ALA diffusion after 4 h at pH 7.0 (Fig. 1A and B). Among all tested cationic lipophilic counter-ions at pH 7.0, only CP showed a 1.6-fold increase in ALA diffusion (Fig. 1A).

Addition of BZ (ALA 7-BZ) had no effect on the cumulative amount of ALA (Fig. 1A). Although ALA 7-CT caused a higher $\log P_{Oct}$ in the range of ALA 7-CP (Table 2), the cumulative amount of ALA was even slightly decreased (Fig. 1A).



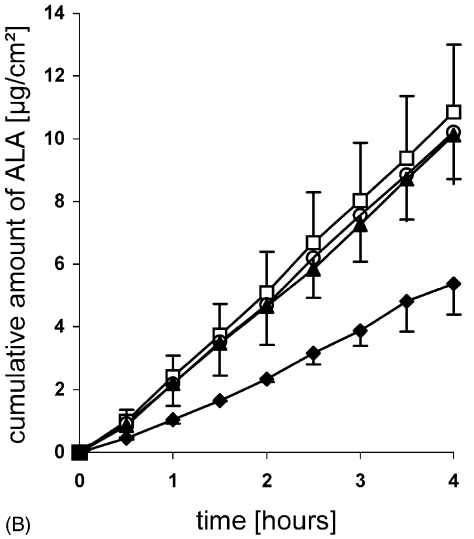


Fig. 1. (A) Permeation of ALA at a pH value of 7.0 through porcine skin. Tests were carried out with ALA in combination with benzalkonium chloride (□; ALA 7-BZ), ALA combined with cetylpyridinium chloride (▲; ALA 7-CP), ALA combined with cetyltrimethylammonium bromide (○; ALA 7-CT) and with ALA in presence of unloaded PC-liposomes (◆; ALA 7). Indicated values are means (±S.D.) of at least three experiments. (B) Permeation of ALA at a pH value of 4.0 through porcine skin. Tests were carried out with ALA combined with sodium-1-octanesulfonic acid (□; ALA 4-OS), ALA combined with sodium-1-heptanesulfonic acid (▲; ALA 4-HS), ALA combined with sodium-1-pentanesulfonic acid monohydrate (○; ALA 4-PS) and with ALA in presence of unloaded PC-liposomes, served as a control (◆; ALA 4). Indicated values are means (±S.D.) of at least three experiments.

Fig. 1B shows the diffusion profiles of ALA at pH 4.0 in the presence of the various anionic lipophilic counter-ions (OS, HS, PS). As can be seen, after 4 h all used counter-ions increased the cumulative amount of ALA on average of 1.9-fold compared to the control.

In conclusion, all tested lipophilic counter-ions, anionic as well as cationic, with the exception of CT, increased the cumulative amount of ALA.

The previous diffusion studies were carried out after skin impregnation with PC-liposomes. It has been previously shown that skin impregnation with phloretin and 6-ketocholestanol can have a slightly positive effect on ALA diffusion at pH 4.0 (Valenta et al., 2002). In the present study, at pH 7.0, we also showed a 1.7-fold increase in the cumulative amount of ALA. As indicated, the effect was in the same range for both 6-ketocholestanol and phloretin (Fig. 2).

At pH 4.0, for all tested donor solutions (Table 1; Fig. 1B), no additional positive effect on the ALA permeation could be detected by skin impregnation with phloretin–PC-liposomes or 6-ketocholestanol–PC-liposomes.

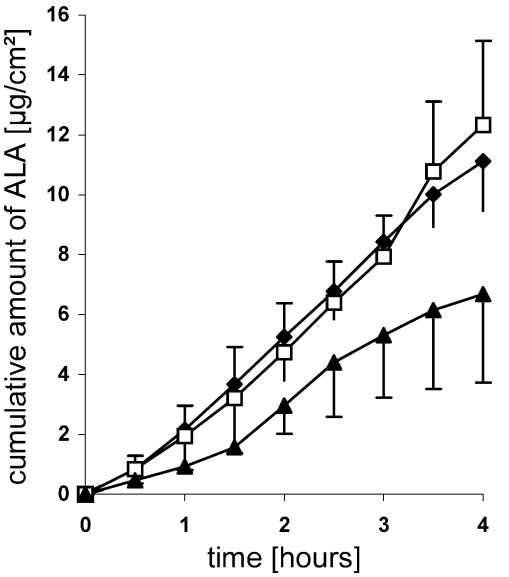


Fig. 2. Permeation of ALA at a pH value of 7.0 through porcine skin. Tests were carried out with ALA 7 in presence of 6-ketocholestanol-loaded PC-liposomes (◆), ALA in presence of phloretin-loaded PC-liposomes (□) and with ALA in presence of unloaded PC-liposomes, served as a control (▲). Indicated values are means (±S.D.) of at least three experiments.

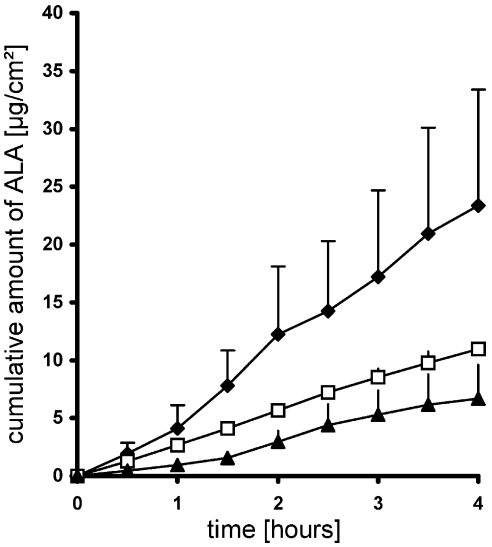


Fig. 3. Permeation of ALA at a pH value of 7.0 through porcine skin. Tests were carried out with ALA 7-CP in presence of 6-ketocholestanol-loaded PC-liposomes (◆), ALA combined with cetylpyridinium chloride with unloaded PC-liposomes (□) and with ALA in presence of unloaded liposomes, served as a control (▲). Indicated values are means (±S.D.) of at least three experiments.

At pH 7.0, a combination of ALA 7-BZ and ALA 7-CT with 6-ketocholestanol–PC-liposomes or phloretin–PC-liposomes had no additional positive effect on the ALA permeation either (data not shown). The optimal combination found was the application of ALA 7-CP with skin pretreated with 6-ketocholestanol–PC-liposomes. As seen in Fig. 3, the further increase in ALA permeation due to the 6-ketocholestanol impregnation was about 3.5% after 4 h. Because the used control of empty liposomes, the positive effect cannot be assigned solely to the liposomes but also to the lipophilic counter-ion as well as the impregnation with 6-ketocholestanol.

4. Discussion

ALA is commonly used in PDT. PDT is an experimental approach in cancer treatment which uses a natural occurring photosensitizer localised in a tumour to provoke local tissue destruction by absorbing an adequate dose of light of an appropriate wavelength (Ortel et al., 1996; Peng et al., 1996). A common technique for PDT, based on the heme biosynthetic pathway, employs ALA as a prodrug. In this biosynthetic pathway, ALA is converted to heme over the highly fluorescent key component PpIX. This is an effective photosensitizer. In the body, ALA is formed from glycine and succinyl CoA in the first step of the heme cycle. The biosynthesis of ALA is kept under feedback control by the cellular heme amount. The final step of heme biosynthesis is the incorporation of iron into PpIX. This feedback mechanism can be avoided by exogenous ALA. The intracellular accumulation of PpIX is induced by topical application of ALA (DeRosa et al., 2000). The disadvantage of ALA is the high hydrophilicity of the molecule, therefore the skin penetration is insufficient. ALA has two pK_a values of 4.0 and 7.9 and thus can form ion pairs with both anions and cations depending on the medium pH.

In order to enhance the skin permeation rate of either the cationic or the anionic form of ALA, both cationic and anionic counter-ions were evaluated. Ion pairs are defined as neutral species formed only by electrostatic attraction between oppositely charged ions (Kraus, 1956), which are sufficiently lipophilic to dissolve in lipoidal medium such as stratum corneum. Therefore, ion-pair formation has several advantages over other approaches: it can enhance the skin transport of ionic drugs without modification of their structure, and without change in skin barrier function (Pardo et al., 1992; Takahashi and Rytting, 2001).

For further conformation of the possible ion-pair skin transport, the effect of counter-ions on partitioning of ALA was assessed at the same pH values used in the diffusion experiments. Although the zwitterion ALA may form ion pairs, the contribution to the increased partition coefficient should be negligible because of additional positive or negative charges.

At pH 7.0 interestingly, only CP was able to increase the ALA permeation although the log P_{Oct} of ALA 7-CT was in the same range (Table 2). This is in agreement with a recently published paper, where the partition of quaternary ammonium drugs was influenced by different lipophilicity, size and flexibility (Takács-Novak and Szász, 1999). However, no significant effect has been found due to the type (e.g. aliphatic or aromatic) of the quaternary N atom.

The higher accumulative amount of ALA 7-CP may be explained by the difference in the lipophilicity and/or association constant of the ion pairs (Lee

and Kim, 1987). It may reflect a higher association constant for ion pairs with CP. The formation of ion pairs is only possible if the ions approach each other and reach a critical separation distance (Kraus, 1956).

In addition to the ion-pair approach, the influence of phloretin and 6-ketocholestanol was determined. Previous studies showed that phloretin incorporated in PC unilamellar liposomes could enhance lignocaine hydrochloride flux (Valenta et al., 2000, 2001b); whereas 6-ketocholestanol could enhance the flux of bacitrcin (Cladera et al., in press). This is in agreement with other studies, where phloretin increased the transport of cations and reduced the rate of anion transfer in model phospholipid membranes; whereas 6-ketocholestanol produced opposite effects from those of phloretin (Franklin and Cafiso, 1993).

The combination of ALA and 6-ketocholestanol with CP was beneficial. It resulted in a 3.5-fold increase in ALA permeation within the short period of 4 h. For CP, no expensive toxicological studies have to be performed. CP is already in use as a preservative (Grant et al., 2002) and is allowed up to 1% in food. To date in additional studies, CP was not only tested as a preservative but also as a penetration enhancer in buccal and ocular preparations (Ali et al., 2002; Monti et al., 2002) and as charge carrier for the production of solid lipid nanoparticles (Olbrich et al., 2001).

A combination of 6-ketocholestanol with CP could be considered as a future permeation enhancer for ALA. Hence, in vivo human tests should be carried out to verify the obtained results.

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