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# Influence of phloretin and 6-ketocholestanol on the permeation of progesterone through porcine skin

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**Abstract**

In this study the effect of phloretin (PH) and 6-ketocholestanol (KC) on the permeation of progesterone through porcine skin has been examined. Both PH and KC were incorporated into unilamellar L-α-phosphatidylcholine (PC) liposomes at different concentrations (7.5, 15, 30 and 60 mol%). In diffusion experiments with porcine skin, both substances, to a different degree, enhanced the steady state flux of progesterone. It was increased up to 2.4-fold using 15 mol% KC, and 1.4-fold using 30 mol% PH.. The results indicate an interaction of these two compounds with the lipid components of the stratum corneum. In order to visualise the interaction, differential scanning calorimetry (DSC) measurements were performed on porcine skin, which had been impregnated with KC and PH. Both showed a lowering ($\sim$5–6°C) in the lipid phase transition temperature that occurs around 75°C in porcine skin. 

*Keywords:* Phloretin; 6-ketocholestanol; Porcine skin; DSC; Diffusion

## 1. Introduction

Over the past few years major advances have been made in the field of dermal and transdermal drug delivery. Increasing numbers of drugs are being added to the list of therapeutic agents that can be delivered to the systemic circulation, in clinically effective concentrations, via the skin. This is perhaps surprising since the stratum corneum is a very effective barrier which prevents xenobiotics entering the body. The first recognised penetration enhancers were simply disruptive keratolytic agents that permanently destroyed the stratum corneum integrity and were generally non-specific. 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 (Barry, 1987; Smith and Maibach, 1995; Walters, 1989). In contrast to this, penetration enhancers with a different, more specifically reversible mechanism would be of great benefit.

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In model membrane studies it has been shown that the membrane dipole potential of bilayers is decreased by phloretin (PH) and increased by 6-ketocholestanol (KC). PH increased the transport of cations and reduced the rate of anion transfer in these model phospholipid membranes. It was noted that KC produced the opposite effects from those from PH (Franklin and Cafiso, 1993). Although artificial membranes (unilamellar phospholipid vesicles) are very simplified structures and cannot be directly compared to the complex structure of skin, this paper examines whether PH or KC has any influence on the passage of progesterone through skin. Recently it was shown that pre-treatment of skin with PH loaded liposomes enhanced the flux of lignocaine hydrochloride through human epidermis. The local anaesthetic lignocaine hydrochloride has a cationic charge at physiological pH (Valenta et al., 2001). The flux of charged FITC-bacitracin through human epidermis was enhanced by KC (Cladera et al., submitted for publication). However, these previous investigations did not clarify whether electrostatic contributions alone or other mechanisms were responsible for this enhancement. If the passage of only charged molecules is influenced by KC and PH, we could conclude that electrical effects are dominant. If the passage of uncharged molecules also is influenced, it would indicate other reasons. Therefore we investigated whether the diffusion of an uncharged compound, progesterone, was influenced by PH or KC. Permeation experiments were conducted with porcine abdominal skin pre-treated with PH or KC compared to a control. Also information about the PH and KC mediated changes to the stratum corneum was examined by differential scanning calorimetry studies (DSC).

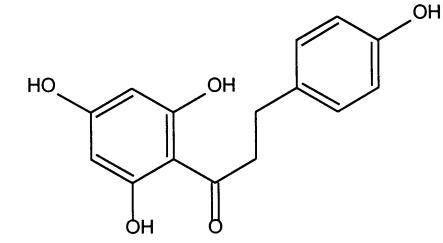
## 2. Material and methods

### 2.1. Materials

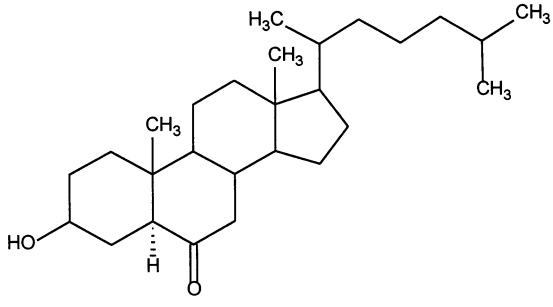
Progesterone; PH (3-[4-hydroxyphenyl]-1-[2,4,6-trihydroxyphenyl]-1-propanone; KC (5α-cholestan-3β-ol-6-one) (Fig. 1); and phosphatidylcholine (PC) (L-α-phosphatidylcholine solution, 100 mg per ml in chloroform) were purchased from Sigma (St. Louis, USA). All other chemicals used in the study were of analytical reagent grade and were used as received without any further purification.

### 2.2. Preparation of unilamellar vesicles (ULVs)

Vesicles with different amounts of PH or KC, together with controls, were prepared by a modified method, which has been previously reported (Mayer et al., 1986; Cladera and O'Shea, 1998). PC was obtained in a chloroform solution (100 mg/ml). PH and KC were dissolved in a 3:1 chloroform / methanol solvent. Either 200 μl PC solution or 200 μl PC–PH solution were mixed in a round flask. The concentrations of KC and PH corresponded to 7.5, 15, 30 and 60 mol%. The mixtures were then carefully dehydrated, using a




(I)




(II)

Fig. 1. Structure of phloretin (I) and 6-ketocholestanol (II).

gentle stream of argon, until a thin homogenous lipid film was formed inside the flask. The films were stored, under vacuum, in a desiccator for 12 h to ensure that all the solvents had evaporated. The lipid films were re-hydrated with 2 ml 10 mM phosphate buffer, pH 7.2. The resulting multilamellar liposomes were frozen and re-thawed five times. They were then extruded ten times through a 100 nm diameter polycarbonate Nucleopore membrane and support (Nucleopore, CA, USA). Nitrogen gas was used to extrude the mixture using a pressure ranging from 200–500 psi. The ten-fold repetition of extrusion produced a virtually homogenous population of unilamellar vesicles with diameters in the range of 110 to 120 nm. This method is a standard technique for producing unilamellar liposomes. Each batch of filters obtained from Lipex had been tested (protocol included with each test certificate) and the diameter of the vesicles measured by quasi-elastic light scattering (Lipex BM application manual).

### 2.3. Skin preparation

Porcine abdominal skin was prepared by removing carefully the subcutaneous fat layer by layer with a scalpel. The skin was shaved, cut in suitable pieces for Franz diffusion cells and stored in a freezer at −20°C. The samples were thawed 2 h prior to use.

### 2.4. Diffusion cell preparation

Permeation of progesterone was investigated using Franz-type diffusion cells. The receptor compartment was filled with propylene glycol: water (40:60 w/w), thermostated at 32°C and continuously stirred using a magnetic bar. The effective area available to diffusion was 1.13 $cm^2$. The excised skin was mounted on the cell, stratum corneum uppermost, with the dermal side facing the receptor compartment. At defined time points the receptor medium was removed for analysis and replaced with fresh receptor medium.

Samples from the permeation experiments were analysed by an HPLC (high performance liquid chromatography) method for progesterone content as described in Section 2.5. All experiments were repeated at least three times. During the experiment the sampling arms were covered with metal foil to prevent evaporation. Firstly the receptor chambers were filled with propylene glycol–water (40:60 w/w) and brought to 32°C. After equilibration the liposomes were applied to the top, stratum corneum surface of the skin. The various pre-treatments were with:

1. 150 μl PC-liposomes
2. 150 μl PH loaded PC-liposomes
3. KC loaded PC-liposomes.

After 15 h affecting time for the liposomes, 1.0 ml of saturated progesterone solution in propylene glycol–water (40:60 w/w) was added to each donor chamber of the cells. Start of the experiment was the time of progesterone application. The receptor phase was renewed at the following time intervals after the start for porcine skin: 2, 4, 6, 8, 10, 12, 14, 24, 26, 28, 30, 32, 34, 36, 49 and 50 h; and after 1, 2, 3, 4, 5, 6, 7, 24 and 48 h for rat skin.

### 2.5. Analytical procedure

Samples were assayed for progesterone content, using a previously reported modified HPLC method (Valenta and Wedenig, 1997) at a flow rate of 1 ml/ min with a UV detector (series 200 LC, Perkin Elmer) at a detection wavelength of 240 nm. The stationary phase was a Nucleosil 100 5C-18 column (240 mm × 4.6 mm). Any impurities were held back on a pre-column (Nucleosil 100-5C-18, 40 mm × 4.0 mm). The mobile phase was methanol–water (90:10). 20 μl samples were injected by an autosampler (ISS-200, Perkin Elmer). The retention time for progesterone was approximately 4.4 min. Calibration curves were calculated on the basis of peak area measurements. The linearity interval established in the diffusion receptor phase of propylene glycol–water (40:60 w/w) was 0.01–54.5 μg/ml ($r^2$: 0.9999) and in methanol 4.75–475 μg/ml ($r^2$: 0.9999).

### 2.6. Solubility

An excess of progesterone was added to propylene glycol–water (40:60; w/w) at 32°C and stirred for 48 h. After filtering (Minisart; Sartorius 0.45

μm) the progesterone content was analysed by HPLC. The solubility of progesterone in propylene glycol–water (40:60 w/w) was 336.6 μg/ml ± 5.11 (standard deviation, SD).

### 2.7. Differential scanning calorimetry (DSC)

Porcine skin samples of approximately 25–35 mg from the cells after a 50 h diffusion experiment were sealed in a sample pan and heated at a rate of 0.75°C per min from ambient temperature to 110°C in a differential scanning calorimeter (Perkin Elmer DSC-7). Three different skin samples were measured.

1. untreated
2. treated with PH-PC-liposomes 30 mol%
3. treated with KC-PC-liposomes 15 mol%.

Gravimetric analysis of the samples prior to DSC experiments showed an average water content of about 69% (w/w) ± 6.0.

### 2.8. Statistical data analysis

Results of the diffusion studies are expressed as the means of at least three experiments ± SD, and for the DSC studies, of four experiments ± SD. Statistical data analysis was performed using a *t*-test with $p < 0.05$ as a minimal level of significance.

## 3. Results and discussion

PH and KC are two possible penetration modifiers in different artificial membranes, and therefore could act as such in skin. PH is the aglycone of phlorizin, a polyphenolic substance occurring in the root bark of apple trees (Windholz, 1983; Burger and Wachter, 1993) and KC is a cholestanol derivative. They have often been used to study interactions with phospholipid vesicles as model membranes (Jendrasiak et al., 1997; Cladera and O'Shea, 1998; Bechinger and Selig, 1991). In these simplified artificial systems it could be shown that PH enhances the transport of cations and KC enhances the transport of anions. One of the reasons for this may be the dipole modifying ability of PH and KC. From electrical measurements in monolayers and work in model bilayers it is known that lipid bilayers possess an internal dipole potential. In bilayers, this is believed to account for the permeability differences of certain organic cations and anions. PH is known to reduce the magnitude of the dipole potential in biological membranes (Franklin and Cafiso, 1993); KC increases this dipole potential.

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 (Swartzendruber et al., 1987; Wertz et al., 1985). Therefore PH as well as KC may have also an effect on the permeability of skin. Recently it has been shown that lignocaine hydrochloride penetration through skin can be enhanced by PH (Valenta et al., 2001). Also it has been shown that treating human skin with liposomes containing KC influences the penetration of the peptidic antibiotic bacitracin (Cladera et al., submitted for publication). Bacitracin is a bactericidal antibiotic commonly used topically. It is a cyclic peptide which contains positively and negatively charged lateral chains at neutral pH and is composed of a mixture of L- and D- amino acids. The diffusion of fluorescein-labelled bacitracin through human skin was monitored using confocal microscopy and complementary fluorescence studies with model membranes using the fluorescent dyes fluorescein-phosphatidylethanolamine (FPE) and di-8-ANEPPS. Pre-treatment of isolated human epidermis with liposomes containing 30 mol% KC enhanced the penetration of the antibiotic bacitracin.

In the current study, the influence of PH and KC on the percutaneous penetration of an uncharged molecule, namely progesterone, was tested. As in the previous studies, PH as well as KC were associated with PC unilamellar liposomes. Because it is easier to prepare rat skin, preliminary experiments were carried out with KC and PH at a concentration of 30 mol% in the liposomes. Rat skin was prepared as previously reported (Valenta and Wedenig, 1997). The rat skin was pre-treated for 15 h prior to diffusion (Fig. 2A). The permeation experiments with rat skin were conducted for 48 h but samples were

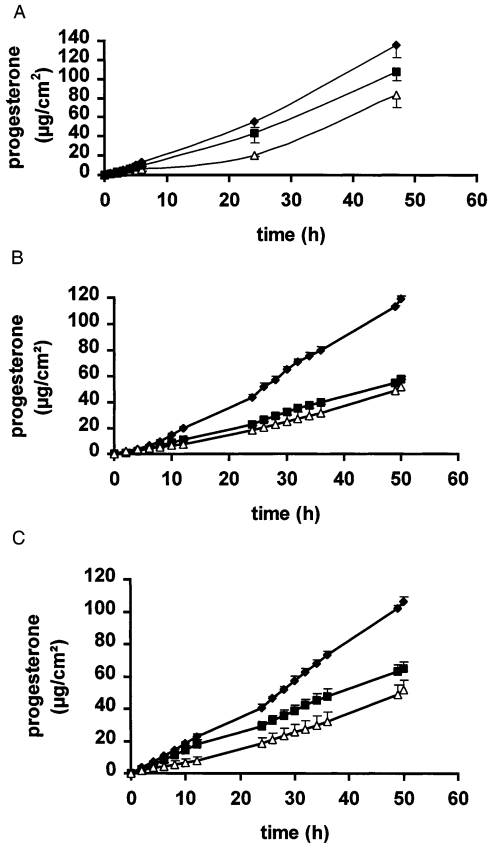



Fig. 2. 2A. Progesterone diffusion profiles through rat skin. Influence of different impregnation protocols. -◆- PC–KC (15 mol%)-liposomes; -■- PC–PH (15 mol%)-liposomes; -△- PC-liposomes (control).2B. Progesterone diffusion profiles through porcine skin. Influence of different impregnation protocols. -◆- PC–KC (15 mol%)-liposomes; -■- PC–PH (15 mol%)-liposomes; -△- PC-liposomes (control).2C. Progesterone diffusion profiles through porcine skin. Influence of different impregnation protocols. -◆- PC–KC (30 mol%)-liposomes; -■- PC–PH (30 mol%)-liposomes; -△- PC-liposomes (control).

only withdrawn at 24 h and 48 h. As can be seen, there appears to be a lag time for the control, and both PH and KC enhanced the progesterone flux. Because rodent skin is much more permeable than human skin, all further studies were done with porcine skin, which has a similar lipid composition to human skin and is therefore thought to be more representative.

The cumulative amount of drug released through the porcine membrane $Q(t)$, at any time, $t$, was determined from the formula:

Table 1
Influence of different amounts 6-ketocholestanol (KC) and phloretin (PH) on the progesterone flux through porcine skin.

| steady state progesterone flux $J$ (µg/cm²/h) | | | |
|---|---|---|---|
| control | mol% | KC | PH |
| $0.91 \pm 0.2$ | 7.5 | $1.19 \pm 0.08$ | $1.16 \pm 0.05$ |
| | 15 | $2.19 \pm 0.05$ | $1.09 \pm 0.04$ |
| | 30 | $1.99 \pm 0.05$ | $1.28 \pm 0.09$ |
| | 60 | $1.02 \pm 0.136$ | $0.68 \pm 0.06$ |

$$Q = (CV)/A$$

where $C$ is the progesterone concentration in the receptor compartment in µg/ml at time $t$, $V$ is the volume of fluid in the receptor phase, and $A$ is the diffusional area of the cell. The slope of the best linear fit line (Table 1) gave the steady state flux $J$ (µg/cm²/h). All diffusion profiles showed a good linear fit, as shown in Fig. 2 (B, C). Although porcine skin is a bio-membrane the SDs are very small.

In order to investigate the optimal concentration, four different concentrations (7.5, 15, 30 and 60 mol%) of liposomes with PH, as well as KC, were tested. Empty liposomes served as a control. As can be seen in Fig. 3, PH exhibited a significant positive influence on the permeation of

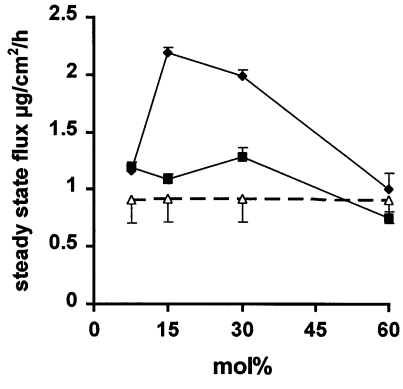



Fig. 3. Influence of PC-liposomes loaded with different amounts of phloretin (PH) and 6-ketocholestanol (KC) in comparison to unloaded PC-liposomes on the steady state flux of progesterone through porcine skin. -◆- PC–KC liposomes; -■- PC–PH-liposomes; -△- PC-liposomes (control).

progesterone only at a concentration of 30 mol% (Table 1). The progesterone flux was increased 1.4-fold compared to the control. In case of 7.5 mol%, 15 mol% and 60 mol% no significant influence could be detected. In contrast, pre-treatment with KC caused a higher increase of flux between 15 mol% and 30 mol%. Pre-treatment with 15 mol% KC liposomes and with 30 mol% KC liposomes resulted in progesterone flux increases of 2.4-fold and 2.1-fold, respectively. In both cases there was a significant difference. 60 mol% PH liposomes even had a small negative influence on progesterone permeation. Results of the diffusion experiments indicated that there could be an interaction with lipid components of the stratum corneum.

Therefore, differential scanning calorimetry (DSC) experiments were conducted to verify this interaction. DSC techniques have been used to evaluate the role of lipid and protein thermal transitions in stratum corneum barrier properties (Potts, 1989; Wertz and Downing, 1989). Treatment of skin with the fatty acid *cis*-9-octadecenoic acid resulted in a shift of the lipid associated transitions consistent with lipid disruption (Golden et al., 1987a; Golden et al., 1987b). Porcine stratum corneum samples were also investigated by DSC techniques. The results obtained were remarkably similar to thermal profiles of human samples. This may be due to the similar lipid composition (Golden et al., 1987a). In these experiments it was proposed that the transitions occurring near 60, 75 and 100 °C, in hydrated samples, were due to intercellular lipid, protein intercellular lipid and keratin respectively (Knutson et al., 1985; Golden et al., 1987b). DSC results showed that water can have a large effect. In particular, results show that at concentrations up to about 30%, water is bound to the stratum corneum, whereas at higher concentrations free water is observed (Inoue et al., 1986). As water increased up to 30%, the lipid phase transition temperature decreases by 5–10°C, remaining constant with further increase of water content.

We selected the peak near 75°C, which belongs to the lipid transition, to compare the different treated skin (Potts, 1989). Changes in DSC–thermal profiles and progesterone flux through porcine skin were measured for samples treated with PH–PC-liposomes (30 mol%), KC–PC-liposomes (15 mol%), and untreated porcine skin as controls, respectively. Skin pieces showing the highest progesterone flux after 50 h diffusion served as samples. Therefore we used pig skin impregnated with 30 mol% phloretin or 15 mol% ketocholestanol, respectively. The DSC profiles are compared in Fig. 4. Treatment with KC as well as PH resulted in a pronounced shift to lower temperature. As can be seen in Table 2, the maximal thermal transition temperature ($T_m$) is shifted from about 79°C to 71°C after treatment with KC, and to 73°C after treatment with PH. This indicates an interaction with the skin lipids. As seen with other penetration enhancers, like monounsaturated acids, the findings also suggest that incorporation of PH or KC into stratum corneum results in a decreased lipid order. This is partly confirmed by recently published DSC-studies with artificial multilamellar vesicles (DMPC), where PH in its neutral form strongly decreased the lipid phase transition temperature and slightly reduced the co-operativity of the phase transition within the lipid bilayer (Cseh et al., 2000). The shift in these multilamellar vesicles was more than 10°C and depended on the pH. In these studies it has been clearly demonstrated that PH decreases the phase transition temperature of lipid membranes. This effect is dependent on the concentration of its neutral form in the lipid phase.

## 4. Conclusions

More studies will have to be performed to investigate the possible mechanisms of interaction of these potential permeability modulators. Both KC and PH have a permeability modifying effect on progesterone permeation through porcine skin, whereas the effect on skin caused by KC is higher. The fact that the effects are on a neutral permeant is suggestive of interactions between PH and KC and structured lipids in the skin. This was confirmed using DSC. Both compounds are lipophilic. PH has a logP of 3.5, and KC about 8. Clearly the latter is extremely lipophilic and is difficult to formulate such that it is dermally available. The

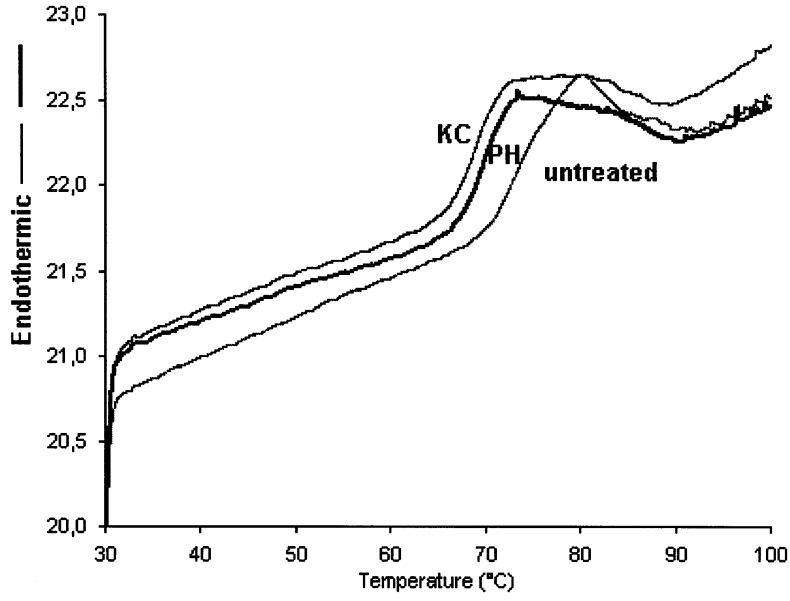



Fig. 4. DSC thermal profiles for porcine stratum corneum. Curves are labelled "KC"-treatment with KC–PC-liposomes; "PH"-treatment with PH–PC-liposomes; and "untreated"-no treatment. Gravimetric analysis of the samples prior to DSC experiments showed an average water content of about 69% (w/w).

Table 2
Thermal changes following treatment of porcine skin with PH–PC-liposomes (PH); KC–PC-liposomes (KC) and untreated[a]

| Treatment | DSC $T_m$ °C |
|---|---|
| PH | $73.4 \pm 1.5$ |
| KC | $71.4 \pm 0.5$ |
| No treatment | $79.6 \pm 0.7$ |

[a] Temperature of the transitions maximum $T_m$, $n = 4$

lipophilicities suggest that if the molecules do permeate the skin they will be incorporated into the structured bilayer domains. The formulation of KC in the liposomes and its subsequent incorporation into the skin suggests that this formulation approach may be useful in promoting the uptake of highly lipophilic species into the stratum corneum.

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