

# PRELIMINARY STUDY WITH PHENOL RED TO EVALUATE THE INTEGRITY OF HUMAN EPIDERMIS IN *IN VITRO* PERMEATION EXPERIMENTS

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## Resumen / Abstract

Se estudia la posibilidad de utilizar rojo fenol como marcador de la integridad de la piel en los ensayos *in vitro* de absorción percutánea. Para ello se realizan ensayos, utilizando piel intacta y piel dañada, en los que se compara la permeación de rojo fenol con otra molécula (aciclovir) de referencia o control. Los resultados obtenidos en este estudio preliminar, apoyan la hipótesis planteada, aunque en cualquier caso deberá confirmarse en estudios posteriores.

*The potential of the Phenol Red (PR) as a marker of skin integrity was studied. The *in vitro* permeability of both, acyclovir (ACV), as a model hydrophilic permeant, and PR were determined on intact and damaged skin. Although the results obtained seem to support this hypothesis, further studies are needed to confirm the utility of PR as skin integrity marker.*

## Introduction

*In vitro* permeability studies are a useful tool for assessing the potential of skin as a route for drug delivery, and when evaluating the potential systemic exposure of test penetrants following cutaneous application. It is thus necessary to control the *in vitro* conditions and minimize, where possible, artefacts (presence of the dermal layer, 1) that may not be representative of the *in vivo* situation. One of the main concerns is to guarantee the epidermal layer integrity that can be damaged during preliminary handling (i.e. dermis removal, 2) or storage (i.e. freezing of skin samples)

Two different strategies are described to accomplish this objective: physical methods and the use of marker compounds. Considering the first type, two methods have been used, i. e. Transepidermal water loss measurement (TWL, 3) and Transcutaneous electrical resistance monitoring (TER, 4). Both have the disadvantage of the equipment requirement. The most common marker for skin integrity test is tritiated water ( $T_2O$ ), whose permeability coefficient ( $K_p$ ) is calculated over a number of hours (5). The main disadvantage of this method is its cost, due to the use of radioactivity.

Phenol red (PR) is generally used as non absorbable marker in *in vitro*, *in situ* and *in vivo* absorption experiments and also has been proposed for assessing the development of epithelial confluence and tight junction formation in epithelial cell cultures (6). A comparative study on serial measurements of transepithelial electrical resistance (TEER) and permeability values of PR showed that an increase on monolayer permeability to PR was associated with a decrease in TEER, that is to say, an increased permeability goes together with decreased integrity (7).

The purpose of this study was to assess the potential of the PR as a marker of skin integrity. The *in vitro* permeability of acyclovir (ACV), as a hydrophilic permeant model, and this of PR have been characterized in several skin sample types. Those include fresh excised skin, skin frozen for 3 months and intentionally damaged skin.

## Materials and Methods

### Material

PR was purchased from Scharlau Química S.A. (Barcelona, Spain) and ACV from Sigma-Aldrich-Química S.A. (Madrid, Spain). All other chemicals were of high-performance liquid chromatography (HPLC) analytical grade.

### In vitro permeation experiments

All permeation experiments were performed on Caucasian abdominal skin samples (female aged 38-48 years), obtained from cosmetic surgical corrections. Excess fatty and connective tissues were removed. Epidermal membranes were prepared by a heat-separation technique (2). The skin was immersed in water at 60° C for 45 s and at this moment the epidermis was carefully separated from the underlying dermis

Three sets of experiments were carried out: In one of them fresh human epidermis was used. In the second set, frozen epidermal membranes for a storage period of 3 months were employed. In the third set, fresh epidermal membranes were intentionally damaged (8) by placing holes (5 per exposed area) using a Terumo® 25-G needle (Terumo Medical Corporation, Elkton, MD), and then used in permeation experiments.

In vitro permeation studies were carried out using unjacketed Franz diffusion cells. Four to six diffusion experiments were performed on every condition, and six cells were mounted at a time. The receptor compartment of the cells was filled with an isotonic solution (phosphate buffer pH 7.4). Then human epidermis was mounted with dermal-side in contact with receptor phase. Subsequently, a mixture of 800 µl of ACV solution (1 mg.mL<sup>-1</sup>) and 200µl of a PR solution (0.5 mg.mL<sup>-1</sup>) was applied in donor compartment and covered with parafilm to prevent evaporation of solvents. Samples (100 µl) were withdrawn at specified intervals from receptor compartment followed by replacement with fresh receptor solution. Quantification of the test compounds in the samples was done by HPLC with a Lichrosorb® RP-8 (250x4.0mm, 10µm). The chromatographic conditions for ACV and PR are detailed in Table 1.

**Table 1.** Chromatographic conditions employed for the analysis of ACV and PR.

Condition	ACV	PR
Mobile phase	Methanol : 1% acetic acid solution (pH=2.6) (10:90, V:V)	Methanol : PBS (pH=7.4) (55:45, V:V)
Flow rate	1 mL/min	1 mL/min
Injection volume	40 µL	40 µL
Detection	Fluorescence (λ excitation=266 nm; λ emission=350 nm)	UV (λ =546 nm)

The cumulative amount of drug permeated through of skin was plotted as a function of time. The linear steady-state expression (Eq 1) was used to fit experimental data (9):

$$Q_{(t)} = A \cdot P \cdot L \cdot C \cdot \left[ D \cdot \frac{t}{L^2} - \frac{1}{6} \right] \quad (\text{Eq 1})$$

where the symbols stand for: Q(t) is the quantity which passes through the membrane and reaches the receptor solution at a given time, t, A represents the actual diffusion surface area (0.78 cm<sup>2</sup>), P the partition coefficient of the permeant between the membrane and the donor vehicle, L the membrane thickness, D is the diffusion coefficient of the permeant in the membrane and C is the concentration of the permeant. The terms P · L and D/L<sup>2</sup> were replaced in Eq. 1 by P' (partition parameter) and D' (diffusion parameter), respectively, and estimated through fitting the theoretical equation to individual in vitro permeation data sets using a computerized non-linear squares method. The permeability coefficients, Kp (= P' · D'), were calculated and used as representative of each drug penetration process.

### Data analysis

Kp values were statistically analyzed by One-way ANOVA and all pairwise multiple comparisons Tukey test at P<0.05.

## Results and Discussion

The purpose of an integrity marker is to ensure that barrier properties of the tissue are maintained not only at the beginning but also during and at the end of the experiments. To assess the potential of the PR as a marker of skin integrity, measures of both ACV and PR values that crossed the membrane versus time were determined.

The permeability profiles of two compounds through fresh, frozen skin for storage (3 months) and damaged epidermis are shown in Figure 1, 2 and 3, respectively. The permeability coefficients from steady state ( $K_p$ ) obtained are summarized in Table 2.  $K_p$  values for ACV on fresh skin are in the same range than those obtained in previous works (10). As can be seen, the loss of the skin integrity increased  $K_p$  values of both de drug ACV and the marker PR.

The cumulative amounts, as a percentage of applied dose of PR (1, 6 and 24 h) for fresh, frozen for storage, and intentionally damaged tissue were also compared (Figure 4). The permeation of PR at 24 h through intact epidermis is 0.71%, which represents a PR concentration of  $1.2 \cdot 10^{-4} \text{ mg}\cdot\text{mL}^{-1}$  in the receptor compartment at the end of experiment (non-visual color). This value can be taken as a reference point to reject any diffusion cells with higher PR concentration. This limit could be meant as an index of the losing of the membrane integrity.

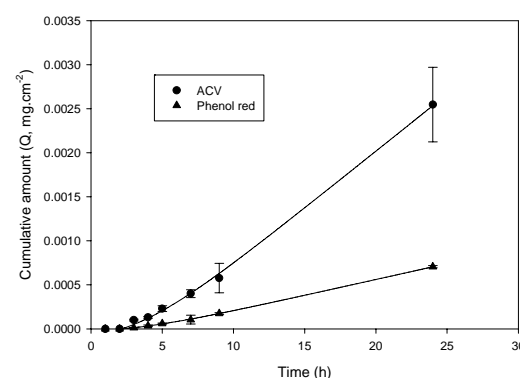
On the other hand, taking into account that PR can be detected visually in the receptor compartment (PBS, pH 7.4) when the concentration is around of  $0.001 \text{ mg}\cdot\text{mL}^{-1}$  this marker can be easily used to quickly reject a particular diffusion cell because of integrity concerns. So that, PR can be considered advantageous in front of another markers (i.e. caffeine).

**Table 2.** Permeability coefficients ( $K_p$ ) for ACV and PR through fresh intact, frozen and intentionally damaged epidermis<sup>a</sup>.

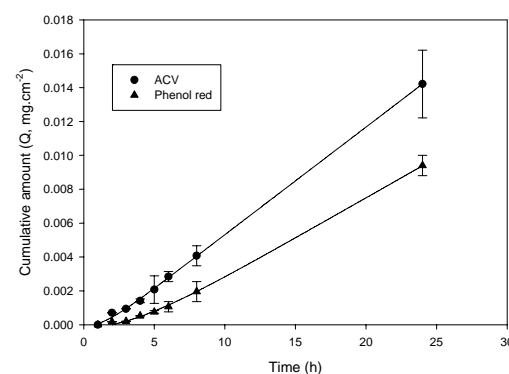
Compound/ Condition	Permeability coefficients ( $K_p \cdot 10^{-4}$ )	
	ACV	PR
Intact epidermis	1.14 (0.09)	0.06 (0.02)
Frozen epidermis	6.29 (0.07)	8.72 (0.34)
Damaged epidermis	27.49 (0.04) <sup>b</sup>	144.1 (10) <sup>b</sup>

<sup>a</sup> Data expressed as mean  $\pm$  SD (n=4-6).

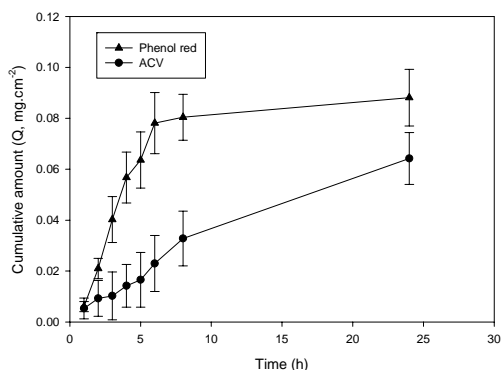
<sup>b</sup> Values of permeability from 0 to 6 h



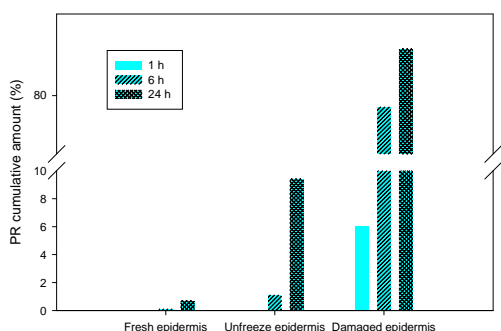
**Figure 1.** Permeation of ACV and PR through intact epidermis. Data expressed as mean  $\pm$  SD (n=4-6).



**Figure 2.** Permeation of ACV and PR through epidermis (frozen three months). Data are presented as mean  $\pm$  SD (n=4-6)



**Figure 3.** Permeation of ACV and PR through intentionally damaged epidermis. Data expressed as mean  $\pm$  SD (n=4-6).



**Figure 4.** Comparison of PR cumulative amount (%) through fresh, frozen for storage (three months) and intentionally damaged epidermis (n = 4-6)

In conclusion, the use of PR as marker seems to be an easy and inexpensive method for assessing the barrier function of the epidermal membrane in light of the results obtained in this preliminary study.

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