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Note

The "in vitro" percutaneous penetration of three antioxidant compounds

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Abstract

Caffeic acid, chlorogenic acid and oraposide, a natural glycoside, are phenyl-propanoid compounds. These natural products have been reported to have antioxidant activities such as the scavenging of superoxide anions and hydroxyl radicals. These compounds could be used in the dermocosmetic field to protect the skin from oxidative stress induced by UV radiation. To this end, the permeation of caffeic acid, chlorogenic acid, and oraposide, through pig-ear skin was evaluated in vitro. The percutaneous permeation of these three compounds through pig skin was measured and compared using Franz diffusion cells. At appropriate intervals, up to 72 h, diffusion samples were analyzed using an HPLC assay. After 48 h of drug contact the permeation was also evaluated with a fluorescent microscope on vertical microtomed pig skin sections. In this study on excised pig skin, the flux value was found to be equal to 0.32 and 0.48 $\mu g\,cm^{-2}\,h^{-1}$ for caffeic and chlorogenic acids, respectively; for oraposide the levels were below the limit of detection and the flux was not evaluated. These results were corroborated by fluorescent microscopy. Caffeic and chlorogenic acids were found in all skin sections, and these might represent a systemic activity, whereas oraposide remained in the upper superficial layer of the skin. This latter phenomenon seems to be interesting for dermocosmetic applications.

Keywords: Antioxidant; Polyphenol; In vitro; Skin permeation

1. Introduction

Cumulative and prolonged exposure to ultraviolet radiations are now recognized to induce deleterious reactions in human skin, including cutaneous ageing, immunosuppression, photo-carcinogenesis and various inflammatory skin disorders (Gilchrest, 1996; Sailstad et al., 2000; Rittié and Fischer, 2002). UVB and UVA are proved to generate DNA damage directly and indirectly through oxidative stress, by increasing the level of reactive oxygen species (ROS) (Nazim et al., 1999; Hattori et al., 1997). Compounds such as phenolic acids can scavenge oxidative radicals, decrease lipid peroxidation and thus reduce the possible damage. Several studies concerning topically applied antioxidant drugs have recently confirmed this fact and it was demonstrated that photo-damage decreased when skin was supplemented with antioxidants (Jurkiewicz et al., 1995; Jin-Yin Lin et al., 2003). Plants also have to defend themselves from UV radiations and the most obvious candidates for total antioxidant outside those naturally present in the body could be plant antioxidants. In this context, topical administration of natural antioxidants from plants could provide an efficient procedure to increase the endogenous cutaneous protection system. Caffeic and chlorogenic acids are compounds that widely exist in fruits and in vegetables (Fig. 1). Oraposide is a natural glycoside extracted from *Orobranche rapum genistae*, and studied by Andary (1975). This polyphenolic compound is a phenyl-propanoid glycoside derivative reported to also have antioxidant properties. Therefore, these products could be good candidates for successful use as protective agents against photo-oxidative damage. However, for this, two conditions are required: the candidates should permeate through the *stratum corneum*, and reach the deeper cutaneous layers, without significant leakage into the systemic route.

The aim of the present study was to determine the in vitro percutaneous penetration across excised pig skin of three natural antioxidant compounds: caffeic acid, chlorogenic acid and oraposide and to evaluate their kinetic permeation profiles.

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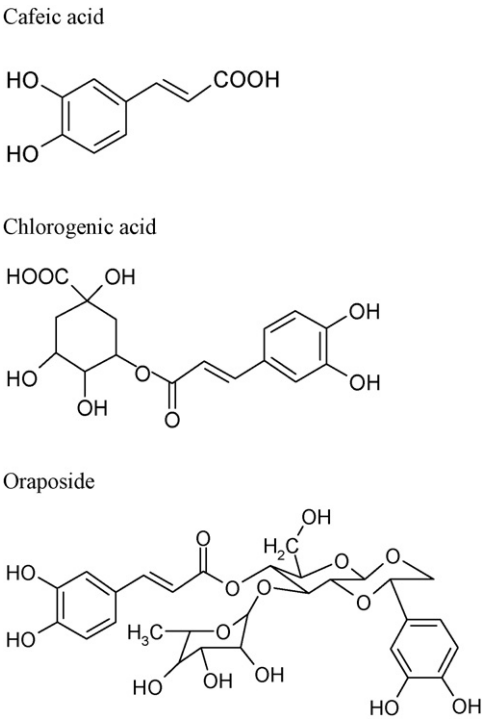

Fig. 1. The chemical structures of the phenyl-propanoid compounds.

2. Material and methods

2.1. Chemicals

Chlorogenic acid and caffeic acid were purchased from Sigma Chemical Company (USA). Oraposide was kindly provided by Professor C. Andary (Laboratoire de Botanique, Phytochimie, Mycologie, UM1, Montpellier, France). *O. rapum genistae* is a broom parasite belonging to the Orobranchacae family. Oraposide was isolated according to the usual methods for extraction and purification (Andary, 1975) in this field. Ethoxydiglycol (Transcutol® Gattefossé, France) and propylene glycol (Prolabo, France) were used as vehicles. Methanol and acetonitrile were HPLC grade (Sigma–Aldrich, France). All commercially obtained chemicals were used as received without further purification. Working solutions for the HPLC assay of the phenolic compounds were first prepared in methanol and then by dilution in water.

2.2. Skin preparation

Porcine ears (three different donors) were obtained from freshly killed animals in a local slaughterhouse (Nîmes, France). After cleaning with cold tapwater, full-thickness, non-dermatomed skin (about 1200–1500 μm; measured with a Mitutoyo 20505 Apparatus) was removed with a scalpel from the cartilage of the outer region. The subcutaneous fat was then removed. Only intact skin discs with an internal diameter of 3 cm were kept and sealed in plastic bags then stored at −20 °C until ready for use, for a period not exceeding 6 weeks.

Pig skin is used because it has similar permeation characteristics to those of human skin. The density of hair follicles in human and porcine skins has already been determined by Bronaug et al. (1982) and has been found similar ($11 \pm 1\ cm^{-2}$). However, the mean diameter is different 97 ± 3 and 177 ± 4 μm, respectively, for human and pig hair follicles. Full-thickness skin is the general rule with pig skin in dermal absorption specifically for the study of cosmetic ingredients, as it is technically more difficult to obtain intact split-thickness skin (SCCNFP, 2003).

2.3. Diffusion study

The permeation behavior of each compound was examined "in vitro" using a glass Franz diffusion cell (Legallais, France) with a surface area available for diffusion of 1 cm^2.

2.3.1. Donor compartment

Fifty microlitre of test formulations [2% drug in transcutol–propylene glycol (1:1, v/v)] were placed on the skin surface in the donor compartment which was then sealed from the atmosphere with a plastic film (Parafilm).

The receptor chamber volume was approximately 10 ml, and the receptor solution (isotonic saline solution (9‰ NaCl, Versol, Aguettant) with 1% gentamycin (gentalline, Schering-Pough), was stirred at 600 ± 1 rpm with a stirring rod (Poly15, Variomag). The pH was measured as 7. The receptor solution was maintained at a constant temperature (37 ± 1 °C) by water circulation (Polystat CC1, Huber). In these conditions the skin disc temperature is 32 ± 1 °C which corresponds to the skin surface temperature in vivo (measured with Thermocouple thermometer, EcoScan).

2.3.2. Kinetic studies

All the receptor solutions were taken after 12, 24, 36, 48, 60 and 72 h and diluted with an appropriate quantity of fresh receptor medium. After each sample selection the Franz cells were filled with the same volume of receptor medium. Samples were kept at −20° until drug analysis.

2.3.3. Evaluation

The absorption of each test-penetrant was studied over a 72-h period and the steady-state flux was calculated by the application of linear regression to the diffusion profile.

Several studies have shown that percutaneous absorption can often be described using Fick's diffusion law, which states that flux J ($\mu g\ cm^{-2}\ h^{-1}$) at steady-state through a basic membrane is related to:

$$J = K_m D \frac{\Delta C}{h} = K_p C_0$$

in which C_0 is the drug concentration in the donor solution and K_m is the partition coefficient of the drug between the vehicle and the skin. The diffusion coefficient (D) is the effective diffusivity of the drug in the stratum corneum. K_p ($cm\ h^{-1}$) is the permeability of the drug through the stratum corneum at steady-state and is defined as $K_m D/h$, where h is the effective path length.

The flux values J at the steady-state were calculated from the slope of the linear segments of the permeation profile, and the permeability K_p (cm h^{-1}) equalled J divided by C_0.

2.4. High-performance liquid chromatography assay

Concentration of each drug in all diffusion samples were analyzed using an HPLC assay developed in our laboratory. Briefly, a Hewlett-Packard (HP) 1050 system, was equipped with a variable wavelength diode UV 6000 LP detector (set at 330 nm), and a C-8, reverse-phase column (Nucleosyl C8, 5 μm; 25 cm × 3 mm i.d., Germany), at 40 °C. The mobile phase was isocratic acetonitrile–water 2% acetic acid (18:80, v/v, for caffeic acid, 20:80 for chlorogenic acid and 10:90 for oroposide) with a flow rate of 0.5 ml min^{-1}. The injection consisted of 25 μl. The amounts of each compound in the receptor were calculated from standard curves of either authentic standards (caffeic and chlorogenic acids), or purified material (oraposide) at their UV maximum wavelength (330 nm). Under these chromatographic conditions, caffeic acid, chlorogenic acid and oraposide were eluted at 5.97 ± 0.025, 8.64 ± 0.073, and 7.38 ± 0.05 min, respectively.

2.5. Fluorescence microscopy

After 48 h of drug contact, vertical microtomed skin specimens (Cryocult 1800, Leica) were biopsied and cut (5 μm skin sections). Skin samples were embedded in a matrix consisting of polyvinyl alcohol and carbowax compounds (Tissue Tek®, Sakura Fitenek Europe) frozen at −80 °C and sectioned to 5 μm thickness. A conventional fluorescence microscope (Nikon Optiphot, excitation: 350 nm) with a 40× and 100× objectives was used to evaluate drug distribution in biopsied pig skin sections. Ten microlitre of Neu reagent (2-aminoethyl dipheny borinate 1% in methanol) (Neu, 1956) was used to differentiate and determine the caffeic ester function. Fluorescence images were then recorded.

3. Results

A linear relationship was obtained between response (y) and the corresponding concentration of each component (X). For caffeic acid $Y = -35.93 + 490.73X$ ($R^2 = 0.999$) and the limit of detection (LOD) (AFNOR-NF V03-110, 1998) was 0.2 μg ml^{-1}. For chlorogenic acid $Y = -3.23 + 277.48X$ ($R^2 = 0.999$) and the LOD was 0.01 μg ml^{-1}. For oraposide $Y = -7.72 + 126.78X$ ($R^2 = 0.999$) and the LOD was 1.2 μg ml^{-1}. The limit of quantification (LOQ) in each case corresponds to 3× the LOD value (AFNOR-NF V03-110, 1998).

Fig. 2 shows the cumulative drug amounts which permeated through excised pig skin. Six diffusion cell experiments were performed for each compound. The permeation parameters were calculated for each individual experiment. The mean values ± standard errors (S.E.) are given in Table 1. The flux value (J), as notified in Section 2.3, is equal to the slope of the linear permeation profile and was calculated from the experimental curves reported in Fig. 2. For caffeic and chlorogenic acids the steady-state flux was found to be equal to 0.48 ± 0.07 and 0.32 ± 0.06 μg cm^{-2} h^{-1}, respectively.

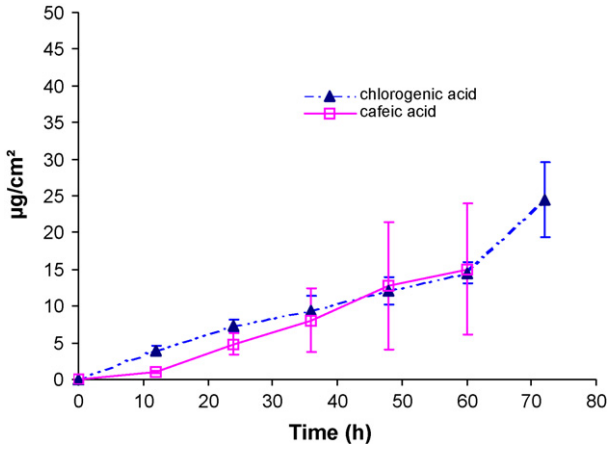

Fig. 2. Cumulative penetration profiles of phenyl-propanoid compounds through the porcine skin (50 μl of a 2% solution in transcutol/propylene glycol).

Extended studies were carried out for up to 60 or 72 h, but it was impossible to determine the flux value for oraposide as drug levels in the receptor compartment were always below the LOD. Although skin viability cannot be maintained in a diffusion cell for the full 72 h of an extended study, the diffusional barrier of the skin appears to remain intact (according to visual analysis: the membrane was neither torn nor damaged) and oraposide was always mainly located in the outermost layer of the skin.

Fluorescence studies were carried out because the HPLC method was not sensitive enough to determine the amount of drugs in each layer of skin. However, the fluorescence method seemed like a good alternative for specifically identifying and locating the drugs.

Analytical results were well corroborated by fluorescent microscopy on biopsied skins. Figs. 3–6 show fluorescent images obtained from mechanical sections after 48 h of treating skin samples. Images obtained with control pig skin, in which the solution applied was the mixture of trancutol–propylene glycol, did not show any fluorescence. An increase of fluorescence in skins treated with caffeic (Fig. 3) and chlorogenic acids (Fig. 4) can be clearly seen with an intense fluorescence in all layers of skin section.

It has been shown that tissues which accumulate these drugs emit a characteristic fluorescence. All the skin sections were fluorescent and mainly down inside the follicular orifice, indicating

Table 1
Physico-chemical parameters and diffusional parameters of three phenyl-propanoid compounds

Antioxidant compounds	MW	log $K_{o/w}$	J (μg cm^{-2} h^{-1})	K_p (cm h^{-1})
Oraposide	622	−1.3	nd	nd
Chlorogenic acid	354.3	−1	0.48 ± 0.07	2.4×10^{-3}
Caffeic acid	180.2	1.1	0.32 ± 0.06	1.6×10^{-3}

Diffusional parameters were determined through linear regression (kinetic study in Franz diffusion cell). log $K_{o/w}$ calculated with the KowWin Program (SRC's Logkow, 2005). nd: not determined.

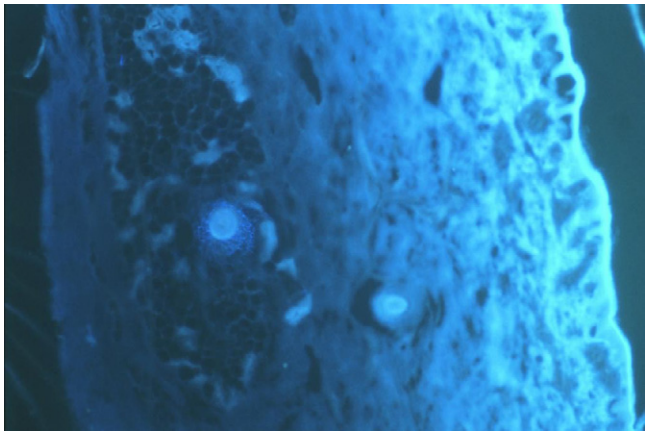

Fig. 3. Fluorescence microscopy of vertical slicing of porcine ear skin sections 48 h after application of caffeic acid (50 μl of a 2% solution in transcutol/propylene glycol). Sections were visualized through a 40× objective. Untreated skin was used to evaluate the intrinsic fluorescence of the tissue.

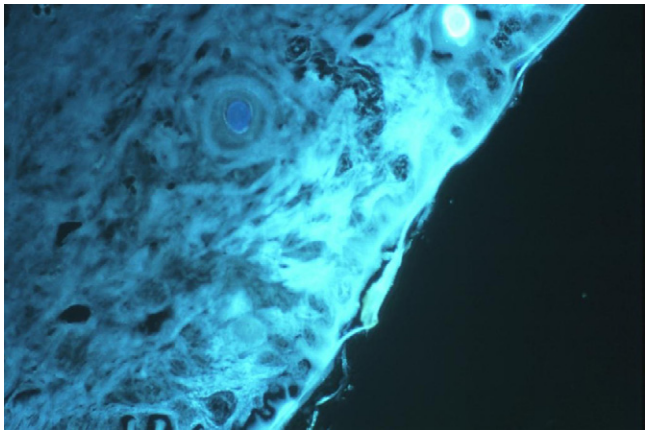

Fig. 4. Fluorescence microscopy of vertical slicing of porcine ear skin sections 48 h after application of chlorogenic acid (50 μl of a 2% solution in transcutol/propylene glycol). Sections were visualized through a 40× objective.

that the transport route is also through these regions of the skin. Moreover fluorescence is visible around skin structures such as corneocytes (Fig. 5).

On the other hand, the fluorescence from skin treated with oraposide appeared to be mainly located in superficial layers where the fluorescence was observed with a similar intensity. In Fig. 6 a fluorescent image of a skin surface biopsy is presented, which demonstrates that the fluorescent dye is only located in the upper part of the skin and not in further areas of the biopsy. The stratum corneum is the outermost layer of the skin, which constitutes the major barrier to percutaneous penetration, and after 48 h in this experiment, oraposide was still mainly located in the layer skin and in viable epidermal layer.

4. Discussion

Porcine skin has been shown to have similar histological properties to human skin, and is also considered as a good model for human skin permeability. Several in vitro penetration/absorption studies in human and pig ear skin yielded similar results (Hueber-Becker et al., 2004). In most cases the pH range of normal human skin is said to be between 5.4 and 5.9, and tissue pH levels deviate from a healthy range 7.0–7.40, and finally 7.30–7.40 in the blood. According to these data a pH 7 was chosen for all experiments and, at this pH, weak acidic drugs were mainly in the ionized form.

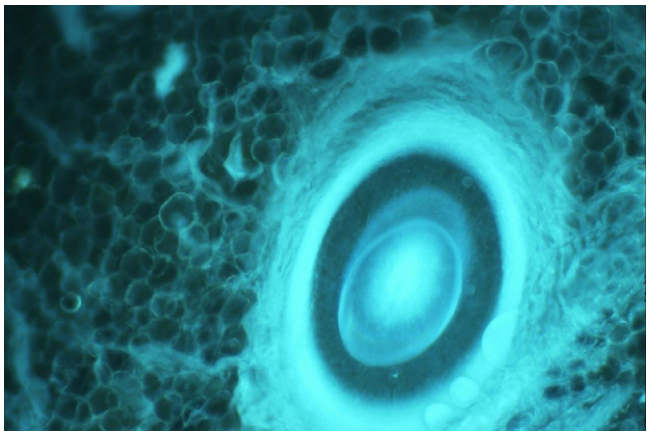

Fig. 5. Fluorescence microscopy of vertical slicing of porcine ear skin sections 48 h after application of chlorogenic acid (50 μl of a 2% solution in transcutol/propylene glycol). Sections were visualized through a 100× objective. Localization of penetrating chlorogenic acid into the hair follicle and around corneocytes.

As clearly evidenced by permeation profiles, flux values and visually by fluorescent microscopy, our findings show that chlorogenic and caffeic acids are able to permeate through the stratum corneum. Skin absorption of a drug is determined by its physico-chemical properties, in particular, molecular weight and lipophilicity which play the major role in the skin permeation process. The permeation requires both lipid and aqueous solubility with an optimal $\log K_{o/w}$; poor absorption is more likely when the $\log K_{o/w}$ is greater than 5 or lower than −1, and when the molecular weight is greater than 500. It appears that chlorogenic acid (molecular weight 354) with a $\log K_{o/w} = -1$ (log octanol/water partition coefficient estimates obtained by

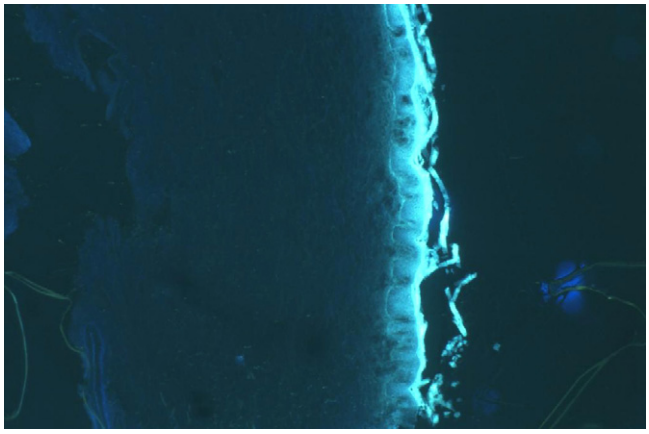

Fig. 6. Fluorescence microscopy of vertical slicing of porcine ear skin sections 48 h after application of oraposide (50 μl of a 2% solution in transcutol/propylene glycol). Sections were visualized through a 40× objective.

KowWin) (SRC, 2005) possesses a slightly superior percutaneous absorption compared to caffeic acid (molecular weight around 180) with a $\log K_{o/w}$ equal to 1.1. Saija et al. (2000) observed comparable results for two biphenols: ferulic and caffeic acids, where fluxes obtained at pH 7.2 were 1.45 ± 1.45 and $0.56 \pm 0.15\ \mu g\,cm^{-2}\,h^{-1}$, respectively. On the other hand, oraposide, with a molecular weight of 624.61 and a $\log K_{o/w}$ to approximately −1.3, seems to remain specifically in the superficial layer of the skin, therefore this antioxidant remains longer on the surface of the skin were it is intended to act.

Caffeic and chlorogenic acids cannot be considered as efficient penetrants like caffeine; for an application dose of 100 μg caffeine, in a multi-centre comparison study, fluxes across human skin were found to be $2.45 \pm 1.43\ \mu g\,cm^2\,h^{-1}$ (Van de Sand et al., 2004). In comparison, fluxes given in the literature and notified in the EDTOX database, for nicotine (EDETOX, 2005), a drug used very often in the form of a patch, which possesses a $\log K_{o/w}$ of 1.2 and a low molecular weight (162), are between 174 and 218 $\mu g\,cm^{-2}\,h^{-1}$ (human skin or pig skin).

One of the strategies for improving skin penetration of drugs is to use a solvent with enhancement properties in the formulation. In our study, 2% of the compounds were incorporated into a simple solution of diethylene glycol monoethylether (Transcutol®) and propylene glycol (50/50, w/w). These ingredients were used as solubilizing agents. Several studies have shown that these vehicles significantly increase the percutaneous penetration of drugs (Touitou et al., 1994; Ganem-Quintanar et al., 1997), and act as enhancers. An enhancer may increase the diffusion coefficient of the drug in the stratum corneum principally by disrupting the barrier, or can improve the partitioning between the formulation and the stratum corneum by altering the solvent nature of the skin membrane. The penetration enhancement effect of solvent on the drug is a complex phenomenon as described by Trottet et al. (2004). In this study it was demonstrated that if a drug does not have an aptitude for penetrating through the skin, such as the case with oraposide, the use of valuable enhancers does not significantly increase percutaneous penetration. This supports our earlier observation (Toutain et al., 2003) on verbascoside, another phenyl-propanoid glycoside, for which we obtained an extremely low flux $0.01\ \mu g\,cm^{-2}\,h^{-1}$ in similar experimental conditions. Verbascoside also stayed mainly in the first layer of the skin.

We consider it particularly interesting to use, at the end of the penetration study, a technique with which it is possible to visualise the depth of penetration by analysing histological skin sections in association with fluorescence. Just as for the visual analysis, the sections also show that the skin remains intact after 48 h of contact with the receptor medium. Similar techniques have recently been developed by Toll et al. (2004) with microspheres and by Schulz et al. (2002) for pigments used as sunscreens.

The use of a combined antioxidant-sunscreen formulation may prevent photo-aging and skin cancer development. Reduction of photo-damage in human skin by the topical application of an antioxidant drug could be an efficient way to enrich the endogenous cutaneous protection system. Guidelines for safe application of sunscreens are being continually reviewed, and if frequent applications of sunscreen formulations to minimize skin damage are recommended, we must add to these guidelines the fact that each compound must have limited systemic absorption and stay in the initial layers of the skin to act in that area.

In conclusion, in this study we have demonstrated limited percutaneous penetration, with no significant difference between caffeic acid and chlorogenic acid, and a localized absorption in the epidermal layer for oraposide.

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