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Use of solid phase extraction (SPE) to evaluate *in vitro* skin permeation of aescin

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The aim of this work was to evaluate the feasibility of assessing aescin *in vitro* permeation through human skin by determining the amount of aescin permeated using conventional HPLC procedures after extraction of skin permeation samples by means of solid phase extraction (SPE). Aescin *in vitro* skin permeation was assessed from aqueous solutions and gels using both Franz-type diffusion cells and flow-through diffusion cells. The SPE method used was highly accurate (mean accuracy 99.66%), highly reproducible (intra-day and inter-day variations lower than 2.3% and 2.2%, respectively) and aescin recovery from normal saline was greater than 99%. The use of Franz-type diffusion cells did not allow us to determine aescin flux values through excised human skin, therefore aescin skin permeation parameters could be calculated only using flow-through diffusion cells. Plotting the cumulative amount of aescin permeated as a function of time, linear relationships were obtained from both aqueous solution and gel using flow-through diffusion cells. Aescin flux values through excised human skin from aqueous gel were significantly lower than those observed from aqueous solution ($p < 0.05$). Calculating aescin percutaneous absorption parameters we evidenced that aescin partition coefficient was lower from the aqueous gel with respect to the aqueous solution. Therefore, the SPE method used in this study was suitable to determine aescin *in vitro* skin permeation parameters from aqueous solutions and gels using a conventional HPLC method for the analysis of the skin permeation samples.

1. Introduction

Aescin, a mixture of saponins derived from the seeds of *Aesculus hippocastanum* L., is widely used in topical pharmaceutical and cosmetic formulations for its anti-inflammatory and anti-oedema activity and its effects on the capillary permeability (Vogel et al. 1970; Przerwa and Arnold 1975; Rothkopf and Vogel 1976; Panigati 1992). However, aescin percutaenous absorption parameters, such as flux through the skin, permeability and diffusion coefficients, have been poorly investigated in humans likely due to aescin poor UV absorbance (Pietta et al. 1989; Kockar et al.1994; Apers et al. 2006) that does not allow its analytical determination by means of simple HPLC methods.
Recently, Apers et al. (2006) developed a new method to determine the aescin content in an herbal medicinal product based on purification of the sample by solid phase extraction (SPE) and analysis of the aescin content by densitometric thin-layer chromatography.
In recent years, SPE has emerged as a convenient extraction method whose application is facilitated by the availability of many commercial SPE columns. As reported in the literature (Fritz et al. 1995; Smith 2003), SPE offers many advantages compared to other conventional extraction methods since it is highly versatile and specific, reproducible, fast, and easy to perform.
In this work, we investigated the feasibility of evaluating aescin permeation through excised human skin by determining the amount of aescin permeated using conventional HPLC procedures after extraction of skin permeation samples by means of SPE.
Aescin *in vitro* percutaneous absorption parameters were assessed from aqueous solutions and gels using both Franz-type diffusion cells and flow-through diffusion cells. This last apparatus has been reported to provide a more reliable assessment of drug percutaneous absorption due to its ability to mimic the effect of blood flow through the skin by taking up and carrying away the permeated drug (Bronaugh et al. 1982).
An aqueous gel was chosen as vehicle to investigate aescin skin permeation since gel formulations are the most common aescin topical products on the market.

2. Investigations, results and discussion

2.1. Solid phase extraction (SPE)

Tests on the SPE column loading, the volume of solvents in the washing step, the volume and the composition of the elution solvents and the completeness of elution of aescin from the cartridges were previously performed in order to optimize the SPE experimental conditions. Aescin

recovery, obtained from normal saline using the SPE method described below, was 99.6 ± 0.5%. The full recovery of aescin from the SPE cartridge was confirmed by repeated elutions with methanol of the eluted cartridge that showed no remaining aescin.

When a known amount of aescin (100 μg) was added to preanalysed samples (100, 200 or 400 μg/ml) and assayed using this extraction method, the observed concentrations (199.8, 297.8 or 499.2 μg/ml) were very close to the expected concentrations (200, 300 or 500 μg/ml), pointing out that the method is highly accurate (mean accuracy 99.66%).

The intra-day and inter-day variations of the extraction method were expressed as coefficient of variation and were found to be lower than 2.3% and 2.2%, respectively, showing that the SPE method used was highly reproducible.

Similar results have been reported by Apers et al. (2006) using SPE as sample preparation step to determine the aescin content in an *Aesculus hippocastanum* dry extract by densitometric thin-layer chromatography.

2.2. *In vitro skin permeation experiments*

Aescin *in vitro* skin permeation was evaluated using the infinite dose technique, i.e. applying a large amount of formulation (500 mg) on the skin surface. Although the use of finite dosing more closely resemble "in use" situations, the use of an infinite dosing in *in vitro* experiments avoids drug depletion from the donor compartment during the experiment, thus ensuring a constant driving force for the permeation process and allowing the achievement of steady-state conditions.

Aescin percutaneous absorption parameters obtained from aqueous solutions using flow-through diffusion cells and Franz-type diffusion cells are listed in Table 1. Aescin mean flux values at steady state from aqueous solutions were found to be 3.93 ± 0.67 μg/cm^2/h using flow-through diffusion cells. Preliminary experiments showed that the flow rate of the receiving phase did not affect aescin flux values.

As shown in Table 1, the use of Franz-type diffusion cells did not allow us to determine aescin flux values through excised human skin. This difference could be explained by taking into account the different receptor phase volume used for determining the amount of drug permeated. Since the receptor phase volume withdrawn from Franz cells was very small (100 μl), the sensitivity of the analytical method (12 μg/ml) did not allow us to detect aescin in the receiving phase until 20–22 h from the beginning of the experiment and no flux could be calculated under these conditions. Therefore skin permeation results obtained using Franz cells were expressed only in terms of cumulative amount permeated after 24 h (Q_{24}) and as percentage of the applied dose permeated through the skin (% dose). Comparing aescin Q_{24} values and % dose obtained using Franz cells and flow-through diffusion cells no significant difference was observed ($p > 0.05$). As shown in Table 1, the percentage of aescin permeated with respect to the dose applied on the skin surface was high (18% for flow-through diffusion cells and 19% for Franz cells). These results are in good agreement with those reported by Lang (1977) who found similar amounts after application of an aqueous solution of radiolabeled aescin on animal skin.

Since most common topical aescin containing products on the market are aqueous gel formulations, we thought it to be useful to evaluate aescin skin permeation parameters from this type of vehicle. The composition and the percentage of aescin used to prepare the aqueous gel tested in this study were similar to those of commercially available products.

Plotting the cumulative amount of aescin permeated as a function of time (Fig.), linear relationships were obtained from both aqueous solution and gel ($r^2 = 0.992$ and $r^2 = 0.993$, respectively) using flow-through diffusion cells. Similarly to *in vitro* aescin skin permeation experiments from aqueous solution, no flux could be calculated from aqueous gel using Franz diffusion cells and skin permeation results were expressed only in terms of Q_{24} and % dose.

As shown in Table 2, aescin flux through excised human skin and Q_{24} values from aqueous gel were significantly lower than those observed from aqueous solution ($p < 0.05$). Aescin lower skin permeation from aqueous

Table 1: Aescin skin permeation parameters from aqueous solutions using flow-through diffusion cells (FTC) and Franz cells

Parameter	FTC	Franz cell
Flux ± S.D. (μg/cm^2/h)	3.93 ± 0.67	N.D.[a]
Lag time (h)	0.73	N.D.
Q_{24} ± S.D.(μg/cm^2)	94.32 ± 16.05	96.77 ± 21.15
K_p (cm/h)	7.86×10^{-3}	N.D.
D_{app} (cm^2/h)	6.39×10^{-7}	N.D.
K	20.65	N.D.
% dose	18.86	19.34

Parameters: flux, lag time, cumulative amount permeated after 24 h (Q_{24}), permeability coefficient (K_p), apparent diffusion coefficient (D_{app}), partition coefficient (K) and percentage of applied dose absorbed through excised human skin (% dose)

[a] N.D. = not detectable under the experimental conditions used

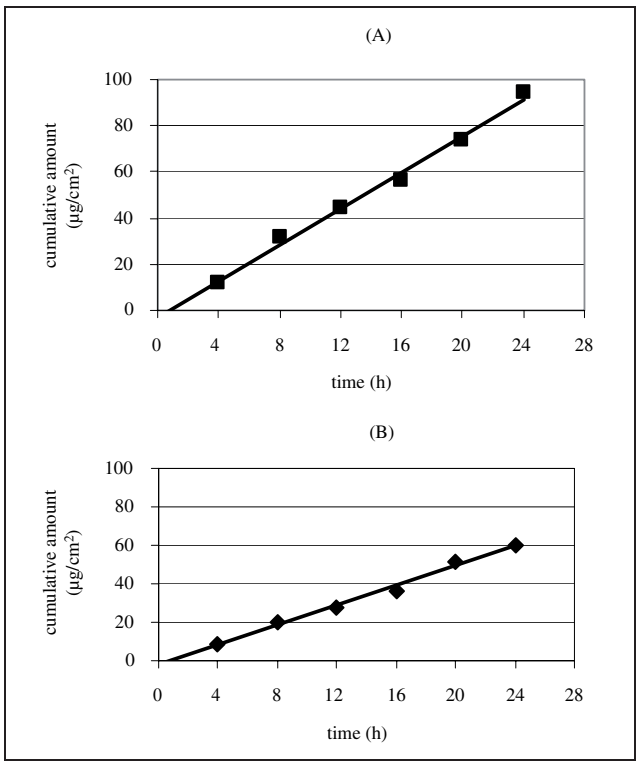

Fig.: Cumulative amount of aescin permeated through excised human skin as a function of time determined using flow-through diffusion cells. (A) aescin skin permeation from aqueous solution; (B) aescin skin permeation from gel formulation

Table 2: Aescin skin permeation parameters from an aqueous gel using flow-through diffusion cells (FTC) and Franz cells

Parameter	FTC	Franz cell
Flux ± S.D. (μg/cm^2/h)	2.56 ± 0.51	N.D.[a]
Lag time (h)	0.85	N.D.
Q_{24} ± S.D. (μg/cm^2)	60.26 ± 12.06	68.43 ± 18.55
K_p (cm/h)	5.12×10^{-3}	N.D.
D_{app} (cm^2/h)	5.49×10^{-7}	N.D.
K	15.66	N.D.
% dose	12.05	13.69

Parameters: flux, lag time, cumulative amount permeated after 24 h (Q_{24}), permeability coefficient (K_p), apparent diffusion coefficient (D_{app}), partition coefficient (K) and percentage of applied dose absorbed through excised human skin (% dose)
[a] N.D. = not detectable under the experimental conditions used

gels could be attributed to its slower release from the gel vehicle or to different drug/vehicle/skin interactions. Preliminary *in vitro* release study showed that aescin release rates from the aqueous solution and the aqueous gel tested were similar and were about 10-fold higher than aescin flux through excised human skin (data not shown). These results suggest that skin permeation could be regarded as the rate-limiting step in the percutaneous absorption of aescin. Similar findings have been previously reported studying *in vitro* drug release from aqueous gel formulations (Bonina and Montenegro 1994). Although Cross et al. (2001) observed that *in vitro* drug skin permeation could be affected by the viscosity of the vehicle depending on the use of finite or infinite dosing, in our experiments the greater viscosity of the gel formulation (60.000 cps) could not be regarded as a key factor in determining the different aescin flux values observed since aescin release rates from both aqueous vehicles were similar.

In order to elucidate the mechanism by which the gel formulation tested decreased aescin skin permeation, percutaneous absorption parameters such as permeability coefficient (K_p), apparent diffusion coefficient (D_{app}) and partition coefficient (K) were calculated for both the aqueous solution and the aqueous gel tested. As shown in Tables 1 and 2, no significant difference was observed comparing aescin K_p and D_{app} values from aqueous solutions and gels while its partition coefficient was lower from the aqueous gel with respect to the aqueous solution. These results suggest that interactions between aescin and the polymer forming the gel network could affect aescin ability to partitioning from the vehicle into the stratum corneum, thus decreasing its permeation through the skin. Interactions between drug and vehicle resulting in a decreased drug skin permeation have already been described for formulations containing gel-like structures (Barry 1993).

Further studies are needed to better elucidate the effects of gel composition on aescin *in vitro* skin permeation.

2.3. Conclusions

The SPE method used in this study allows us to determine aescin *in vitro* percutaenous absorption parameters from aqueous solutions and gels by analyzing with a conventional HPLC procedure the skin permeation samples obtained from flow-through diffusion cells. Further studies are planned in order to evaluate the possibility of using SPE methods to investigate skin permeation parameters of aescin after its topical administration in humans.

3. Experimental

3.1. Materials

Aescin was obtained from Acef (Milan, Italy). Carbopol 934 was supplied by Biochim (Milan, Italy). Acetonitrile and water used in the HPLC procedures were of LC grade and were purchased from Merck (Darmstadt, Germany).

The cartridges used for solid phase extractions (Supelclean LC-18) were obtained from Supelco Inc. (Bellafonte, USA). All other reagents were of analytical grade.

3.2. Preparation of aescin aqueous gel

Aescin aqueous gel was prepared by dispersing 0.8% w/w Carbopol 934 in water containing 1.0% w/w aescin with constant stirring. The dispersion was then neutralized and made viscous by the addition of 0.9% w/w triethanolamine. The gel was stored at room temperature for 24 h under air-tight conditions prior to use. The viscosity of the gel was measured using a Brookfield RVT viscosimeter equipped with a spindle n° 7 rotating at 20 rpm. Samples of the gel were left to settle over 30 min at the assay temperature (32 °C) before measurements were taken.

3.3. Solid phase extraction (SPE)

An extraction procedure similar to that described by Apers et al. (2006) was used. SPE cartridges were conditioned by flushing them twice with 1 ml of methanol and twice with 1 ml of water before being used for extraction. The sample containing aescin was passed through the cartridge and then the cartridge was washed with 2 ml of water, sucked dry and eluted three times with 0.5, 0.5 and 0.3 ml of methanol respectively. The eluate was evaporated to dryness under nitrogen at 30 °C and reconstituted with 500 μl of methanol. Amounts of the reconstituted sample were assayed by HPLC for quantitative evaluation of aescin.

The SPE method was validated for extraction recovery, accuracy and precision. The recoveries of aescin from normal saline containing a known amount of aescin were calculated by comparing peak areas at low, medium and high concentration levels with those obtained from the analysis of corresponding standard dilutions injected into HPLC directly.

The accuracy of the method was investigated by means of a recovery experiment, by adding a known amount of aescin to normal saline containing different amount of aescin. The drug-spiked samples were prepared freshly on three different times of the same day and on three different days, and extracted using the SPE method described above.

3.4. HPLC analysis

The HPLC apparatus consisted of a Hewlett-Packard model 1050 liquid chromatograph (Milan, Italy), equipped with a 20 μl Rheodyne 7125 injection valve (Rheodyne, Cotati, Ca, USA) and an UV-VIS detector. The chromatographic analyses were performed on a Lichrosphere® 100 C_{18} RP column (particle size 5 μm; 250 × 4 mm I.D.; Merck, Darmstadt, Germany), equipped with a 5 μm Lichrosphere® 100 C_{18} RP guard column (4 × 4 mm I.D.; Merck Darmstadt, Germany), and eluted isocratically at room temperature. The mobile phase consisted of a 37 : 63 (v/v) mixture of acetonitrile/water containing 0.1% trifluoroacetic acid (pH 2.2). Drug detection was carried out at λ_{max} 210 nm and the flow rate was set at 1 ml/min. The calibration curve for quantitative evaluation of aescin was linear in the range 62.5–500 μg/ml ($r^2 = 0.9999$). The lower limit of HPLC drug quantification was found to be 12 μg/ml.

3.5. In vitro skin permeation experiments

Samples of healthy adult human skin (mean age 35 ± 8 years) were obtained from abdominal reduction surgery. Membranes of stratum corneum and epidermis (SCE) were prepared in accordance with the method reported by Kligman and Christophers (1963). Briefly, subcutaneous fat was removed and the skin was immersed in distilled water at 60 ± 1 °C for 2 min. SCE samples were stored and assessed for the barrier integrity as previously described (Bonina and Montenegro 1992).

Skin permeation of aescin was measured using both Franz cells and flow-through diffusion cells (LGA, Berkeley, CA) by applying 500 μl of aescin aqueous solution (1 mg/ml) or 500 mg of aescin aqueous gel (1% w/w) on the skin surface.

Franz type diffusion cells had a diffusion surface area of 0.75 cm^2 and a 4.5 ml receiving chamber which was filled with normal saline. The receiving phase was constantly stirred (700 rpm) and thermostated at 35 °C so as to maintain the membrane surface at 32 °C. Samples of the receiving solution (100 μl) were withdrawn at intervals and analyzed for their aescin content by the HPLC method described above. The sample volumes were replaced with normal saline pre-thermostated at 35 °C.

The flow-through diffusion system had a receiving chamber volume of 3.0 ml and the area available for diffusion between compartments was 1.0 cm^2. The receiving chamber was kept at 35 ± 1 °C and the receptor

phase was stirred during the experiments. Normal saline was pumped into the receiving chamber at a flow rate of 18 ml/h. Using flow-through diffusion cells, the effluent from the receiving chamber was collected in flasks using a fraction collector at 4 h intervals over 24 h. Each solution sample collected over a 4 h interval was extracted by SPE and the eluate was analyzed by HPLC as described above in order to determine the amount of aescin permeated.

Each skin permeation experiment was made in triplicate.

3.6. Data analysis

Aescin fluxes through excised human skin were calculated using linear regression analysis, by plotting the cumulative amount of aescin permeated against time and dividing the slope of the steady state portion of the graphs by the area of the skin surface through which diffusion took place. The lag time (t_L) was calculated from the x-intercept values of the regression lines.

The apparent diffusion coefficient of a compound that permeates through the skin can be estimated from the lag time values according to the following Eq. (1) (Flynn et al. 1974):

$$D_{app} = \frac{h^2}{6\,t_L} \quad (1)$$

where h is the barrier thickness. Since the stratum corneum is the main rate-limiting barrier, h value is 16.8 μm for human skin (Bronaugh et al. 1982). The permeability coefficient (K_p) can be calculated by dividing the flux value by the donor phase concentration of the drug. Therefore, the SC/vehicle partition coefficient (K) can be calculated indirectly from the following Eq. (2):

$$K = \frac{K_p \cdot h}{D_{app}} \quad (2)$$

Statistical analysis of the data was performed using Student's t-test.

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