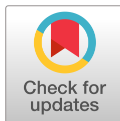

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Research Communication

A comparison of the *in vitro* permeation of 3-O-ethyl-l-ascorbic acid in human skin and in a living skin equivalent (LabSkin™)

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The EU Scientific Committee on Consumer Safety (SCCS) and the Cosmetic Ingredient Review (CIR) Expert Panel have listed a number of requirements for safety evaluation of cosmetics (1-3). These include data on the percutaneous absorption of selected cosmetic ingredients, such as actives, colouring agents, UV filters and preservatives. For such studies, the European Centre for the Validation of Alternative Methods (ECVAM), the European Medicine Agency (EMA) and the UK National Centre for the 3Rs (NC3Rs) have encouraged the replacement of animal testing with alternative *in vitro* models (4-6). In 2003, a ban on *in vivo* animal testing for cosmetic products was introduced by Directive 2003/15/EC of the European Parliament and of the Council, and became law a few years later with the Cosmetics Regulation (EC) No. 1223/2009 (1). Currently, percutaneous absorption of cosmetic ingredients may be determined in human or porcine skin *in vitro* according to procedures described by the Organisation for Economic Co-operation and Development (OECD), SCCS and COLIPA - The European Cosmetic and Perfumery Association (1, 7-9). The use of porcine tissue offers advantages for routine permeability testing given the financial and safety considerations associated with sourcing human tissue. However, this testing still requires consideration of ethical and animal welfare issues. Consequently, various human skin equivalents (HSEs) have been developed as alternative sources of tissue for skin permeation experiments (10).

HSEs are constructed by culturing human keratinocytes and fibroblasts and are designed to comprise either only the epidermis or both the dermis and epidermis (full thickness HSEs) (11). The use of HSEs for *in vitro* testing is generally encouraged by COLIPA (12) and several research studies have shown that these models may be useful for examining the effects of cosmetic products on skin hydration, or protein and lipid synthesis (13-16). The use of HSEs has also been included in OECD guidance for the conduct of skin absorption experiments (17). Accordingly, several studies in the literature have attempted to

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evaluate different types of HSEs, representing either human epidermis (Episkin®, EpiDerm®, Skinethic®) (18-23) or full-thickness skin (Phenion®) (24). The barrier properties of the HSEs have generally been reported to be inferior to those of human skin. Therefore skin absorption in human skin is likely to be overestimated by these models and thus their relevance for percutaneous absorption studies remains to be established (25).

Labskin™ is a full-thickness HSE. The dermal layer comprises viable fibroblasts embedded in a fibrin matrix, and an epidermis that is developed by seeding keratinocytes on the dermis (26). LabSkin™ has been used for investigating the wound healing process and also for analysing the skin microbiome (26-28). In the current work we aimed to determine the permeation of a model compound, 3-O-ethyl-l-ascorbic acid (EA), in human epidermis and in LabSkin™. EA is widely used in personal care products and its ability to penetrate the skin has recently been confirmed (29, 30). The formulation examined consisted of propylene glycol (PG), propylene glycol monolaurate (PGML) and isopropyl myristate (IPM). These substances are commonly used in cosmetic formulations as solvents and skin conditioning agents (31-33), and they have been reported act as penetration enhancers for several compounds (34, 35). Recently, we investigated the skin delivery of EA from various binary and ternary solvent mixtures *in vitro* and a PG:PGML:IPM vehicle was the most effective in promoting skin permeation of the active (36). This vehicle, therefore, was selected for the present study.

All reagents used were analytical grade unless otherwise stated. Isopropyl myristate (IPM) and propylene glycol (PG) were supplied by Sigma-Aldrich (Dorset, UK). Lauroglycol™ 90 or propylene glycol monolaurate Type II (PGML) was donated by Gattefossé (Saint-Priest, France). Phosphate buffered saline (PBS) tablets were purchased from Oxoid Limited (Cheshire, UK). Ortho-phosphoric acid (H_3PO_4), HPLC grade water and methanol were obtained from Fisher Scientific (Leicestershire, UK). Abdominal human skin was obtained following plastic surgery from a single donor with institutional ethical approval (Research Ethics Committee reference 07/H1306/98). Labskin™ full thickness living skin equivalent (Batch number: 190117) was supplied by Innovenn Ltd. (York, UK).

Permeation of EA was determined *in vitro* using vertical glass Franz-diffusion cells according to OECD and SCCS guidelines (1, 7, 17). The experiments were carried out using heat separated human epidermis (37, 38) and LabSkin™ according to procedures described elsewhere (29, 39). A volume of 5 µL of 2% EA (*w/w*) in PG:PGML:IPM (0.65:0.30:0.05, *w/w*) was applied to the skin surface using a micropipette. Samples of 200 µL of receptor fluid (phosphate buffered saline (PBS), pH = 7.3 ± 0.2) were collected at different time intervals up to 24 h, and an equal volume of fresh temperature-equilibrated PBS was added to the

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receptor compartment. At the end of the permeation experiments, mass balance studies were performed to recover the amounts of EA remaining on and in the skin membrane. The skin surfaces were washed once with 1 mL of methanol and three times with 1 mL of water:methanol (50:50, *v/v*), consecutively, followed by swabbing with a cotton bud. Skin samples were placed in Eppendorf® tubes with 1 mL of water:methanol (50:50, *v/v*) in a thermostatically controlled orbital shaker (Orbital Mini shaker, VWR International Limited, Leicestershire, UK) for at least 5 h. All samples were analysed using a validated HPLC method for EA (29, 36).

The data were analysed using Microsoft® Excel 2016 (Microsoft Corporation, Redmond, Washington, DC, USA) and GraphPad Prism Statistics (version 8.3.0, San Diego, CA, USA). Results are presented as the mean ± standard deviation (SD). The Shapiro-Wilk test was used to assess the normality of the data. For parametric data, statistical analysis was performed by one-way analysis of variance (ANOVA) and multiple comparisons between groups by post hoc Tukey test. For non-parametric data, the Kruskal-Wallis test was performed. Statistical significance was assumed when the p value was less than 0.05. For non-parametric data, the Mann-Whitney U test was used to test statistical significance between two groups. For ≥ 3 groups, the Kruskal-Wallis test was performed.

The permeation profiles of EA in human skin and LabSkin™ are shown in Figure 1. Permeation of EA was evident from 2 h for LabSkin™; however, for human epidermis, EA was not detected in the receptor phase until 5 h. Additionally, the amount of EA that permeated at 5 h was significantly higher in LabSkin™ (19.7 ± 2.3 μg cm^{-2}) compared with human skin (7.0 ± 4.3 μg cm^{-2}, $p < 0.05$). The shape of the permeation profile for LabSkin™ was similar to that for human skin, as shown in Figure 1. The cumulative amounts of EA that permeated through LabSkin™ at 8, 10, 12 and 24 h were comparable to the respective amounts for human skin ($p > 0.05$). Similar cumulative amounts of EA permeated at 24 h in both tissues, namely. 41.3 ± 2.0 μg cm^{-2} for LabSkin™ and 49.4 ± 4.1 μg cm^{-2} for human skin ($p > 0.05$).

Results for the mass balance studies are shown in Figure 2. The percentage permeation of EA from LabSkin™ at 24 h was 55.1 ± 1.8 % of the dose applied. This value was similar to the percentage permeation of EA in human skin (58.0 ± 4.2 %, $p > 0.05$). With regards to skin retention of the active, the percentages of EA extracted from LabSkin™ were significantly higher (13.6 ± 1.3 % of applied dose) compared with human skin (9.7 ± 1.5 % of applied dose, $p < 0.05$). This might be attributed to the fact that LabSkin™ comprises both dermis and epidermis, while the heat-separated human skin consists only of the epidermal layer. The dermis is generally regarded a more hydrophilic structure compared to the

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epidermis (40), and the hydrophilic nature of EA ($\log P_{(o/w)} = -1.1$) (29) suggests that the compound will be present in this layer. Total recovery of the applied EA from LabSkin™ (103.1 ± 6.3 %) was similar to human epidermis (93.0 ± 3.7 %, $p > 0.05$). Both values were within the limits recommended by the OECD (90 – 110 %) and SCCS guidelines (85 – 115 %) (1, 7).

Several studies in the literature have attempted to evaluate how the permeability of various HSEs compares with human skin *in vitro* (41, 42). Asbill et al. (43) investigated the permeation of several actives, including caffeine, across several skin models: a full-thickness human skin equivalent, a model of reconstructed human epidermis (EpiDerm™) and dermatomed human skin. These researchers conducted diffusion studies over 24 h following application of infinite doses (25 μL cm^{-2}) of suspensions of caffeine in PG. The volume applied was 16.2 μL and the diffusional area of the Franz cells was 0.64 cm^2, as reported in previous work (44). The permeation parameters of caffeine (steady state flux, cumulative amount of caffeine permeation and lag time) in the various tissues were calculated from the linear portion of the permeation curve, and these values were used to assess the permeability of the HSEs against human skin. The flux and the cumulative amount of caffeine permeation were 3.2 μg cm^{-2} h^{-1} and 24.2 μg cm^{-2} for the full thickness HSE. Both values were reported to be statistically similar to the corresponding values obtained for human skin, 0.7 μg cm^{-2} h^{-1} and 11.0 μg cm^{-2} respectively ($p > 0.05$). As regards the EpiDerm™ model, values of both flux and cumulative permeation of caffeine were found to be more than ten times greater than corresponding values for human skin ($p < 0.05$). The lag-times for human skin, full-thickness HSE and EpiDerm™ were 5.5, 4.1 and 0 h respectively, although statistical comparisons were not reported. The authors concluded that the permeability of the full-thickness bio-engineered HSE was similar to human skin and suggested that it is a more acceptable model for percutaneous permeability testing than EpiDerm™. However, it should be noted that the permeability of the tissues was examined under infinite dose conditions. Consequently, the data have limited applicability for the performance of these models under finite dose conditions. A further issue is that the skin membranes were exposed to 8 μL of neat PG for 1 h prior to the diffusion experiments. PG is a solvent that has been known to alter skin barrier function (45, 46), and therefore the permeability of the models may have been affected by this pre-treatment step.

In a multi-laboratory study, Schäfer-Korting et al. (47) also investigated the permeation of caffeine in commercially available HSE models (SkinEthic®, Episkin® and EpiDerm™), as well as in heat-separated human epidermis. These researchers conducted Franz diffusion experiments and applied infinite doses (0.5 mL of a 0.1 % caffeine solution in PBS) to the donor compartment that was subsequently occluded. This volume corresponded

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to a caffeine dose of 281.4 μg cm^{-2}. The permeability of the tissues was evaluated by calculating the permeation parameters of lag time, permeability coefficient and cumulative permeation of caffeine. These researchers reported similar permeability coefficients for the EpiDerm™ model (0.2×10^{-6} cm s^{-1}) and human epidermis (0.1×10^{-6} cm s^{-1}, $p > 0.05$). The permeability coefficient values for Episkin® and SkinEthic® were significantly higher ($p < 0.05$), and were 2.8×10^{-6} cm s^{-1} and 3.6×10^{-6} cm s^{-1}, respectively. The lag time for human skin was calculated as 1.7 h, while corresponding values for the HSEs were 0.3 h for EpiDerm™, 1 h for Episkin® and 0 h for the SkinEthic® model. The cumulative amounts of caffeine permeated after 6 h were 4.9, 51.3 and 73.7 μg cm^{-2} for EpiDerm™, Episkin® and SkinEthic® respectively, while the value for human epidermis was 1.1 μg cm^{-2}. Statistical comparisons for these values were not reported.

As these data were obtained under infinite dose conditions, the same researchers conducted a later study in EpiDerm™ and human epidermis using finite doses (48). Caffeine was applied as either a 1 % or 0.1 % *w/v* solution in PBS and dosed at 10 μL cm^{-2} to the donor compartment that was occluded. The comparisons of the various models for the finite dose experiments were performed by assessing the percentage of the active permeated over 6 h for EpiDerm™ and over 24 h for human skin. The researchers did not provide a rationale for comparing caffeine permeation at different time points in the two tissues. The percentage permeation of caffeine from EpiDerm™ after 6 h was similar for both concentrations tested (0.1 % and 1%), 57.3 and 59.3 % respectively. Total permeation of caffeine in human skin at 24 h was approximately 20 %, however, the exact values were not documented and statistical comparisons between the two models were not reported. Although the evaluation of the HSEs was performed under finite dose conditions, it should be noted that the occlusion of the donor chamber is expected to affect the barrier function of the tissues. Skin occlusion has been reported to increase *stratum corneum* water content and affect percutaneous absorption by various mechanisms, such as the swelling of corneocytes and disruption of the *stratum corneum* lipid organisation (49-52). An additional issue is that occlusion prevents evaporation of the vehicle. The depletion of the vehicle may affect the concentration and the residence of the formulation on the skin surface and may also impact the permeation of the active.

Dreher et al. (53) determined the permeation of caffeine and α-tocopherol in two HSEs (EpiDerm™ and Episkin®) and in excised human skin. The application dose was 10 mg cm^{-2} and *in vitro* permeation experiments were conducted up to 24 h. Permeation of 1 % *w/w* caffeine was investigated from O/W and W/O emulsions, a hydrogel and an aqueous solution. For α-tocopherol, the formulations tested were 0.5 % *w/w* of active in O/W and W/O emulsions, a liposome dispersion and a hydrogel. The various HSE models were evaluated

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by comparing the percentage permeation or skin uptake of the actives after 24 h. The percentage permeation of caffeine across human skin (up to 20% of the dose applied) was similar for all formulations. However, as regards the HSEs, percentage permeation of caffeine from the hydrogel was significantly lower (60% in EpiDerm; 45% in Episkin®) compared with the other formulations (~90% in EpiDerm; 70-80% in Episkin®). Permeation of α-tocopherol to the receptor solution was low for all models (0.01 - 0.03%). The hydrogel promoted significantly greater percentage retention of the active in human epidermis (10.4%, $p < 0.05$) compared with the other formulations (2.5 – 4.4 %). However, for the HSEs, the W/O emulsion resulted in significantly greater epidermal uptake (2.8% for EpiDerm; 3.5% for Episkin®) than the other formulations (0.8 – 1.1% for EpiDerm; 0.3 – 0.7% for Episkin®). The HSE models were generally more permeable than human skin for both compounds. The authors highlighted that permeation of caffeine was greater than α-tocopherol in all membranes, and they suggested that the HSEs may be useful for predicting the rank order of permeability for different solutes. Additionally, it was noted that the various vehicles had different effects on the permeation of the actives in the HSEs than in human skin. This was attributed to the different intercellular lipid organisation of the HSEs and the increased hydration of the *stratum corneum* in the HSEs compared to human skin.

In the present work, the evaluation of LabSkin™ permeability to EA in relation to human epidermis was based on the time required for the active to permeate, the permeation profiles as well as the total permeation of the active in the two models. Overall, the findings indicate that although EA permeated across the LabSkin™ tissue more rapidly, the cumulative permeation of the active was similar for both membranes over 24 h. This is the first published study to compare the permeability of LabSkin™ with human skin *in vitro*. Results are encouraging for the use of this skin model for assessing percutaneous absorption of other personal care actives. Future work will focus on additional experiments with EA to enable further assessment of the reproducibility and reliability of the model. Additionally, the range of molecules and vehicles used with this HSE tissue will be expanded to include caffeine as well as other substances with varying physicochemical properties.

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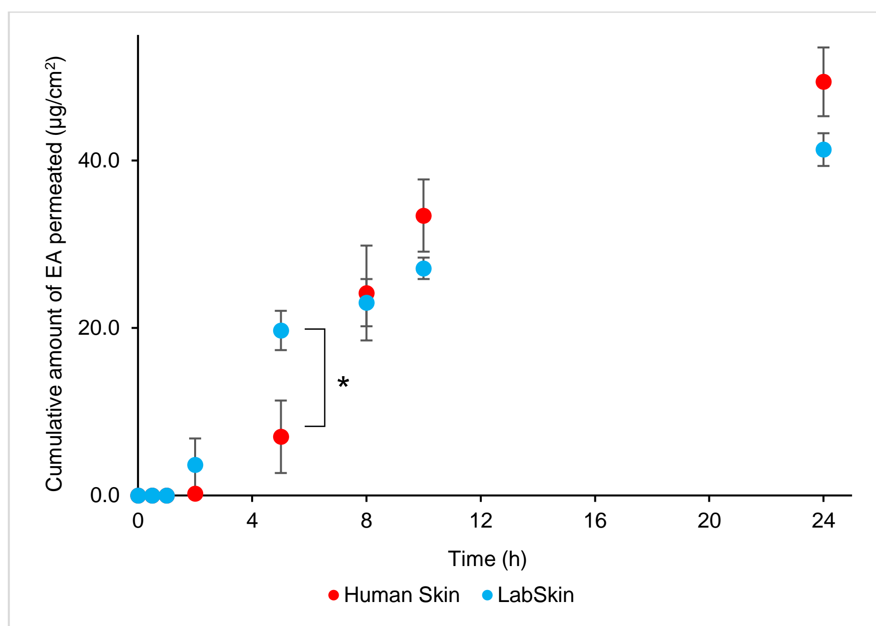

Figure 1 – Cumulative amount of 3-O-ethyl-l-ascorbic acid that permeated from PG:PGML:IPM in vitro under finite dose conditions ($4 \geq n \geq 3$; mean ± SD, $^*p < 0.05$)

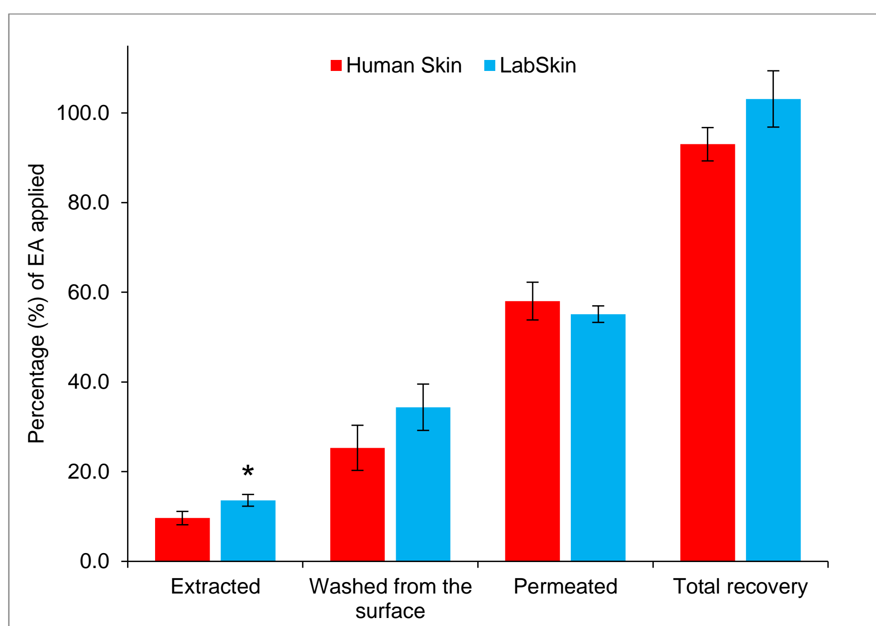

Figure 2 – Percentage (%) permeation, skin extraction, recovery from skin surface and total recovery of EA in LabSkin^{TM} and in human skin ($4 \geq n \geq 3$; mean ± SD, $^*p < 0.05$)

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