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Skin Concentrations of Topically Applied Substances in Reconstructed Human Epidermis (RHE) Compared with Human Skin Using *in vivo* Confocal Raman Microscopy

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Abstract: Detailed knowledge about the skin concentration of topically applied substances is important to understand their local pharmacological activity. In particular since *in vitro* models of reconstructed human epidermis are increasingly used as models for diseased skin. In general, diffusion cell experiments are performed to determine the diffusion flux of test substances through either skin models or excised skin both from humans and animals. Local concentrations of the test substances within the skin are then calculated applying diffusion laws and suitable boundary conditions. In this study we used a direct approach to reveal the local concentrations of test substances within skin using confocal Raman microscopy. This non-invasive method can also be applied *in vivo* and therefore we directly compared *in vivo* concentrations with those obtained from commercially available reconstructed human epidermis (RHE). Hydrophilic and lipophilic test substances with log P_{ow} from –0.07 to 5.91 were topically applied on human skin *in vivo* and RHE from SkinEthic was used as the commercial skin model. Local concentration profiles in the stratum corneum (SC) showed substantial differences between the RHE model and the *in vivo* situation. Differences between RHE models and human skin *in vivo* were also observed in their molecular composition, in particular in terms of their water profile, lipid content and the presence of natural moisturizing factor (NMF). Confocal Raman is shown to be a powerful non-invasive method for qualitative and quantitative comparative studies between RHE models and human skin *in vivo*. This method can also be applied to validate RHE models for future use in clinical studies.

Keywords: Concentration profiles · *in vivo* · Raman spectroscopy · Reconstructed human epidermis · Skin penetration

Introduction

In vitro models of reconstructed human epidermis (RHE) have become an essential tool for research and development in the cosmetic and pharmaceutical industry. Today, several RHE models are commercially available and theses models have been validated for irritation, corrosion, or photo toxicity testing.

However, no such validated protocols exist for the pharmacokinetically relevant adsorption testing using RHE, despite economic interest, ethical concerns, and increasing regulatory restrictions such as the prohibition of animal testing for cosmetic ingredients within the European Union[1] or the OECD guideline 428,[2] which encourage the replacement of human and animal tests by RHE models. The main reason could be the overall higher permeability of RHE models compared with human epidermis, which was observed in an elaborate validation study for the commercial RHE models SkinEthic RHE, EpiSkin (now both SkinEthic, F), and EpiDerm (MatTek, USA)[3] and also concluded by others (for selected reviews see refs [4–6]). Percutaneous penetration through RHE has been tested for a variety of hydrophilic and lipophilic compounds such as caffeine and testosterone, drugs, vitamins and essential oils.[7–13] Factors controlling skin barrier properties, such as the highly ordered structure of lipids and proteins in combination with specific variations in molecular composition, have been intensively investigated; see for example refs [14–17].

While percutaneous penetration is important for the systemic uptake of drugs or potentially toxic cosmetic ingredients, less is known about the skin concentration of topically applied substances in RHE. However, skin concentration matters for the pharmacological activity of dermal drugs and skin concentration will affect the results of the validated irritation and corrosion tests for RHE. Even though skin concentration can be expressed theoretically from percutaneous penetration data by Fick's second law of diffusion,[18] selecting the correct boundary conditions is crucial when calculating skin concentration from penetration data.

In this study, we investigated skin concentration profiles for both human skin *in vivo* and RHE models. Hydrophilic and lipophilic test substances (log P_{ow} from –0.07 to 5.91) were selected. Concentration profiles were determined by confocal Raman spectroscopy. This non-invasive label-free technology has been developed for skin penetration studies[19,20] and it is even applicable to human skin *in vivo*.[21,22] By focusing a laser sequentially into the skin sample, depth-resolved Raman spectra were recorded and concentration profiles of the test substance of interest were then extracted from the respective spectra. Furthermore, confocal Raman microscopy enabled us to investigate changes in the molecular composition of skin during penetration. Test substances showed a strong dependence of concentration based on their lipophilic/hydrophilic character and different concentration profiles for RHE models and human skin *in vivo* were observed. Differences in the molecular composition of RHE models and human skin *in vivo*, particularly for water, lipids and natural moisturizing factor (NMF) content may explain these different absorption properties.

Materials and Methods

Human Skin in vivo and RHE

Investigations with human skin *in vivo* were conducted on the volar forearm which is the preferred body site for sensory tests in cosmetic industry because this skin is relatively well protected against environmental influences (*e.g.* sun exposure and contact with allergens). Five volunteers, male and female, Caucasian skin, aged from 22 to 37 years, took part in this study upon written consent. The volunteers were told not to use any cosmetic product on their forearm within the last 24 hours before conducting the experiments.

Reconstructed human epidermal skin substitutes (SkinEthic RHE) were purchased at SkinEthic® Laboratories, F. We received the skin models at day 18. The SkinEthic RHE models were

kept in an incubator for 18 h (37 °C, 5% CO_2) according to the recommendation of SkinEthic®.[23,24] Afterwards, the models were used for the Raman experiments within three days. The typical thickness of the SC at the days of experiment was 80 μm, as determined from histological paraffinic sections of the RHE models, which had been stained with hematoxilin and eosin (nucleus and cytoskeleton staining, respectively).

Test Compounds

Chemicals were obtained from Sigma-Aldrich and used without further purification. The test substances were applied as solutions in ethanol except for caffeine that was dissolved in ethanol/water (1:1) due to its low solubility in pure ethanol. The concentrations are given in Table 1. The concentrations were selected based on the test substances' solubility and Raman scattering cross section. The lower concentrations for the slowly penetrating 4-methylbenzylidene camphor (MBC) and octyl methoxy cinnamate (OMC) were enough to maintain reservoir conditions at the skin's surface during the period of application.[25,26] Water–octanol partition coefficients in Table 1 were taken from the literature.[25,27,28]

Table 1. Applied test substances, their logarithmic octanol-water partition coefficient log P_{ow} their molar mass M, and the mass fraction w of the solution.

Test substance	log P_{ow}	M [g mol^{-1}]	w [%]	Solvent
Caffeine (Caf)	–0.07	194	2	ethanol/ water 1:1
Benzoic acid (BA)	+1.87	122	2	ethanol
Salicylic acid (SA)	+2.26	138	2	ethanol
4-Methylbenzylidene camphor (MBC)	+5.13	254	1	ethanol
Octyl methoxy cinnamate (OMC)	+5.91	290	1	ethanol

Penetration Experiments

Human Skin in vivo

20 μl cm^{-2} of solution were applied onto the volar forearm using self-sticking allergy patches (*Van der Bend,* area of 1 cm^2) that had been filled with 20 μl of the test solution. The patches were removed after 30 min and the skin was carefully dried with a cellulose cloth. Raman measurements started 5 min thereafter. Each test substance was tested on two volunteers at least. Untreated skin and skin treated with ethanol only served as control for each volunteer.

RHE

SkinEthic RHE models were removed from the incubator about 1 h before application. Then the models were removed from the medium, washed with distilled water, carefully blotted dry with a cellulose cloth, and solutions (20 μl cm^{-2}) were directly applied to the surface. After 15 min, the skin models were blotted dry, cut out from the inset, and immediately placed on the acquisition window of the Raman spectrometer. They were covered to avoid dry-out. Three batches of SkinEthic RHE containing 12 models each were used for the penetration experiments. For each batch, untreated and ethanol treated models were analyzed as control.

Confocal Raman Spectroscopy

The Raman measurements were made with an inverse confocal Raman microscope (Model 3510 SCA, *River Diagnostics)*. The Raman microscope was equipped with a 60x oil-immersion objective. The 785 nm excitation laser was used to acquire spectra in the fingerprint region (400–1800 cm^{-1}, integration time 5 s) and the 671 nm excitation laser was used for the high wavenumbers (2500–4000 cm^{-1}, 1 s). Raman spectra were acquired starting from the skin's surface down to a depth of 60 μm (step size of 2 μm). The acquisition was repeated at five positions at least, both for the finger print region and at high wavenumbers. Averaged concentration profiles of skin components and test substances were extracted by fitting the Raman spectra with reference spectra of keratin (as representative of all proteins), water, ceramide 3 and cholesterol (as representatives of all lipids), NMF (fixed composition of natural moisturizing factor), lactate, urea, test substance, and ethanol (solvent). Concentrations of test substances are given as mass fraction relative to keratin rather than correcting for the Raman signal attenuation by mathematical models.[29] Response factors had been determined against BSA solutions assuming similar Raman cross-sections for keratin and BSA as described in the literature.[19] The cumulated amount of test substance was calculated from the area under the curve of the depth profile. Error bars represent the combined measurement uncertainty which is caused by both local inhomogeneity within the skin and variability between volunteers or RHE models. The boundary between the SC and the viable epidermis was determined based on changes in the keratin signal. The water content was calculated from Raman spectra at high wavenumbers according to Caspers *et al*.[19]

Results

Penetration Profiles for Human Skin in vivo

Raman-based concentration profiles of caffeine, benzoic acid, salicylic acid, MBC, and OMC 30 min after application to the volar forearm are shown in Fig. 1. For human skin, the highest concentration of test substance is found within the first 6 μm from the surface followed by an exponential-like decrease approaching zero concentration close to or before the boundary between the SC and the viable epidermis (ED). This boundary was determined to be between 15 and 20 μm, which is close to the 22.6 μm, observed by Egawa *et al*.[30] After 30 min of penetration the higher concentrations are observed for the hydrophilic test substances with log P_{ow} close to zero while concentration of the lipophilic ones such as MBC or OMC are lower.

Penetration Profiles for SkinEthic RHE Models

Two borderline cases are observed, when comparing the concentration profiles of caffeine, benzoic acid, salicylic acid, MBC, and OMC in SkinEthic RHE (Fig. 1). For MBC and OMC, the test substances concentration is highest at the surface followed by an exponential-like decrease. These profiles are similar to the *in vivo* situation in terms of the shape but the absolute amount of test substance is 10 to 100 times higher. The test substances caffeine, benzoic acid, and salicylic acid, on the other hand, are already homogeneously distributed within the entire model after 15 min. For these rapidly penetrating compounds, it would be desirable to record penetration profiles within the first minutes after application, but there are limits due to the required handling of the samples and the signal to noise of the Raman spectra. Therefore, we have selected 15 minutes as the shortest interval.

Characterization of Untreated Human Skin and of SkinEthic RHE Models

To understand the significant difference in permeability between SkinEthic RHE and human skin, molecular concentration profiles of their main constituents were determined.

In human skin, the water, natural moisturizing factor (NMF),

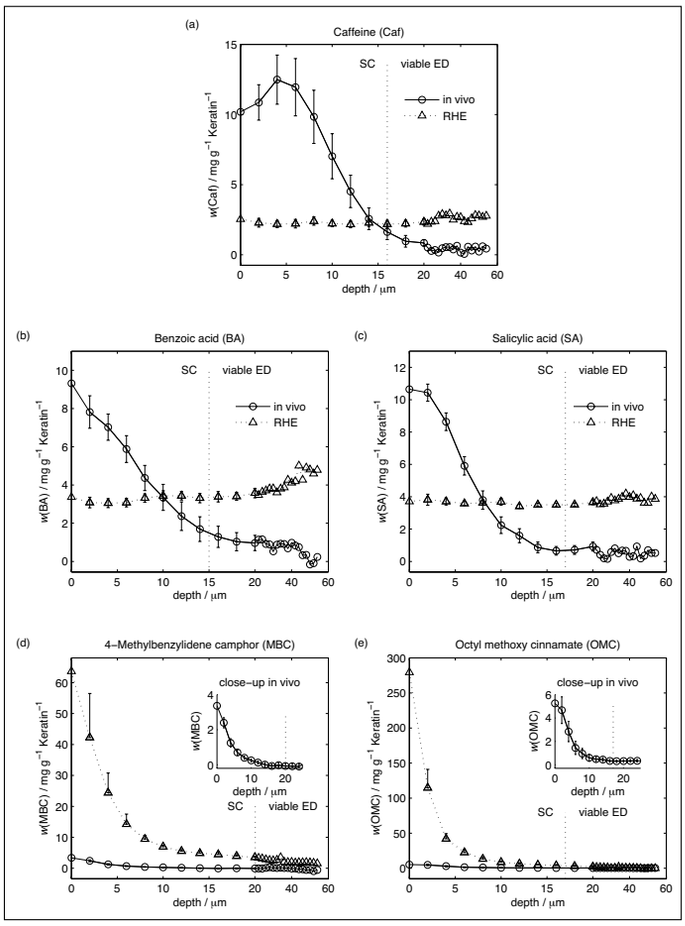

Fig. 1. Concentration profiles for human skin *in vivo* (circles) (30 min penetration) and for SkinEthic RHE (triangles) (15 min). Mass fractions, *w*, are given relative to keratin. They are normalized to 1 wt% solutions. The depth is given from the skin's surface down to 60 µm and the scale is condensed between 20 and 60 µm to improve visibility. For human skin, the boundary between SC and viable epidermis (ED) is indicated by a vertical dashed line. The typical thickness of the SC in SkinEthic RHE was 80 µm. The combined measurement uncertainty is indicated by error bars.

and ceramide concentration is correlated with the evolution of stratum corneum (SC). *In vivo*, the water content increases from 20–30% at the skin's surface to a constant value of 60–70% in the epidermis (see Fig. 2a). The water content in SC depends amongst other factors on the relative humidity and on the concentration of NMF, a highly hygroscopic and water-soluble mixture of amino acids found in the SC. NMF is a derivative of amino acids and specific salts produced in SC by proteolysis of filaggrin.[31] The NMF has a maximum close to the skin's surface and drops close to zero at the border between SC and the viable epidermis, (Fig. 2b). Similar profiles for the individual components of NMF are reported for the thenar.[19] Fig. 2c shows the ceramide content that decreases from the skin surface. Ceramides and other skin lipids are released during the differentiation from keratinocytes to corneocytes and consequently, their content increases in the SC.

In SkinEthic RHE, the water, NMF, and ceramide concentration profiles are very different. The water content is already >60% at the model's surface – a value that is only found in human for the viable ED, (Fig. 2a). Both the high humidity in the incubator during cultivation and a reduced inside-out barrier function in the RHE model may account for this increased water level. The ceramide content at the surface is similar to the *in vivo* situation, but remains high in deeper skin layers (Fig. 2c).

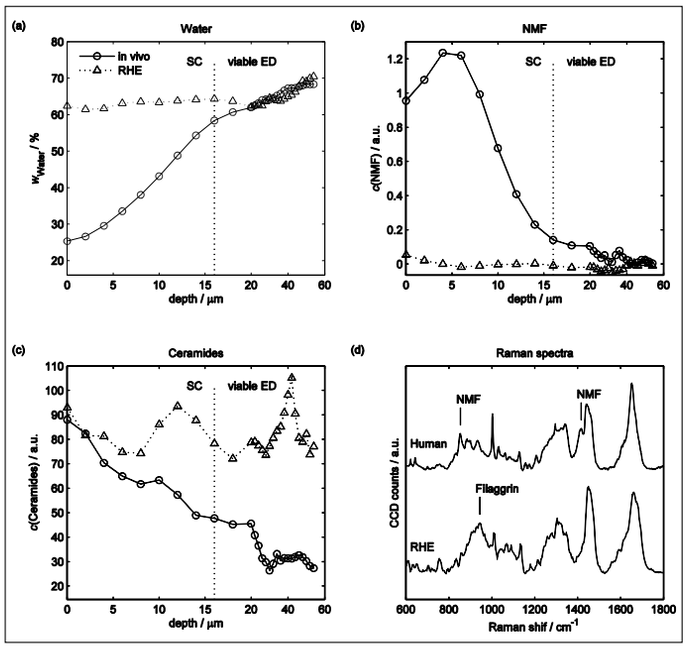

Fig. 2. (a) Water, (b) ceramide, and (c) NMF content of untreated human skin *in vivo* (circles) and of untreated SkinEthic RHE models (triangles). (d) Raman spectra of untreated human skin and of untreated SkinEthic RHE models. The characteristic peaks for NMF and Filaggrin are indicated.

This is consistent with Ponec *et al*. who found higher ceramide content in skin models.[32] In addition, we found lipid droplets by Raman microscopy in a previous study.[33] NMF is not present in the skin model, but filaggrin (the precursor of NMF) is observed. In the Raman spectrum, the NMF can be identified by two distinctive peaks at 855 and 1415 cm^{-1} while filaggrin shows a distinctive, broad peak at 880 cm^{-1}.[20,34] At these positions, the Raman spectra of human SC *in vivo* and of SkinEthic RHE differ (Fig 2d). Filaggrin is present in skin models – signaling that its proteolysis has not yet started – and NMF is missing.

Discussion

The different concentration profiles for SkinEthic RHE and the *in vivo* situation can be understood using a single-layered diffusion model and applying Fick's second law of diffusion with different boundary conditions.[18] Fig. 3a shows the concentration profile at steady-state level for sink conditions on the receiver side ($c = 0$ at $x = l$) and Fig. 3b for equilibrium conditions, where

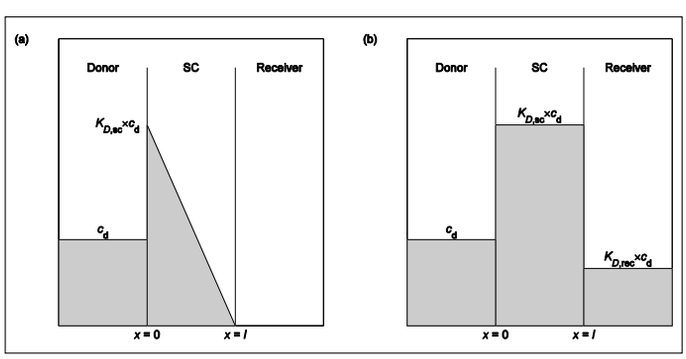

Fig. 3. Schematic concentration-distance profiles for a single layered diffusion membrane with different boundary conditions: (a) steady state sink conditions, (b) equilibrium conditions; *l* is the thickness of the membrane, c_d is the donor concentration and $K_{D,sc}$ and $K_{D,rec}$ are the partition constants.

$K_{D,sc}$ and $K_{D,rec}$ are the partition constants of the active from the donor to the stratum corneum and the receiver, respectively.

When comparing the schematic diagrams in Fig. 3 with the experimental concentration profiles in Fig. 1, it is obvious that equilibrium conditions were met for SkinEthic RHE in the case of caffeine, benzoic acid, and salicylic acid, since the concentration of the test substances remained constant throughout the analyzed first 60 µm of the SC. The time required until equilibrium is reached depends on the volumes and concentrations of the donor and receiver reservoirs as well as on the diffusion constant and the thickness of the skin. Interestingly, for SkinEthic RHE short lag-times – *i.e.* the time until pseudo steady state conditions are met – between 0.02 and 0.17 hours were reported for molecules with a log P_{OW} between –0.08 and 1.90.[3]

This is different for the *in vivo* situation: in Fig. 1 the three compounds caffeine, benzoic acid, and salicylic acid exhibit concentration profiles, which are close to the steady state sink conditions illustrated in Fig. 3a. The reported lag-times for compounds with similar log P_{OW} for pig skin were 4.47 to 11.05 hours,[3] which explains the different profiles between the *in vivo* situation and the SkinEthic RHE. For caffeine and salicylic acid, the concentration at the skin's surface is less than expected from the theoretical profile (Fig. 3a). Such profiles with a moving front were already reported for caffeine, where 15.5 mg g^{-1} keratin^{-1} were found when applying a 1.8% caffeine solution in ethanol:water 1:2 for 60 min.[35] The model in Fig. 3a assumes an infinite reservoir, however, if the reservoir is not infinite, c_d will drop and a moving front can be observed.

For the lipophilic molecules MBC and OMC with log P_{OW} 5.13 and 5.91, penetration profiles according to Fick's second law are observed for both the SkinEthic RHE and for human skin *in vivo*. This is consistent with an overall lower flux of the lipophilic compounds.[36] Schäfer-Korting *et al.* found in their validation study smaller permeation coefficients for clotrimazole than for caffeine,[3] which is consistent with our data, given the similar physicochemical characteristics between clotrimazole (P_{ow} = 5.74, M = 345 g mol^{-1}) and MBC or OMC. The obvious difference between SkinEthic RHE and human skin is the significantly higher concentration. This can be explained by a higher partition constant $K_{D,sc}$ in case of SkinEthic RHE.

The Raman-based concentration profiles are in line with diffusion cell based penetration studies, where permeability of RHE was compared to excised human skin or pig skin: in general, the permeability of RHE was higher, but the ranking of compounds is similar.[3,12,37,38] Typical vehicles for penetration studies using RHE models were phosphate buffered saline (PBS), partially supplemented with solubilizers such as EtOH or Igepal®, alcoholic solutions (MeOH/water or EtOH/miglyol® 812) or o/w, w/o-emulsions, and fatty ointments. When investigated, a clear role of the vehicle on penetration was observed.[10,11,39] This is in line with the schematic diagrams in Fig. 3, since the vehicle will affect the $K_{D,sc}$ and consequently also the concentration in the SC. Here, we used EtOH as the solvent to account for the poor water solubility of the lipophilic test substances.

The different molecular constitution of human skin and SkinEthic RHE given in Fig. 2a–c may also explain the different concentration profiles observed for the test substances. The SkinEthic RHE shows a water content in the SC that is similar to the viable ED of human skin. This is surprising, since the SkinEthic RHE had been grown at the air–water interface and therefore it should be partially dehydrated. This is even more surprising, since NMF that accounts for a balanced water level at the air/skin interface in human skin, is missing as well. The simplest explanation could be an overall weak water barrier within the SkinEthic RHE that allows constant water supply from the medium. If the water barrier is weak, it may also be inferred that hydrophilic substances such as caffeine or salicylic acid will easily penetrate. The situation is different for the lipophilic MBC and OMC. These substances were accumulated at the surface layers of the SkinEthic models which should be explained with a higher partition constant $K_{D,sc}$. The higher partition constant is consistent with a higher level of lipids as can be observed in case of ceramides (Fig. 2c). Lipid droplets, observed in SkinEthic RHE[33] may also account for a higher partition constant.

Acquiring Raman-based concentration profiles at steady state sink conditions for the highly permeable hydrophilic compounds at lag times shorter than 15 min is mostly limited by RHE handling. However, lipophilic compounds show such profiles in RHE after 15 minutes. On the other hand, *in vivo* Raman microscopy will be suited to determine penetration profiles of RHE models that are closer to the in vivo situation in terms of permeability. The development of RHE models with improved barrier properties is ongoing and advances in that respect have been reported recently.[7] Raman microscopy will also be suited to characterize these models in terms of their molecular concentration profiles.

The integral under the curves in Fig. 1 is also very revealing and pharmacologically relevant. The area under the curve is the persistent concentration of the dosed compounds at application time t, which was 30 min for human skin *in vivo* and 15 minutes for RHE (Fig. 4). The observed dependence of the persistent concentration on the log P_{ow} of the test substance illustrates the selectivity of the applied *in vivo* Raman spectroscopy for studying *in vivo* concentration profiles of test substances. In our case, the hydrophilic test substances show a higher persistent concentration after 30 minutes than the lipophilic ones (MBC and OMC). For the SkinEthic RHE models, only the persistent concentration for the slowest test substances MBC and OMC is given, since the hydrophilic substances had already fully penetrated after 15 minutes. Compared to the *in vivo* situation, the persistent concentration is increased by at least one order of magnitude. This may either be due to an impaired barrier or due to a higher content of lipids and the presence of lipid droplets in the SkinEthic RHE models.[33] Accounting for the difference in persistent concentration is important when drawing conclusions on the pharmacological activity of drugs applied to skin models.

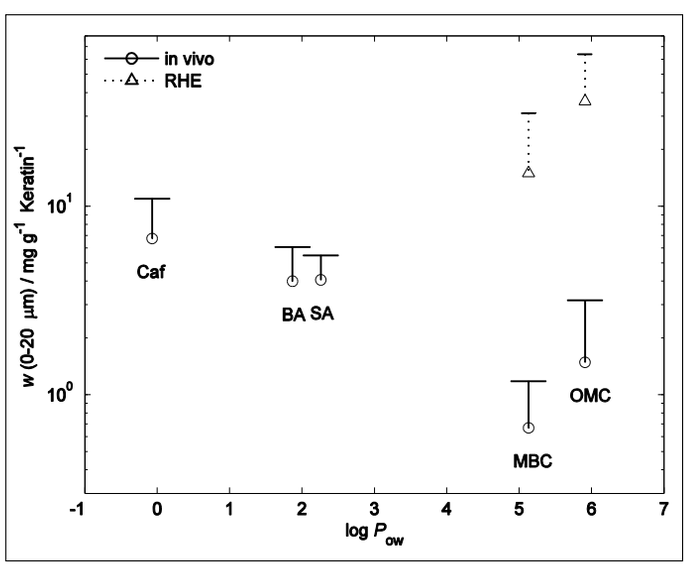

Fig. 4. Persistent concentration of the dosed compounds at lag time t for human skin *in vivo* (t = 30 minutes) and for SkinEthic RHE models (t = 15 minutes) against their log P_{OW} within the top 20 µm of the skin.

Conclusion

Confocal Raman spectroscopy is a powerful non-invasive method for qualitative and quantitative skin permeation studies

in RHE and in human skin. Actives of varying lipophilicity are applicable. RHE from SkinEthic shows substantial differences, both in composition and intake of topically applied actives with respect to human skin. Furthermore, confocal Raman spectroscopy can be applied to the validation of artificial skin models for future use in clinical studies.

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