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Research paper

In vitro vs. in vivo tape stripping: Validation of the porcine ear model and penetration assessment of novel sucrose stearate emulsions

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ABSTRACT

Porcine ear skin is frequently used as a substitute for human skin in dermatological research and is especially useful for tape stripping experiments where the penetration of active substances into the uppermost skin layers is investigated. However, certain differences between the surface properties of these skin types exist, and reports on the comparability of tape stripping data obtained in vitro using porcine ear skin and data obtained in vivo on human forearm skin are scarce. Thus, we performed comparative tape stripping experiments in which the skin penetration of curcumin and fluorescein sodium from conventional microemulsions and hydrogels was investigated. In this context, the skin penetration potential of novel semi-solid macroemulsions and fluid nanoemulsions based on sucrose stearate was evaluated as well. The removed corneocytes were quantified by NIR-densitometry using recent correlation data for human and porcine proteins. The trends observed for the skin penetration into porcine ear skin were highly representative for the in vivo situation on human skin, confirming that the porcine ear is an excellent in vitro model for tape stripping experiments. Moreover, the validity of the NIR-densitometric approach for the quantification of both human and porcine stratum corneum proteins was confirmed in this study for the first time.

1. Introduction

Tape stripping is a well-established method to investigate the skin penetration of topically applied substances. The superficial layers of the stratum corneum (SC) are removed with adhesive films, which are subsequently analysed for their drug and protein content. Thus, the amount of penetrated drug and the penetration depth into the skin can be determined [1,2]. Recently, near-infrared densitometry (NIR-densitometry) has been shown to possess great potential for the analysis of the SC proteins removed with each tape and has subsequently been validated for in vivo [3] and in vitro [4] tape stripping on human skin. However, in vivo studies may be associated with significant organisational and legislative issues, and the sources for excised human skin for in vitro studies are limited. In tape stripping experiments, the excised porcine ear has been shown to be a suitable skin substitute for human skin in vivo [2] since the ear skin, which remains on the cartilage during the experiments, does not contract [5]. In fact, this renders porcine ear skin even more suitable for in vitro tape stripping than excised human skin or excised porcine skin from other areas [6]. Thus, the NIR-densitometric method of protein quantification has been validated for this application as well [7].

Valuable information on the porcine ear skin model in general can be found [5]. However, only few reports deal with the use of porcine ear skin for in vitro tape stripping [8,9] and quantification of the porcine proteins removed with the tapes [2,10]. There is a surprising lack of comparative studies, which deal with conventional vehicles applied in a non-occluded fashion, although it may be assumed that a vast majority of topical formulations is applied in this manner. Validation of the porcine ear model under these conditions using different formulations of practical relevance seems essential to legitimate its use for in vitro tape stripping.

Abbreviations: SC, stratum corneum; NIR-densitometry, near-infrared densitometry; TEWL, transepidermal water loss.

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Detailed comparative information should be obtained through parallel tape stripping on human and porcine skin with substances of different log*P* values.

Thus, the aim of this study was to compare the skin penetration of two well-established model substances, namely the lipophilic dye curcumin and the hydrophilic dye fluorescein sodium, from different formulations applied on porcine ear skin in vitro and human skin in vivo. Classical formulations such as a microemulsion and an alcoholic hydrogel were employed as vehicles for this task. Recently developed skin-friendly O/W macroemulsions and nanoemulsions were additionally tested for their drug delivery potential in vivo. Interestingly, these formulations of identical composition had yielded highly comparable skin penetration rates in vitro despite significant differences in particle size and viscosity [11]. Thus, in vivo tape stripping should reveal whether this phenomenon was confined to the realms of in vitro set-ups or would be observed in vivo as well.

In summary, the present work aims to validate the porcine ear skin model for in vitro tape stripping experiments while using the novel technique of NIR-densitometry for protein quantification of both porcine and human skin. Since differences in the pattern of wrinkles, the corneocyte distribution and the pseudo-absorption at 850 nm of porcine and human corneocytes have been reported [7,12], the present study should elucidate whether these factors would impair the comparability of porcine and human skin penetration profiles. Determination of the transepidermal water loss (TEWL) as a biophysical skin analysis technique was additionally employed to ensure integrity of the skin of both volunteers and porcine ears. Long-term monitoring of the TEWL of human volunteers was conducted to determine the physiological TEWL range and to ensure representative skin properties for later tape stripping.

2. Materials and methods

2.1. Materials

Standard Corneofix® adhesive films with a square area of 4.0 cm^2 were obtained from Courage + Khazaka GmbH (Cologne, Germany). Curcumin (CAS: 458-37-7), fluorescein sodium salt (CAS: 518-47-8) and potassium sorbate (CAS: 24634-61-5) were purchased from Sigma Aldrich (St. Louis, USA). Sucrose stearate (Ryoto Sugar Ester® S-970) was supplied by Mitsubishi-Kagaku Food Corporation (Tokyo, Japan). PCL-liquid (cetearyl ethylhexanoate/isopropyl myristate, CAS: 90411-68-0) and propylene glycol (1,2-propanediol, CAS: 57-55-6) were purchased from Dr. Temt Laboratories (Vienna, Austria). Oleic acid (CAS: 112-18-1), carbopol® 940 polymer (CAS: 9063-87-0) and TRIS (tris(hydroxymethyl)-aminomethane 2% w/w buffer solution, CAS: 77-86-1) were obtained from Herba Chemosan Apotheker-AG (Vienna, Austria). The lecithin mixture lipoid S-75 was kindly donated by Lipoid GmbH (Ludwigshafen, Germany). Ultraphil® amphiphilic cream was purchased from Intendis Austria GmbH (Vienna, Austria). According to the manufacturer, the compounds of this cream are glycerol distearate, glycerol monostearate, liquid paraffin, polyoxyethylene 100 stearate, polyoxyethylene 2 and 21 stearyl alcohol, white vaseline, distilled water, benzyl alcohol and perfume oil. The traditional Austrian product spiritus vini gallici 60% (Franzbranntwein) was prepared according to the Codex Alimentarius Austriacus (Chapter B33) by diluting an extract (Urkörper Gallhuber 60%, Herba Chemosan Apotheker-AG, Vienna, Austria) with appropriate amounts of ethanol, menthol and distilled water (1:10/0.2/10 w/w). All further chemicals such as the solvents ethanol (CAS: 64-17-5) and isopropanol (CAS: 67-63-0) used were of analytical reagent grade and used as obtained from Sigma Aldrich (St. Louis, USA).

2.2. Formulations

Established formulations were employed for the comparative tape stripping experiments on porcine ear skin in vitro and human skin in vivo, namely a lecithin-based microemulsion [13] and an alcoholic hydrogel. The amphiphilic cream ultraphil® was employed for preliminary in vitro studies. The novel sucrose stearate-based emulsions were evaluated only by in vivo tape stripping since their penetration behaviour in vitro had already been established [11]. The composition of the individual formulations is given in Table 1.

The microemulsion was prepared by dissolving the lecithin mixture in isopropanol, ethanol and oleic acid. Distilled water was slowly added during magnetic stirring.

The hydrogel was prepared by dissolving the polymer carbopol® 940 in a mixture of spiritus vini gallici, propylene glycol and isopropanol. A homogeneous gel network was formed upon addition of the TRIS buffer solution under mild mechanical stirring.

The sucrose stearate-based macroemulsions and corresponding nanoemulsions were prepared as described [11]. The separate oil and aqueous phases were mixed and stirred with an ultra-turrax (Omni 500, 2500 rpm, 4 min). In case of the fluid nanoemulsions, the mixture was further treated with a high-pressure homogeniser (EmulsiFlex C3, Avestin) for 20 cycles at 750 bars.

The model drug curcumin was incorporated into all above mentioned formulations at 0.5% (w/w), respectively. The hydrophilic model drug fluorescein sodium was dissolved in the microemulsion for comparative investigation at 0.5% (w/w). The drug content of all formulations was determined after preparation to confirm drug incorporation. In case of curcumin, 10 mg of the respective formulation was dissolved in 1 ml of ethanol (96% v/v). The samples were homogenised in an ultrasonic bath at 30 kHz (US Starsonic 60, Liarre, Italy) for 20 min and then centrifuged for 6 min at 12,000 rpm (Hermle Z323 K, MIDSCI, USA). Subsequently, 0.5 ml of each sample was further diluted with ethanol (96% v/v, 1:10) and analysed by UV/Vis spectroscopy as described in Section 2.10. In case of fluorescein sodium, the same procedure was conducted with phosphate buffer (pH 7.4, 0.012 M) instead of ethanol. For drug quantification, 100 µl of the diluted samples was transferred into a microtiter plate in triplicate and analysed by fluorescence spectroscopy as described in Section 2.11. Samples were always taken at least in triplicate ($n \geqslant 3$).

2.3. Skin tissue

Fresh porcine ears were obtained from the Clinic for Swine at the University of Veterinary Medicine Vienna, Austria. The age of the slaughtered pigs was around 6 months. To ensure integrity of the skin barrier, the ears were removed before the carcass was exposed to any high-temperature cleaning procedure [9]. The ears were washed carefully under cold running water and blotted dry with soft tissue. All ears were stored at −20 °C and thawed prior to the experiments since the main penetration barrier, the non-vital superficial SC layer, is not influenced by frozen storage [4,14]. All experiments were carried out at room temperature. The skin remained on the isolated ear. In addition, porcine abdominal skin was tested in preliminary studies, but was found unsuitable for tape stripping experiments. The SC of porcine abdominal skin is thinner than that of the dorsal side of porcine ears [15], thus rendering the acquisition of accurate skin penetration data difficult.

2.4. Test persons and experimental conditions

Long-term evaluation of the TEWL on the volar forearms and the forehead of human volunteers was performed on a population

Table 1
Composition of all investigated formulations in % (w/w) and abbreviations.

Microemulsion		Hydrogel		Macroemulsion/nanoemulsion	
Excipients	% (w/w)		% (w/w)		% (w/w)
Lipoid S-75	40	Propylene glycol	10	PCL-liquid	20
Isopropanol	35	Isopropanol	10	Sucrose stearate	5
Oleic acid	9.5	TRIS 2%	5.1	Potassium sorbate	0.1
Ethanol 96% v/v	5	Carbopol® 940	0.6	Model drug	0.5
Model drug	0.5	Model drug	0.5	Distilled water	74.4
Distilled water	10	Spiritus vini gallici	73.8		

Abbreviations: ME microemulsion; GE hydrogel; E macroemulsion; NE nanoemulsion.

of 26 male and female volunteers aged between 18 and 44. Written informed consent for this non-invasive procedure was obtained from all participants. Measurements were taken over 4 months.

For the tape stripping experiments, twelve healthy volunteers aged 18–44 were included after giving their written informed consent. They did not suffer from skin diseases or allergies. The study was approved by the ethics committee of the medicinal university of Vienna (EK-Nr. 503/2011). All experiments were performed according to the same protocol. The volunteers were required not to apply cosmetic or skin care products up to 12 h prior to the experiment and to avoid excessive consumption of coffee, tea or alcohol [16]. Furthermore, they were asked not to engage in physical activity prior to the experiments and to remain seated for 15 min after entering the test area with constant ambient temperature (24 ± 1 °C) and humidity (50 ± 4% relative humidity).

The steps of the working protocol for in vitro and in vivo experiments were the same: after application of a formulation, the penetration behaviour of the respective model drug was determined by tape stripping and subsequent analysis of both protein content and drug amounts found on the individual tape strips. The tape stripping procedure in combination with sensitive analytical methods allows an exact localisation of the applied drugs in the SC [17].

2.5. Transepidermal water loss (TEWL)

The TEWL of the skin was determined using the closed-chamber device AquaFlux® (Biox Ltd., London, UK) and the AquaFlux® V6.2 program for data analysis. In case of porcine ear skin, TEWL measurements were taken as a quality control to exclude areas with altered skin barrier function. Although the TEWL of excised porcine ears is hardly representative of the in vivo situation, it can serve to monitor the defrosting process and to ensure a standardised working protocol [7,8,18]. The presence of small lesions and scars can be detected since such areas exhibit noticeably higher TEWL values.

Prolonged storage of fresh or defrosted porcine ears at ambient conditions will inevitably lead to dehydration of the tissue and thus to a constant decrease in the TEWL. However, there is a sufficiently long time frame for fresh or defrosted porcine ears with average TEWL values around 15 g m^{-2} h^{-1} that represents a suitable starting point for tape stripping experiments. Therefore, the TEWL of the porcine ears was determined for all samples before the tape stripping procedure. Assessment of the TEWL after tape stripping provided additional information about changes in skin barrier function [15].

In case of human skin in vivo, determination of the TEWL is an established method for skin integrity testing [19,20]. Substantiated TEWL monitoring of human volunteers was performed to establish a database of physiologically acceptable TEWL values for different skin areas such as the volar forearms and the forehead. These data served to provide a basis for comparison to identify unsuitable skin areas or skin types prior to the tape stripping experiments. Measurements were taken in regular intervals over a period of 4 months. Volunteers were advised not to treat their skin differently than usual and not to use skin care products immediately before the measurements. The influence of parameters such as age, sex, the frequent use of skin care products, inclination to sweat, skin type and the season on the skin barrier function was evaluated in this context. Information on subjective parameters, such as the use of skin care products and the inclination to sweat of the individual volunteers, was gained by means of a questionnaire.

2.6. Preliminary experiments: optimisation of tape stripping working protocol

Preliminary experiments were performed to identify the most suitable working protocol for in vitro tape stripping. The effect of different application times was investigated. To this end, tape stripping was performed after 1 and 4 h following the application of the respective formulation. The selected model formulations used were the microemulsion, the hydrogel and the amphiphilic cream ultraphil® with curcumin.

2.7. In vitro tape stripping

In vitro skin penetration profiles were established for the curcumin-loaded microemulsion, hydrogel and ultraphil® cream as well as for the microemulsion with fluorescein sodium. At least six individual tape stripping experiments were performed for each formulation on porcine ear skin ($n \geqslant 6$, respectively). After defrosting and cleaning, the skin was blotted dry with soft tissue and freed from visible hair with scissors. The ears were stretched out on styrofoam plates and fixed with needles. Intact, representative skin areas of 15 cm^2 were indicated with a permanent marker. The TEWL of the skin was determined with the AquaFlux® device (Biox Ltd., London, UK) to confirm an intact skin barrier function [7]. When the TEWL reached values between 15 and 20 g m^{-2} h^{-1}, the respective formulation was applied onto the skin by means of a saturated glove finger at a concentration of 2 mg cm^{-2} and gently distributed for 30 s. After an application time of 1 or 4 h, the tape stripping procedure was started.

The adhesive films employed to remove the superficial SC layers were standard Corneofix® tapes. Care was taken to ensure a reproducible working procedure [3,21]. The outline of the first adhesive film was indicated with a permanent marker to facilitate subsequent tape stripping on the same location. Pressure was applied with the thumb covered in a vinyl glove [22] to ensure a rolling movement and thus minimise the influence of wrinkles [1]. While a roller is among the most suitable devices to flatten out the skin during in vivo tape stripping, the skin of excised pig ears provides less space for handling a roller. Experience has shown that a rolling movement performed by the thumb leads to the most efficient and homogeneous removal of porcine corneocytes in vitro [7,22]. Thus, this working protocol was

adopted for the in vitro experiments. A piece of paper was placed between the tape and the thumb to avoid a transfer of emulsion from the adjacent skin parts to the adhesive film [23]. The experiment was performed on a balance to ensure a constant pressure of 49 N (5 kg) [7]. After applying pressure for 3 s, the tape was removed in a single rapid movement. In case of curcumin-loaded formulations, 20 sequential tape strips were removed. Since a higher penetration depth was anticipated for fluorescein sodium, 40 tape strips were removed in this case.

2.8. In vivo tape stripping

In vivo skin penetration experiments were performed on 12 healthy test persons. The penetration profiles were established for the curcumin-loaded microemulsion and hydrogel as well as for the microemulsion with fluorescein sodium. Moreover, the novel curcumin-loaded macroemulsion and nanoemulsion were investigated in the same fashion. Six individual experiments on the human flexor forearm were performed for each formulation ($n = 6$, respectively).

Representative intact skin areas of 15 cm^2 were indicated with a permanent marker. The TEWL of the skin was determined as described above. The respective formulation was then applied onto the skin by means of a saturated glove finger at a concentration of 2 mg cm^{-2} and gently distributed for 30 s. After an application time of 30 min, the tape stripping procedure was conducted. In this case, pressure was applied with a roller [1,23]. Except for the means and intensity of pressure application and the application time, the working procedure was identical to that employed for the porcine ears.

2.9. Protein quantification by NIR-densitometry

We recently validated NIR-densitometry for the quantification of porcine skin during in vitro tape stripping [7]. In the present work, we tested the proposed method for in vitro tape stripping experiments on porcine skin for comparison with in vivo data, for the first time using different formulations.

The amount of corneocytes removed with the individual adhesive films was analysed using the infrared densitometer SquameScan™ 850A (Heiland electronic GmbH, Wetzlar, Germany). The removed tape strips were measured at a wavelength of 850 nm as previously described using an empty tape strip as a reference [3,4]. Thus, the optical pseudo-absorption of the adhesive films that is caused by an interaction of the corneocyte aggregates with light via scattering, diffraction and reflection was determined [23]. Since the device has been validated separately for porcine and human skin, the respective calibration data were used for protein quantification. In case of porcine skin, the mass of SC proteins (m) can be calculated after determination of their pseudo-absorption (A) for a normalised tape area of 1 cm^2 by employing the equation $m = A/0.41$ (in μg cm^{-2}) [7]. In case of human proteins, the equation derived from the respective calibration curve can be employed for this task, namely $m = (A - 2.703)/0.623$ (in μg cm^{-2}) [3]. The mean cumulative amount of removed SC proteins was employed to establish the penetration depth of the applied drugs in relation to the complete SC thickness. Experience has shown that the SC thickness of both human forearm skin and pig ear skin can vary considerably for each individual [7,9]. Since removal of the entire SC in each case is too much organisational effort and an additional strain for the volunteers, we employed well-established reference values for the entire SC thickness in accordance with the literature [9]. Thus, the thickness of the SC of porcine ear skin was assumed with 8.2 μm and the thickness of human SC of the volar forearm with 11.0 μm.

2.10. Curcumin quantification by UV/Vis spectroscopic analysis

After NIR analysis, the individual tapes containing corneocytes and curcumin were dissolved in 4 ml of ethanol (96% v/v). The samples were homogenised in an ultrasonic bath at 30 kHz (US Starsonic 60, Liarre, Italy) for 20 min and centrifuged for 6 min at 12,000 rpm (Hermle Z323K, MIDSCI, USA). The resulting curcumin solutions were analysed by UV/Vis spectroscopy. The quantification was performed as previously reported [17,24] using a double beam UV/Vis spectrophotometer (Spectrophotometer U-3010, Hitachi, Japan). A calibration curve was established for standard solutions of curcumin in ethanol (96% v/v) ranging from 0.31 μg/ml to 5.01 μg/ml with $R^2 = 0.9952$. The curcumin content of the samples filled into quartz cuvettes was determined at 425 nm against a control sample of an extracted blank tape strip. Samples containing higher curcumin content were diluted prior to analysis until values within the linear range of the calibration curve were obtained. This was occasionally the case for samples extracted from the first adhesive film of the experiments.

2.11. Fluorescein sodium quantification by fluorescence spectroscopy

After NIR analysis, the tapes containing corneocytes and fluorescein sodium were dissolved in 4 ml of phosphate buffer (pH 7.4, 0.012 M). As described above for curcumin, the samples were homogenised and centrifuged. The resulting fluorescein sodium solutions were analysed by fluorescence spectroscopy. The quantification was conducted using a microplate reader (Tecan™ infinite 200, Tecan Ltd., Maennedorf, Switzerland) at an excitation wavelength of 485 nm, an emission wavelength of 535 nm and a constant gain of 78. A calibration curve was established for standard solutions of fluorescein sodium in phosphate buffer (pH 7.4, 0.012 M) ranging from 0.04 μg/ml to 5.07 μg/ml with $R^2 = 0.9992$ to quantify the drug content of the samples. Samples containing a higher content of fluorescein sodium were diluted prior to analysis. For drug quantification, 100 μl of the diluted samples was pipetted into a microtiter plate in triplicate and analysed against control solutions of extracted blank adhesive films.

2.12. Statistical data analysis

Results are generally expressed as means of three or more experiments ± SD. Statistical data analyses were performed with the program GraphPadPrism3 while using $P < 0.05$ as a minimum level of significance in all cases. Parametric data were analysed using the Student's t test or ANOVA, while non-parametric data were analysed using the Mann–Whitney test or the Kruskal–Wallis test.

3. Results and discussion

In summary, both visual inspection and analysis of the drug content of the formulations confirmed their suitability for the subsequent experiments (data not shown). The following sections deal with the results of the in vitro experiments, followed by the results obtained in vivo and conclusive remarks on the similarities and differences between the data.

3.1. In vitro experiments: porcine ear skin

3.1.1. Transepidermal water loss (TEWL)

The TEWL of fresh or defrosted porcine ear skin in vitro can be employed as an indicator for the intactness of the SC barrier function [25,26]. Since the excised ear does not possess any metabolism, the determined values will of course differ from those

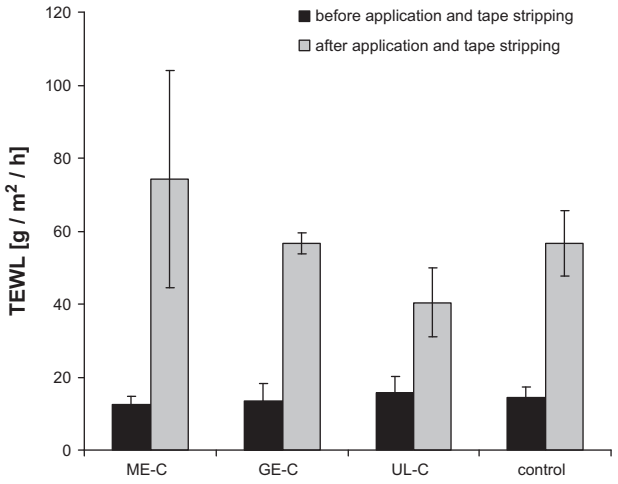

Fig. 1. TEWL of porcine ear skin in vitro measured before and after tape stripping. The influence of the applied formulation on the extent of SC barrier disruption is demonstrated. The TEWL before and after tape stripping of the area treated with the microemulsion containing curcumin is given on the left (ME-C, 20 tapes), followed by the corresponding data for the curcumin-loaded hydrogel (GE-C, 20 tapes) and ultraphil® (UL-C, 20 tapes). The respective values represent the means ± SD of at least seven experiments ($n \geqslant 7$). An untreated skin site, respectively, served as a control (20 tapes, $n = 23$).

in vivo. Nevertheless, experience has shown that lesions or irregularities in the SC barrier function can be identified. The results of the TEWL measurements conducted during in vitro tape stripping are given in Fig. 1. The TEWL value of intact porcine ear skin before tape stripping was always between 10 and 20 g m^{-2} h^{-1}. The experiments were then started immediately to prevent dehydration of the tissue. Upon removal of 20 adhesive films from untreated skin regions, the TEWL increased significantly ($P < 0.05$). However, as can be derived from the large standard deviation, this increase can vary to a large extent due to inter-individual differences. Such differences tend to become negligible upon further tape stripping of the same area. After the removal of 40 tapes from the untreated skin site, the TEWL values reached a peak around 80 g m^{-2} h^{-1}. It may be assumed that the majority of the SC was removed at this point. Removal of further tapes until the complete SC was removed only led to minor increase in the TEWL after this point (data not shown). These findings confirm previous data [7].

Apart from these observations regarding the TEWL of untreated tape stripped skin, the effect of the different formulations applied before tape stripping was investigated (Fig. 1). Apparently, the different formulations influenced the extent of SC barrier disruption during tape stripping in accordance with the employed excipients. The microemulsion, containing large amounts of surfactant and solvents, led to a significantly larger increase in the TEWL after the removal of 20 tapes when compared to the corresponding control site ($P < 0.05$). In contrast, the TEWL values obtained after the removal of 20 tapes from skin sites treated with the hydrogel were comparable to the control ($P > 0.05$). The lowest increase in TEWL was observed after treatment for the skin with ultraphil® cream, which was even lower than the value of the control site ($P < 0.05$). The extent of the SC barrier disruption in vitro can thus be ranked as ME > GE/Control > ultraphil®.

3.1.2. Influence of application time on in vitro skin penetration

Different application times of 1 and 4 h were tested in corresponding experiments with the curcumin-loaded microemulsion, the hydrogel and the ultraphil® cream. The results of these studies are given in Table 2. No significant differences in penetration depth or recovered drug amounts were found for the microemulsion or the hydrogel after 1 and 4 h of application time ($P > 0.05$, respectively). However, a slight tendency towards lower values of penetration depth and drug amounts was generally observed. This indicates that the prolonged application time in vitro does not necessarily yield higher skin penetration. On the contrary, lateral diffusion of the drug may occur [27–29] and dehydration effects may take hold. It was thus decided that 1 h of application time was sufficient for the envisioned experiments. Comparable working protocols have been successfully employed in previous in vitro studies [2]. The phenomenon of the potential lateral spreading effect after prolonged application will be subject of further studies.

In case of the ultraphil® cream, the extent of skin penetration was highly unsatisfying after 1 h. Merely, a prolonged application time of 4 h led to noticeable skin penetration of the drug while a significant amount of formulation still remained on the skin surface. It was apparent that the penetration depth of curcumin when applied in this vehicle was significantly lower even after 4 h of application time than from the other formulations ($P < 0.05$, respectively). Besides, remnants of the formulation still impaired the tackiness of the first adhesive films. Due to its lack of penetration and spreading, ultraphil® cream was thus excluded from further experiments. Modification of the working protocol towards cleaning of the skin surface was not deemed appropriate within the context of the envisioned comparative studies, but this aspect will be investigated separately at a later point.

Regarding the rank order of penetration depth of the compared formulations, an obvious tendency was apparent, namely microemulsion $\geqslant$ hydrogel > ultraphil® cream. Both the microemulsion and the hydrogel yielded significantly higher skin penetration depths than the ultraphil® cream ($P < 0.05$). A statistically significant difference between the penetration depth of the microemulsion over the hydrogel was only reached after 4 h of application time ($P < 0.05$).

Summarising our experiences with different application times, only a slightly longer treatment for the skin than for in vivo experiments seemed most appropriate. The excised porcine ear lacks circulation and is subjected to constant dehydration during the experiment. Insufficient application times might lead to erroneously low penetration depths, which do not represent the in vivo situation. Prolonged application times may yield representative results, but may also promote unphysiological dehydration of the tissue and lateral spreading [27,30]. The decreased skin hydration, accompanied by lower TEWL values, leads to decreased skin diffusion of drugs. This effect might annihilate the positive effect of a longer application time [31]. Although the isolated porcine ear is a suitable and representative model for short-time studies, it appears to be less adequate for longer application periods, especially for highly lipophilic drugs. Appropriate sink conditions may not be reached due to the lack of cutaneous circulation [9]. Thus, decreased penetration rates of model drugs after prolonged penetration times may occur [32].

3.1.3. In vitro skin penetration profiles

The results of the in vitro tape stripping experiments for both curcumin and fluorescein sodium after 1 h of application time are summarised in Table 2. Overall, the amount of penetrated drug was highly comparable for all in vitro experiments irrespective of the lipophilicity of the investigated drug and the type of formulation ($P > 0.05$). In contrast, the penetration depth of the different drugs was influenced by both the excipients of the formulations and the log P value of the drug. The hydrophilic fluorescein sodium achieved deeper skin penetration than the lipophilic curcumin from the corresponding microemulsion ($P < 0.05$), reaching values around 50% of the entire SC. This might be due to the different solubilities of the drugs within the microemulsions. After

Table 2
In vitro skin penetration data for the different investigated formulations after application of 2 mg/cm^2 on porcine ear skin. The influence of different application times of 1 and 4 h on skin penetration of curcumin is shown. The given values are means ± SD of at least six individual experiments for each formulation ($n \geqslant 6$).

Formulation	Application time (h)	Penetrated drug amount (μg/cm^2)	Penetration depth (μm)	Penetration depth (% of SC)
ME curc	1	7.48 ± 1.32	2.26 ± 0.85	27.56 ± 10.37
ME curc	4	6.71 ± 1.76	1.82 ± 0.65	22.20 ± 7.93
GE curc	1	8.25 ± 3.84	1.26 ± 0.69	15.37 ± 8.41
GE curc	4	7.26 ± 1.97	0.91 ± 0.36	11.10 ± 4.39
UL curc	1	Not feasible		
UL curc	4	6.58 ± 1.57	0.39 ± 0.14	4.76 ± 1.71
ME fluor	1	7.65 ± 1.56	3.82 ± 0.88	46.59 ± 10.73

application of the formulations, volatile compounds evaporate and the drugs may re-crystallise due to the destruction of the formulation structure. Since crystallised drug is no longer available for penetration, this might hinder the achieved penetration depth especially in case of lipophilic drugs that are mainly dissolved by the involved solvents (verbal communication with Dr. Thomas Franz, 2011). In case of the different curcumin-loaded formulations, an effect of the vehicle on the penetration depth of the lipophilic drug was noticeable. As already discussed, higher penetration of curcumin was obtained with the microemulsion than with the hydrogel. After 1 h of application time, these differences did not quite reach significant levels due to the strong inter-individual variability within the experiments ($P > 0.05$, with $P = 0.0505$). Nevertheless, the general tendency was apparent.

3.1.4. Influence of the formulation on the amount of removed SC proteins in vitro

An additional aspect that was investigated was the influence of the applied formulation on the amount of removed SC proteins. Fig. 2 shows the comparison between the amount of SC proteins removed from untreated porcine skin as a control and the amount of SC proteins that was removed after application of the different formulations. As can be seen in Fig. 2a, the microemulsion rapidly penetrated into the skin without leaving significant traces of formulation on the skin surface. Thus, the removal of SC proteins was not hindered by the application of the formulation when compared to the control ($P > 0.05$). In case of the hydrogel (Fig. 2b), a significant influence of the formulation on the amount of removed corneocytes was found until the 7th removed adhesive film ($P < 0.05$, respectively). Slightly lower amounts of SC were removed, possibly due to decreased tackiness of the tapes. Interestingly, no weakening of the cohesion between corneocytes caused by the ethanol within the microemulsion and the hydrogel was observed, which would have led to higher amounts of removed corneocytes [30]. The subsequent tapes were not impaired in their tackiness by the remnants of the formulation when compared to the control ($P > 0.05$). In case of ultraphil®, significantly less corneocytes were removed with all 20 adhesive films as opposed to the control ($P < 0.05$, Fig. 2c). This pronounced influence of the formulation on the tape stripping process can be ascribed to the insufficient penetration of the formulation, in particular the lipophilic compounds, which remained on the skin surface and impaired the tackiness of the tapes [30]. In such cases, cleaning of the skin surface prior to the tape stripping process appears essential to obtain reliable results. The cleaning of the skin surface with solvents may likewise influence the results obtained with the first adhesive tapes. Thus, an acceptable compromise has to be found in accordance with the respective formulation properties.

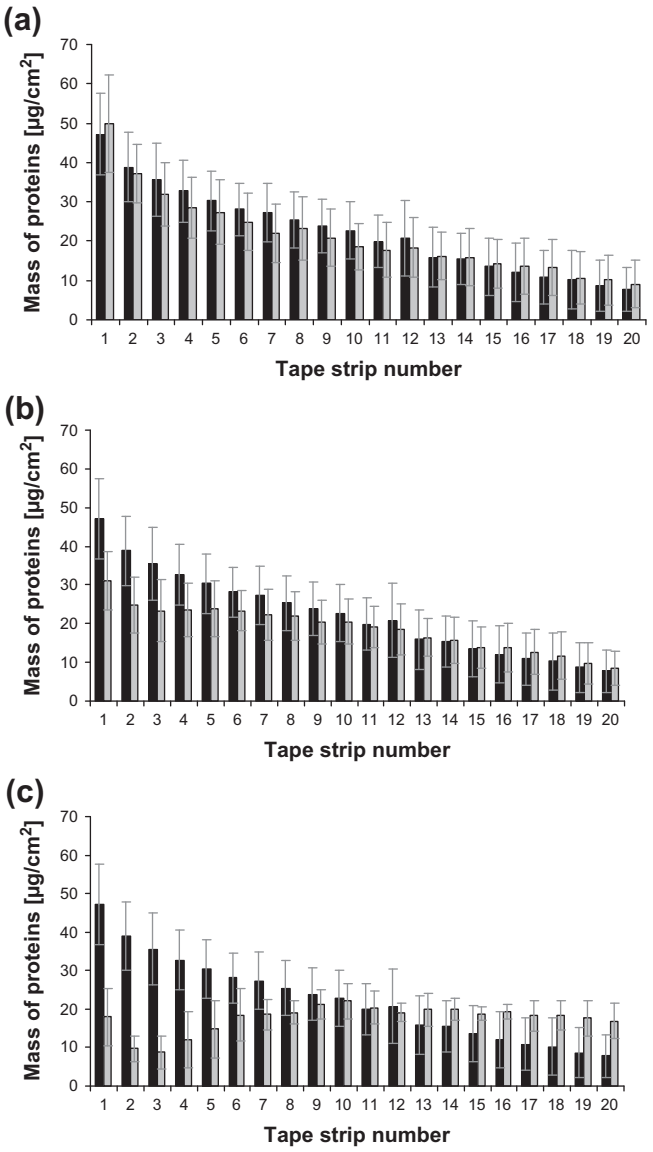

Fig. 2. Influence of the applied formulation on the amount of removed porcine SC proteins in vitro. The black bars, respectively, represent the amount of SC proteins removed from untreated skin areas (control). The grey bars, respectively, represent the amounts of SC proteins removed after the application of a microemulsion (a, after 1 h), a hydrogel (b, after 1 h) or ultraphil® cream (c, after 4 h). The given values represent the mean of at least six experiments ($n \geqslant 6$).

3.2. In vivo experiments: human forearm skin

3.2.1. Transepidermal water loss (TEWL)

The TEWL can be employed to characterise the skin barrier function of human volunteers in vivo [25,26]. The results of the long-term in vivo monitoring of the TEWL of human volunteers are given in Table 3 panels a and b. The TEWL of each volunteer was determined over the course of 4 months in regular intervals both on the forehead as well as the left and right forearm. At least 29 independent measurements were taken for each person

Table 3
TEWL of human volunteers. The TEWL of each volunteer was determined over the course of 4 months by means of at least 29 independent measurements per person ($n \geqslant 29$), which were conducted in regular intervals. The mean of these TEWL values was, respectively, considered as representative for each individual. Table 2a shows the mean TEWL values in g m^{-2} h^{-1} ± SD as determined for all volunteers. The data were evaluated in regard to different criteria as detailed below. Table 2b demonstrates the mean TEWL in g m^{-2} h^{-1} ± SD of all measurements conducted during the course of each month.

Panel 3a				
Investigated group	Number of volunteers	Mean TEWL (g/m^2 h) ± SD		
		Left forearm	Right forearm	Forehead
All volunteers	n = 26	11.67 ± 2.68	12.31 ± 2.85	24.16 ± 4.04
Female Volunteers	n = 15	10.79 ± 1.85	11.11 ± 1.97	24.37 ± 3.74
Male Volunteers	n = 11	12.87 ± 3.23	13.95 ± 3.12	23.89 ± 4.60
Age 18–25	n = 12	12.10 ± 3.47	12.52 ± 3.71	24.19 ± 4.09
Age 26–55	n = 14	11.42 ± 1.83	12.10 ± 2.04	24.06 ± 4.31
Skin care products	n = 18	11.00 ± 2.05	11.64 ± 2.56	24.14 ± 3.54
No skin care product	n = 08	13.16 ± 3.44	13.82 ± 3.04	24.23 ± 5.28
Sweating	n = 15	11.90 ± 2.25	12.69 ± 2.91	24.40 ± 4.00
No sweating	n = 11	11.35 ± 3.27	12.00 ± 2.94	24.35 ± 4.12
Skin type I	n = 03	10.58 ± 1.11	11.67 ± 0.80	20.94 ± 1.65
Skin type II	n = 10	11.69 ± 3.38	11.96 ± 3.14	23.18 ± 3.71
Skin type III	n = 10	12.05 ± 2.55	13.08 ± 3.19	25.65 ± 4.26
Skin type IV	n = 03	11.03 ± 0.62	11.44 ± 0.78	24.94 ± 4.96
Panel 3b				
Month	Number of measurements	Mean TEWL (g/m^2 h) ± SD		
		Left forearm	Right forearm	Forehead
February	n = 85	10.81 ± 3.32	11.54 ± 3.70	22.36 ± 5.50
March	n = 109	12.05 ± 3.18	12.44 ± 3.34	23.86 ± 5.61
April	n = 170	11.67 ± 3.32	12.48 ± 3.74	24.43 ± 5.26
May	n = 155	10.80 ± 2.88	11.60 ± 3.46	25.24 ± 5.51

($n \geqslant 29$). The mean of these TEWL values was considered as representative for each volunteer. The resulting final TEWL values of the volunteers were then evaluated in regard to different criteria to elucidate the potential influence of parameters such as sex, age, the use of skin care products, sweating, skin type (Table 3 panel a) or the season (Table 3 panel b) on the skin barrier function.

As expected, the TEWL of the different investigated skin regions varied for the forehead and the forearm regions. The determined absolute values were around 11–12 g m^{-2} h^{-1} for the forearm skin and around 25 g m^{-2} h^{-1} for the thinner skin region covering the forehead, which is in accordance with previous findings [26]. The higher TEWL of regions with increased permeability can be related to the smaller corneocytes size [26,33], the smaller number of SC cell layers [20] and the density of eccrine sweat glands [16].

No significant difference was found between the TEWL of the left and right forearm ($P > 0.05$). Likewise, no significant difference was found between the TEWL of the respective regions for male and female or younger and older volunteers ($P > 0.05$, respectively). This indicates that neither gender nor age influence the TEWL, which is in accordance with previous reports dealing with the effect of ethnicity, age and sex on the TEWL [26,34]. However, other opinions can be found [35]. None of the other investigated criteria, such as the regular use of skin care products, the inclination to sweating or the skin type of the participants according to the Fitzpatrick scale [36] proved to be of significance regarding the TEWL ($P > 0.05$ in all cases).

Monitoring of the TEWL results for all volunteers as determined during the course of several months revealed a slight, if significant variation of the TEWL during this time ($P < 0.05$, Table 3 panel b). In case of the forehead, it may be assumed that the increasing external temperature might have an effect on the TEWL despite air conditioned laboratory surroundings. In case of the forearms, however, no clear tendency in this respect could be observed, which indicates that a slight variation of the TEWL values can be regarded as normal during long-term observation. The results of evaporimetric methods even with closed-chamber systems may be influenced by the microclimate near the skin surface, which in turn is inevitably influenced by the present overall climate at a given time of the year [37]. In summary, no significant effects of the different subject-related variables on the TEWL were found in the long-term study apart from the obvious difference between the anatomical sites. The season of the measurements was found to have a slight impact on the TEWL, as confirmed by literature where seasonal variations in biophysical and biological characteristics of the SC have been reported [35,38].

Apart from this long-term observation, the TEWL before and after tape stripping was determined as an indicator for the barrier function of the skin [17]. The TEWL of untreated skin was in accordance with the results of the long-term monitoring and previous

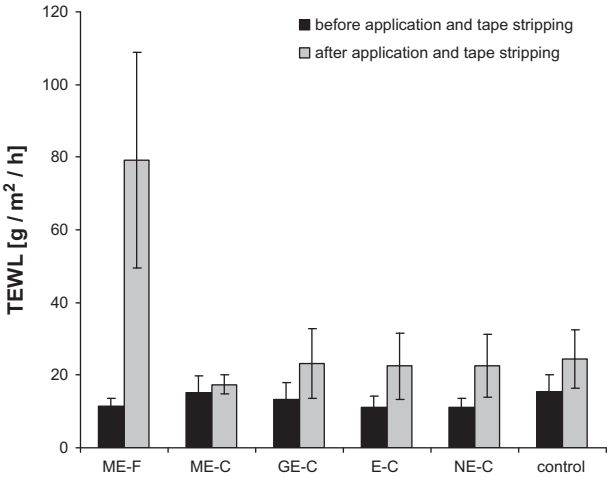

Fig. 3. TEWL of human forearm skin in vivo measured before and after tape stripping. The TEWL before and after tape stripping of the area treated with the microemulsion containing fluorescein sodium is given on the left (ME-F, 40 tapes), followed by the corresponding data for the curcumin-loaded microemulsion (ME-C, 20 tapes), hydrogel (GE-C, 20 tapes), macroemulsion (E-C, 20 tapes) and nanoemulsion (NE-C, 20 tapes). An untreated skin site served as a control. Values represent the means ± SD of six experiments ($n = 6$).

studies [26,33]. Interestingly, the in vivo TEWL values observed after the removal of 20 adhesive tapes were highly comparable for both untreated skin and skin treated with the different formulations (Fig. 3). Thus, in contrast to the in vitro results, only a slow increase in TEWL was observed in vivo, which was not influenced by the presence of topical formulations. Literature confirms that a slow and variable increase in TEWL of human skin is observed during the initial tape stripping process. Only when a major part of the SC is removed, the TEWL increases strongly [39], as can be seen for the microemulsion with fluorescein sodium where 40 adhesive tapes were removed (Fig. 3). The initial changes depend largely on the condition of the SC as well as on the exact tape stripping procedure. Inter-individual variations might be related to site-dependent differences in spontaneous desquamation and SC cohesion [20].

3.2.2. In vivo skin penetration profiles

The results of the in vivo tape stripping experiments are given in Table 4. As for the in vitro experiments, the skin penetration of fluorescein sodium from the microemulsion was higher than that of curcumin from the same or other vehicles ($P < 0.05$). Overall, the same tendencies as for the in vitro experiments were found. The penetration of curcumin tended to be higher from the microemulsion than from the hydrogel or the macro- and nanoemulsions. However, none of these differences reached a significant level ($P > 0.05$, respectively). As already observed in previous in vitro studies [11], the skin penetration behaviour of the macroemulsion and the nanoemulsion was highly comparable despite the marked difference in particle size and rheological properties ($P > 0.05$). For all formulations, no statistically significant differences were found when comparing the results of the tape stripping experiments performed on female and male volunteers ($P > 0.05$).

3.2.3. Influence of the formulation on the amount of removed SC proteins in vivo

The observed tendencies were in agreement with previous in vivo investigations reporting an obvious influence of the applied formulation on the amount of removed corneocytes [29]. In case of the microemulsion and the hydrogel, slight if significant differences were noticeable for the first few tapes ($n = 6$, $P < 0.05$, respectively) in accordance with the effects discussed and depicted for the in vitro data. In case of the macroemulsion and the nanoemulsion, significant differences were also noticeable for the first few tapes ($n = 6$, $P < 0.05$, respectively). However, more corneocytes were apparently removed with the first few tapes after application of these formulations when compared to the control. It may be assumed that the cohesion of the corneocytes might have been decreased after application of the highly hydrophilic systems. Moisturising O/W creams can cause acute hydration of the SC, an increase in TEWL and weakened SC cohesion. Thus, higher amounts of protein are removed though tape stripping [16,20,22].

In summary, the amount of removed corneocytes both in vivo and in vitro may be influenced in different ways. The applied formulations may lead to reduced tackiness of the tapes, especially if highly lipophilic creams with slow skin penetration or solvents are applied. In this case, modification of the working protocol towards cleaning of the skin should be considered. On the other hand, moisturising or occlusive formulations may lead to increased skin hydration, thus causing facilitated removal of corneocytes due to reduced cohesion. These findings once again confirm the need for the quantification of the removed corneocytes on each individual tape.

Table 4
In vivo skin penetration data for the different investigated formulations after application of 2 mg/cm^2 on human volar forearm skin for 30 min. Six experiments were performed for each formulation ($n = 6$), values are given as means ± SD.

Formulation	Penetrated drug amount (μg/cm^2)	Penetration depth (μm)	Penetration depth (% of SC)
ME curc	6.45 ± 2.28	3.08 ± 1.13	28.00 ± 10.27
GE curc	8.68 ± 1.08	2.70 ± 0.68	24.55 ± 06.18
E curc	7.03 ± 1.17	2.52 ± 0.47	22.91 ± 04.27
NE curc	6.60 ± 1.50	2.36 ± 0.62	21.45 ± 05.64
ME fluor	12.89 ± 2.81	5.61 ± 1.35	51.00 ± 12.27

3.3. Validity of the porcine ear model

Different in vitro models exist to mimic human skin penetration in vivo. The benefits of ex vivo investigations are manifold: ethical approval is not required, multiple replicate experiments are more easily performed and toxic compounds can be evaluated [9].The difference between in vivo and in vitro data using excised human skin may become very small if working protocols are harmonised [40]. However, in vitro studies using excised human skin usually rely on material obtained from the abdomen after liposuction, while in vivo studies are conducted on the human forearm. Thus, the obtained data will exhibit certain differences, especially if the skin has been subjected to heat-separation [40]. Therefore, porcine ear skin may be equally if not more suitable as an in vitro model, delivering good correlations [41]. Pig skin shares essential permeation characteristics with human skin, especially for lipophilic components [18]. The physiological conditions of porcine ear skin can be kept almost constant since the porcine ear skin remains on the ear cartilage, the skin barrier is not interrupted, dehydration is decelerated and no contraction occurs [1]. The accuracy of the data depends on the use of proper methodology that has to be standardised and optimised for each in vitro set-up [41]. Previous studies have shown that porcine ear skin is a suitable substitute for human skin when performing in vitro experiments [2,9,42].

In the present study, comparable tendencies were observed after in vivo and in vitro tape stripping, which confirms that the porcine ear model is suitable for conducting comparative studies to evaluate skin penetration of different vehicles. Fig. 4 shows the respective penetration profiles of the curcumin-loaded microemulsion in vivo and in vitro. Both penetration depth in regard to the entire horny layer thickness and the recovered drug amounts were highly comparable. As can be seen from the profiles, very similar amounts of corneocytes were removed with every tape during the tape stripping process from the human forearm skin in vivo (Fig. 4A). As a consequence of this very constant removal process, the drug amounts on the individual tapes were equally homogeneously distributed, as can be derived from the very homogeneous thickness of the individual bars in Fig. 4A. In contrast, the corneocytes of the porcine skin surface appeared to be less densely packed. As can be seen in Fig. 4B, large amounts of corneocytes and, consequently, adherent penetrated drug were removed with the first adhesive films. The number of removed corneocytes and drug per tape decreased constantly with increasing tape number, which might be ascribed to the increasing corneocyte adhesion in the deeper cell layers and an increasingly homogeneous skin structure. Indeed, this more irregular surface structure of the porcine skin with loose cell clusters and canyons has already been reported [7] and is well reflected in the presented results.

Despite these general differences regarding corneocyte removal, the overall trends regarding skin penetration were remarkably comparable for in vitro and in vivo experiments (Fig. 5), especially in the case of the microemulsions both with fluorescein sodium and curcumin. A certain level of variability in individual skin penetration depths was observed both in vivo and in vitro, which should however be acceptable for practical application [9,43]. Determination of six individual samples is generally sufficient to

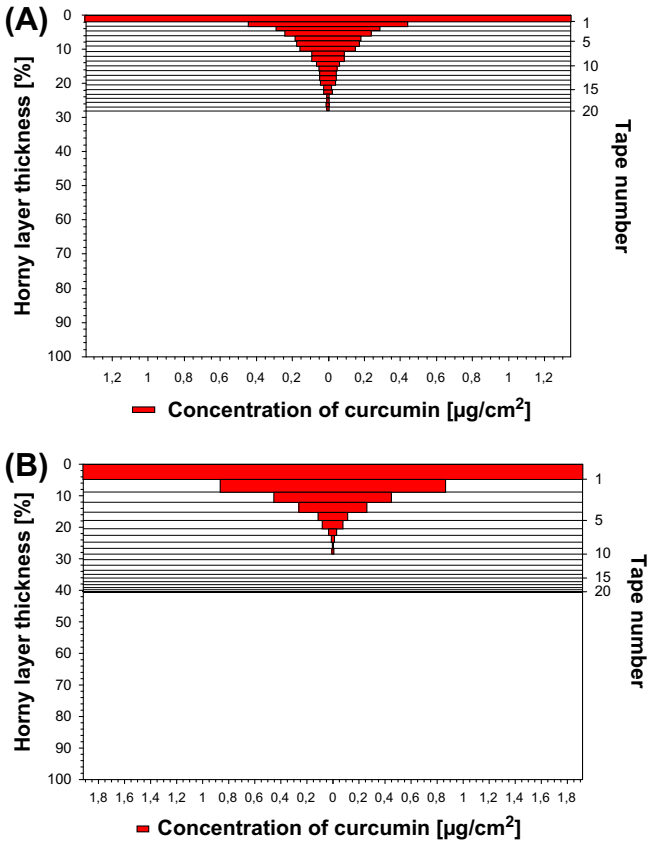

Fig. 4. Skin penetration profiles of curcumin from a microemulsion: comparison of data obtained on human forearm skin in vivo (A) and porcine skin in vitro (B). The depicted values are means of six individual experiments ($n = 6$, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

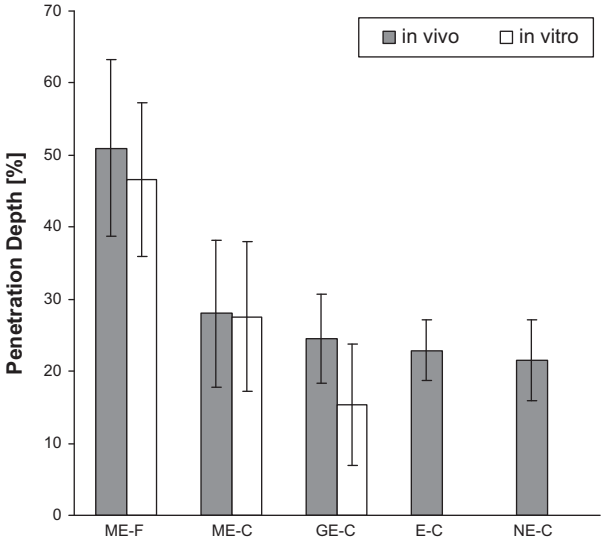

Fig. 5. Comparison of in vivo and in vitro skin penetration of the model drugs fluorescein sodium (F) and curcumin (C) from different formulations. The penetration depth of fluorescein sodium from the microemulsion is given on the left (ME-F), followed by the corresponding penetration data for curcumin from the microemulsion (ME-C), the hydrogel (GE-C), the macroemulsion (E-C) and the nanoemulsion (NE-C). The given values, respectively, represent the means ± SD of 6 individual experiments for both in vitro and in vivo studies.

obtain representative tendencies [2]. Regarding the penetrated drug amounts, the respective values obtained with the curcumin-loaded microemulsion and hydrogel were highly comparable for the in vitro and in vivo studies ($P > 0.05$, respectively). This confirms that the delivered drug load is primarily dependent on passive diffusion, which appears to be in the same order of magnitude for porcine and human skin at the chosen experiment times. Merely, in case of the fluorescein-loaded microemulsion, a slight if statistically significant difference was found between the permeated drug amounts in vitro and in vivo ($P < 0.05$). The higher penetrated drug amount in vivo might be related to the physiological skin hydration, which is continually decreasing in the excised porcine ear tissue and influences the penetration of hydrophilic drugs more than lipophilic ones [17].

When regarding the numerical values represented in Fig. 5 and detailed in Tables 2 and 4, the microemulsion with fluorescein sodium penetrated the SC in vitro to an extent of 46.59 ± 10.73%, while the in vivo penetration reached values of 51.00 ± 12.27%. Thus, the in vitro penetration achieved 91.34% of the actual in vivo values. In case of the microemulsion with curcumin, the in vitro values represented 98.43% of the actual in vivo penetration. In case of the hydrogel, a slight underestimation was reached in vitro with 62.60% of the penetration depth in vivo. Such an underestimation may be observed in vitro with porcine ear skin especially if the follicular pathway of penetration is involved, which is impaired in vitro due to the lack of physiological movement that may favour penetration via this route [42]. Dehydration of the tissue may play an additional role. The macroemulsion and the nanoemulsion, which were evaluated for in vivo application, achieved only slightly lower penetration depths than the other formulations.

Overall, the good in vivo/in vitro correlation obtained for the microemulsions and the hydrogel suggests that in vitro tape stripping experiments may be sufficiently representative to replace in vivo tape stripping for further studies with these formulations.

3.4. Skin penetration of macroemulsion vs. nanoemulsion in vivo

Colloidal systems stabilised by carbohydrate-based surfactants possess great potential for dermal drug delivery [44,45]. Recently, both macroemulsions and nanoemulsions stabilised by sucrose stearate were developed [11]. Interestingly, their in vitro skin penetration behaviour was highly comparable despite the significant differences in particle size and viscosity. Since the outcome of in vitro studies may be influenced by the experimental set-up, we decided to confirm the obtained results by tape stripping experiments conducted in vivo. Overall, few reports deal with the actual skin penetration potential of submicron-sized emulsions in vivo and no such data exist for the novel semi-solid and liquid formulations based on sucrose stearate.

The presented in vivo results confirmed the tendencies observed in vitro [11], namely identical skin penetration potential of both systems ($P > 0.05$, Fig. 5). This is in contrast to numerous reports on the superior skin penetration of nanosized colloidal systems [46–48]. Possibly, the large droplets of the macroemulsion exhibit a larger contact area that fosters penetration of drugs into the skin [15]. This aspect might make up for the comparatively smaller droplet surface area of the macroemulsion in comparison with the nanoemulsion, which is usually held accountable for improved skin penetration of nanosized vehicles. Surprisingly, both systems achieved skin penetration rates that were comparable to those of the microemulsion and the hydrogel ($P > 0.05$) although the latter formulations contain large amounts of surfactants and solvents. It may thus be assumed that these emulsions represent a skin-friendly alternative to the classic vehicles.

4. Conclusion

The presented data confirm that porcine ear skin is a highly suitable model for in vitro tape stripping. The penetration profiles of curcumin and fluorescein sodium obtained after a finite-dose application of different vehicles were in excellent agreement with the corresponding in vivo data. The results can be regarded as a complementary validation of the different NIR-densitometric calibration data for the quantification of human and porcine SC proteins. Moreover, it was confirmed that the skin penetration potential of recently developed semi-solid sucrose stearate-based macroemulsions is comparable to that of fluid nanoemulsions of identical composition despite significant differences in particle size and viscosity.

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