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Research paper

Comparison of stratum corneum penetration and localization of a lipophilic model drug applied in an o/w microemulsion and an amphiphilic cream

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Abstract

Vehicle dependent effects on the penetration behavior of drugs following topical application are well known from the literature. In this context, many reports concerning the enhancing activities for hydrophilic as well as lipophilic substances by colloidal drug carrier systems, particularly microemulsions, are available. However, there is little knowledge about the localization of the drugs within the skin and the stratum corneum, respectively. In the present study, the lipophilic dye curcumin incorporated in an oil-in-water microemulsion and in an amphiphilic cream was applied onto the skin of human volunteers. Using the method of tape stripping to remove the stratum corneum (SC), the depth profiles of the dye within the horny layer were compared. Applying the microemulsion, a deeper part of the SC was accessible by a number of 20 tapes removed and significantly smaller amounts of curcumin were found on the skin surface. Also differences in the distribution and localization of the dye within the stratum corneum were observed by laser scanning microscopy. Furthermore, curcumin was detected in hair follicles. It was obvious that the microemulsion led to a penetration into the complete follicular infundibula, whereas, following application of the cream, a fluorescence signal was only received from the follicular orifices.

Keywords: Microemulsion; Penetration; Curcumin; Tape stripping; Stratum corneum; Follicle

1. Introduction

Human skin is an important target site for the application of drugs. Especially in the treatment of local diseases, a topical drug delivery is an appropriate strategy to restrict the therapeutic effect on the affected area and to reduce systemic incrimination. In order to reach therapeutic drug concentrations in certain skin layers, the uppermost barrier, the stratum corneum (SC), has to be overcome. This process is affected by various factors, e.g., the physicochemical properties of the drug and the vehicle used for application [1–10]. The focus of our research work is to increase dermal availability of lipophilic drugs. From in vivo studies it is known that they are preferably localized on the skin surface and in the superficial SC after topical administration [1,7]. However, using the optimal vehicle, even for lipophilic substances it is possible to minimize this accumulation.

Modern drug carrier systems are microemulsions (MEs). These are thermodynamically stable, low viscous, transparent and optical isotropic formulations with a dynamic microstructure that form spontaneously by combining appropriate amounts of a lipophilic and a hydrophilic ingredient, as well as a surfactant and a co-surfactant. During the last years, a number of investigations have been carried out which demonstrated that drugs incorporated into

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microemulsions penetrate efficiently into the skin and through the SC-barrier [11,12]. Important features are their high drug solubilization capacity, which leads to high concentration gradients towards the skin and a microstructure that allows free and fast drug diffusion [12,13]. Dependent on the physicochemical properties of the active substance, different types of microemulsions can be the optimal carrier. Lipophilic drugs are preferably solubilized in o/w microemulsions whereas w/o (water-in-oil) systems seem to be the better choice for hydrophilic drugs [14].

In the present study, the in vivo penetration of the model drug curcumin was investigated in volunteers. Curcumin is a lipophilic fluorescent food dye, which has already been used to examine the reservoir function of the SC [15] and the distribution of topically applied substances within this layer [3,16].

Vehicle dependent effects on amount and distribution of curcumin within the stratum corneum were studied by means of tape stripping. Therefore, the dye was incorporated into an o/w microemulsion (ME) and an amphiphilic cream (Cremor basalis DAC). The latter does not show a typical droplet-like microstructure. According to Junginger, it represents an amphiphilic system of three coherent phases which are water, lipophilic ingredients and lamellar ordered surfactants [17]. In order to obtain reliable depth profiles also the amount of removed corneocytes was determined.

Additionally, differences in the localization of curcumin within the stratum corneum due to the vehicles were investigated by laser scanning microscopy (LSM). Since the follicular pathway might play a more significant role in dermal drug uptake [15,18–22], LSM was also applied to visualize a possible participation of the appendages, i.e., the follicles, on the penetration process.

2. Materials and methods

2.1. Volunteers

The study was performed on the flexor forearms of 6 healthy volunteers, 4 male and 2 female, aged between 21 and 31 years (mean age: 24.7 ± 3.8 years), with skin photo-types I–III [23]. Approval for these experiments had been obtained from the Ethics Committee of the Charité and the volunteers had signed informed consent forms.

2.2. Applied formulations

Curcumin (Merck-Schuchardt, Hohenbrunn, Germany) was the lipophilic model drug. As its pK_a is in the region of 8 [24], the protonated, sparingly soluble state of curcumin dominates in all chemical environments occurring in the study, e.g., in the formulations used and the SC (pH 4.5–6.9 [25]). The partition coefficient within the relevant pH-range is given by $\log D \geqslant 2.9$ [24].

0.5% of this fluorescent dye was incorporated in an amphiphilic cream (Cremor basalis according to *Deutscher Arzneimittel Codex 2003* [26], purchased from Synopharm, Barsbüttel, Germany) as well as in an o/w microemulsion.

The amphiphilic cream contains 4% glycerol monostearate, 6% cetyl alcohol, 7% polyoxyethylene glycerol monostearate, 7.5% medium-chain triglycerides, 10% propylene glycol, 25.5% white soft paraffin, and 40% distilled water.

The microemulsion consisted of 8% Tagat® O2 (polyoxyethylene glycerol mono-oleate, kindly provided by Frankenchemie, Wendelstein, Germany), 12% Synperonic® PE/L 101 (poloxamer 331, kindly provided by C.H. Erbslöh KG, Krefeld, Germany), 5% Pelemol® BIP (eutectic mixture of *N*-butylphthalimide and *N*-isopropyl-phthalimide, kindly provided by Phoenix Chemical Inc., Somerville, NJ, USA), 50% propylene glycol (Caesar & Loretz GmbH, Hilden, Germany) and 25% distilled water. The formulation was obtained simply by stirring the prepared surfactant blend and the lipophilic component. Subsequently, the mixture of propylene glycol/water was added up to 100%. The percentage values given represent % (w/w).

2.3. Application

The study was performed under controlled conditions, which means constant temperature and humidity. Both formulations were tested on the same volunteer on the same day.

The flexor forearms of the volunteers were prepared by rinsing with hand-warm water and drying using soft tissue. Then, two application areas of 5 cm × 6 cm were marked with a permanent marker and a silicon barrier was applied around these areas in order to avoid lateral spreading of the formulations [7]. Afterwards, 2 mg/cm^2 of either the ME or the amphiphilic cream was applied to these areas and distributed homogeneously using a glove-finger saturated with the formulation [27]. During the penetration time of 1 h, which is a common period for standard tape stripping procedures, the volunteers remained seated without covering the skin areas with textiles.

2.4. Tape stripping

One hour after application, the tape stripping procedure was performed utilizing an adhesive film (*Tesa* film No. 5529, Beiersdorf, Hamburg, Germany) with a width of 19 mm. A piece of this adhesive film of approx. Five centimeters in length was applied onto the treated skin and pressed with a roller to stretch the skin surface [27]. Thus, the influence of the furrows and wrinkles on the tape stripping procedure is negligible [28]. Each tape strip was removed with a quick movement and fixed onto a slide frame for handling. For the first volunteer, the horny layer had been removed completely using 80 tape strips [29]. After the removal of 20 tape strips for both formulations, only small amounts of curcumin were detected in the deeper layers of the SC. Therefore, only 20 tape strips were taken in the further experiments performed on 5 additional

volunteers. In these experiments, 10 tape strips were removed from the non-treated skin area next to the application sites. In this way, a relative SC amount of 38% was removed [30]. This value was used to calculate the penetration profiles.

2.5. UV/VIS spectroscopic measurements

UV/VIS spectroscopic measurements were performed using a double beam spectrometer (Lambda 20, Perkin-Elmer, Überlingen, Germany) equipped with a quadratic beam of $10 \times 10\ mm^2$. The pseudo-absorption of the corneocytes, which correlates with the amount on the removed tape strips [31], was detected at 800 nm. At this wavelength, the pseudo-absorption is not disturbed by the absorption band of the formulation's components and the curcumin.

Subsequent to the spectroscopic measurement of the corneocytes, the tape strips were cut to a constant size of $1.5\ cm \times 2.9\ cm$ and extracted with 4.35 ml ethanol. The extracts were treated with ultrasound (Sonorex Super RK102H, Bandelin Electronic, Berlin, Germany) for 10 min and centrifuged at 4000 rpm for another 10 min at 20 °C (Centrifuge MR1812, Jouan GmbH, Unterhaching, Germany).

Thereafter, spectra of the clear supernatant were recorded between 300 nm and 600 nm, and the curcumin concentration of each extract was determined at the maximum of the absorbance at 430 nm using the software UVWinLab version 2.70.01 (Perkin-Elmer). The extract of an empty tape strip was used as a reference.

2.6. Penetration profile

The amount of corneocytes detected via the pseudo-absorption at 800 nm on the single tape strips was used to determine the horny layer profile for volunteer No. 1. In the case of the volunteers Nos. 2–6, the sum absorbance of the corneocytes sticking to 10 tape strips removed from the non-treated skin area was determined *via* the pseudo-absorption measured for each individual strip. This value corresponding to 38% of the removed SC was used to calculate the horny layer profiles [30]. The concentration of curcumin was related to this horny layer profile via the position of the corresponding tape strip. In this way, penetration profiles of the dye were obtained as described previously [2].

2.7. Cyanoacrylate surface biopsy

Cyanoacrylate surface biopsies were removed from the treated skin areas to investigate the follicular penetration. After partial removal of the horny layer with 20 tape strips, a small quantity of cyanoacrylate glue (UHU GmbH & Co. KG, Bühl, Germany) was homogeneously applied onto the treated skin area. Subsequently, a piece of adhesive tape was applied onto the glue and pressed with a roller. After approx. 3 min, the tape was removed with one quick movement and the follicular casts together with corneocytes from the deeper SC remained on this tape strip. These experiments were performed exemplary on the volunteers Nos. 3–6.

2.8. Laser scanning microscopy

The distribution of the fluorescent dye curcumin on the removed tape strips (volunteer No. 1) and in the follicular casts of the cyanoacrylate surface biopsies was investigated using laser scanning microscopy (LSM 2000, Carl Zeiss, Jena, Germany). The fluorescence of curcumin was excited at a wavelength of 488 nm and detected at a wavelength of approximately 590 nm [16]. No fluorescent signal could be detected on the tape strips or the cyanoacrylate surface biopsies, if the skin area had not been treated with the curcumin-containing formulations.

2.9. Statistics

The recovery rates of the fluorescent dye curcumin were calculated as the ratio of the substance amount found on the tape strips [$\mu g/cm^2$] to the substance amount applied inside the application area [about $10\ \mu g/cm^2$], multiplied by 100%.

For an adequate statistical analysis with a number of 6 volunteers, non-parametric tests were applied using the software program SPSS®. We utilized the Kolmogorov–Smirnov test to demonstrate that the data are not non-normally distributed ($p > 0.05$). On this basis, mean values and standard deviations were calculated. The Wilcoxon test ($n = 6$, two-sided, paired data) was utilized to analyze the amounts of curcumin and corneocytes removed by the first tape strips after application of both formulations as well as the amount of corneocytes sticking to 20 tape strips.

2.10. Saturation data

Spectroscopic analysis of the diluted clear supernatant after a 24-h shaking of different media containing curcumin in excess was performed at 425 nm (Spectronic 601, Milton Roy Company, USA, $n = 3$). The following media were tested: Pelemol®BIP, propylene glycol and a propylene glycol–water mixture at a ratio of 2:1 (m/m).

3. Results

3.1. Distribution of curcumin within the stratum corneum

3.1.1. Penetration profile

Complete penetration profiles of the lipophilic curcumin were obtained by removing the entire stratum corneum with 80 tape strips. The penetration profiles resulting after application of the dye in the o/w microemulsion and the amphiphilic cream are presented in Fig. 1. In the case of the amphiphilic cream, the main amount of the dye was localized on the skin surface. Decreasing amounts were

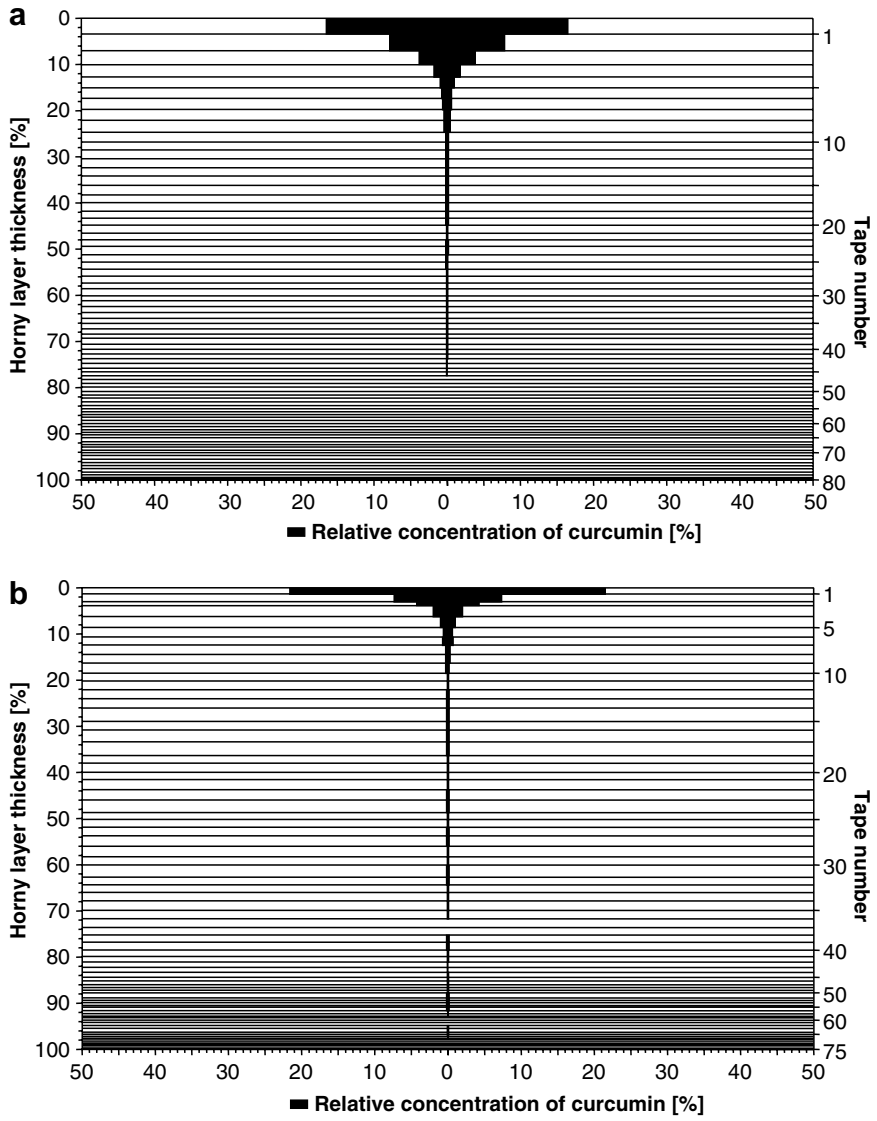

Fig. 1. Complete penetration profiles of curcumin by removing the entire stratum corneum with 80 tape strips after application: (a) in the o/w microemulsion, (b) in the amphiphilic cream (volunteer No. 1).

detected with increasing depth of the SC up to the last tape strip (Fig. 1b). On the other hand, the application of the o/w microemulsion caused a smaller amount of curcumin on the skin surface and decreasing amounts up to a relative SC depth of about 80% (Fig. 1a). Within the following tape strips the curcumin content fell below the limit of quantification (0.02 μg/ml).

After the removal of 20 tape strips, for both formulations only small amounts of curcumin were detected in the deeper layers of the SC. Therefore, only 20 tape strips were removed in further experiments. A similar distribution of the dye was observed in all volunteers. Significantly higher relative rates of curcumin ($p < 0.05$) were detected on the first tape strip after application in the amphiphilic cream ($36.5 \pm 11.1\%$) than in the o/w microemulsion ($26.5 \pm 3.9\%$).

In addition, the pseudo-absorption of the corneocytes removed by the first tape strips was significantly higher ($p < 0.05$) after application of the o/w microemulsion (0.080 ± 0.029) than that of the cream (0.040 ± 0.012).

The amount of corneocytes sticking to all 20 tape strips relative to the total SC was $38.2 \pm 9.6\%$ and $57.2 \pm 10.1\%$ in the case of the cream and the ME, respectively. These values are significantly different ($p < 0.05$) (Fig. 2).

3.1.2. Laser scanning microscopy

The distribution of curcumin within the SC was studied using laser scanning microscopy. The images presented in Fig. 3 were obtained from the first tape strips. Applying the microemulsion, the dye was mainly located around the corneocytes – the fluorescent signal is most intensive around the corneocytes – (Fig. 3a), whereas, in the case of the amphiphilic cream, curcumin was detected in association with the corneocytes and showed a homogeneous distribution of the fluorescent signal over the whole cellular area (Fig. 3b).

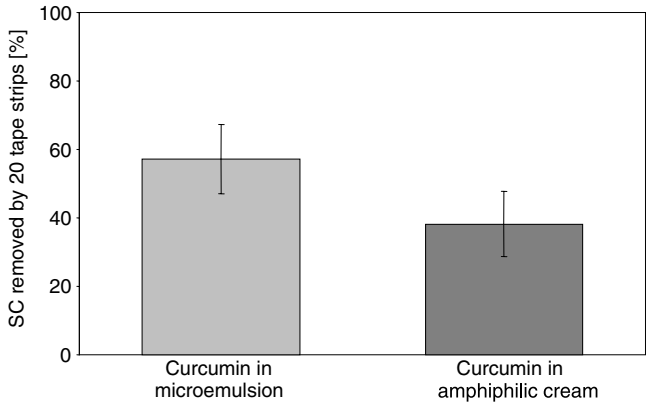

Fig. 2. Amount of stratum corneum removed with 20 tape strips after application of the microemulsion and the amphiphilic cream (mean ± SD, $n = 6$).

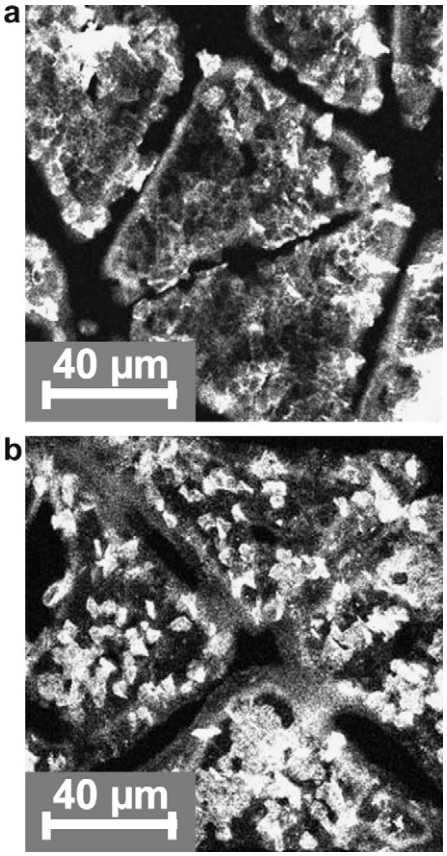

Fig. 3. Fluorescence of curcumin detected on the tape strips using laser scanning microscopy after application: (a) in the o/w microemulsion, (b) in the amphiphilic cream (volunteer No. 1).

3.2. Detection of curcumin in the hair follicles

The penetration of curcumin into the hair follicles was also investigated. Therefore, cyanoacrylate skin surface biopsies were removed from the skin of 4 volunteers after application of the formulations followed by the removal of 20 tape strips. The cyanoacrylate biopsies were investigated using laser scanning microscopy. The images obtained are shown in an exemplary manner (Fig. 4). After the treatment of the skin with the o/w microemulsion, the fluorescent dye was detected in the furrows and in the complete follicular infundibula (Fig. 4a and b), which can be seen by an intensive fluorescent signal in the follicular infundibulum. In the case of the application of the amphiphilic cream, the dye was located in the furrows and only in the follicular orifices. Deeper parts of the follicular casts were free of fluorescent dye (Figs. 4c and d).

3.3. Saturation data

Among the media tested, Pelemol®BIP provided the highest solubility for curcumin (26.88 ± 0.44 mg/ml), followed by propylene glycol (2.61 ± 0.05 mg/ml). Addition of water to the latter led to a further decrease in the saturation level. It was 0.07 ± 0.01 mg/ml for a mixture of propylene glycol and water at a ratio of 2:1 (m/m).

4. Discussion

Vehicle effects on the penetration of topically applied substances were reported in the literature. In particular, microemulsions are known to provide increased penetration rates into deeper skin layers and decreased lag times compared to conventional formulations [5,6], altering both the lipophilic and the polar pathway by synergistic interactions of vehicle components with the SC [32–34].

The results of the present study also show distinct differences in the concentration profile, the localization of curcumin within the SC and the hair follicles dependent on the vehicle.

Compared to the amphiphilic cream (Fig. 1b), the dye was detected in significantly smaller amounts on the skin surface, which was represented by the first tape strip (formulation was not wiped off before tape stripping), when the ME was used. Simultaneously, more corneocytes stuck to this tape (Fig. 1a). This result indicates a more effective penetration of the dye into the SC, if it was applied in the microemulsion.

DelgadoCharro et al. presented a model of multiple factors influencing transdermal drug delivery from microemulsions [32]. It was based on different partitioning processes between ME droplets, continuous phase and skin. Resulting penetration represents the sum of the drug's relative activities in these fractions. Additionally, diffusion of single constituents into the skin is possible, which may reduce the barrier function of the SC by diverse interactions. The formulation can also extract some horny layer components and a new physical entity might realize drug release then. With some restrictions this can be assumed for the amphiphilic cream. However, the diffusion and partitioning processes might occur much slower and to a less extent since this formulation obtains a more rigid and viscous structure.

Consistently, Kriwet and Mueller-Goymann determined low effective diffusion coefficients of the model drug

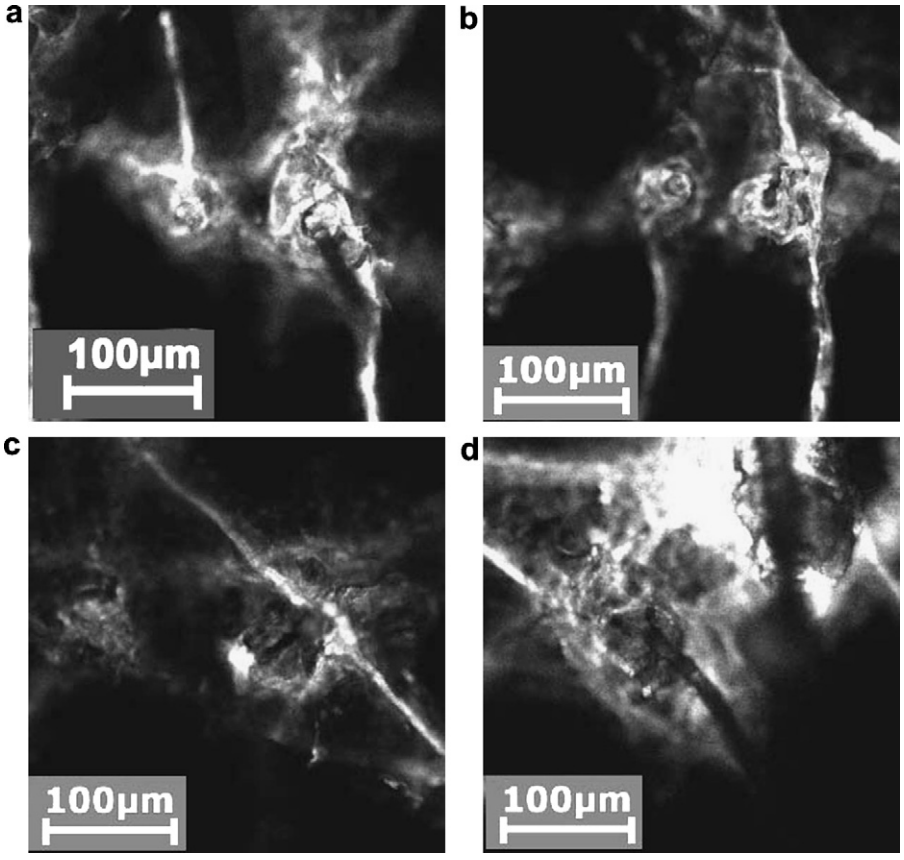

Fig. 4. Fluorescence of curcumin detected on cyanoacrylate skin surface biopsies using laser scanning microscopy after application: (a,b) in the o/w microemulsion, (c,d) in the amphiphilic cream (volunteer No. 3).

diclofenac diethylamine in the case of higher viscous anisotropic gels. Indeed, the rate limiting step for drug transport across SC turned out to be the release from the vehicle. On the other hand, for formulations with high releasing rates (e.g., MEs), the diffusional resistance inside the horny layer was higher than that in the vehicle and, hence, the permeability was controlled by the SC [6].

In general, the low interfacial tension and the continuously and spontaneously fluctuating interfaces of MEs are supposed to facilitate transition of the drug to the skin [12]. Deeper inside into dynamics within the colloidal formulations was provided by NMR-studies of Kreilgaard et al. [13] and Hua et al. [35]. They found a relationship between drug mobility resulting from a certain microstructure and drug flux. Moreover, viscosity of the formulation and permeability were linearly correlated.

Since a molecule in an uncharged state penetrates a lipophilic barrier more easily, pK_a of curcumin which is around 8 [24] and pH values of the formulations provide useful information. In the ME (pH 4.9) as well as in the amphiphilic cream (pH 4.7), the non-dissociated, sparingly soluble state of the dye dominates, also within the SC (pH 4.5–6.9 [25]).

Due to the high solubilization capacity of MEs, a large concentration gradient towards the skin can be reached, which is the driving force in passive diffusion processes. Curcumin was completely solubilized within the ME, however, the vehicle was not fully saturated, whereas in the amphiphilic cream a part of the substance remained suspended as observed microscopically. Although the thermodynamic activity of the drug in the cream is obviously higher than in the ME, the steeper concentration gradient provided by the ME seems to play a more important role. This is in accordance with the literature, where the drug delivery potential of MEs was found to be attributable to large concentration gradients enabled by the high drug load capacity without concurrent increase in the affinity of the drug to the vehicle. Moreover, enhancing effects of the individual ingredients have to be considered [12]. Both formulations contain propylene glycol (PG), which is known to act as cosolvent and penetration enhancer. Different mechanisms are mentioned such as solvent drag effect and favoring of drug partitioning into the skin. Also, solvating of the α-keratin from the corneocytes as well as interactions with polar headgroups of the SC-lipids are discussed [36]. According to Barry and Bennett, its effectiveness seems to depend on the solubility of the drug in propylene glycol [37]. Regarding the ME, curcumin is 10 times more soluble in Pelemol®BIP than in pure propylene glycol. In a propylene glycol–water mixture (ratio 2:1 as used in the ME) the solubility again decreases sharply by factor 37. Therefore, a partitioning of curcumin towards

the colloidal compartment of the ME is assumed and, hence, at least an enhancer effect by a solvent drag mechanism or a facilitated partitioning towards the SC due to PG is unlikely. However, a disordering of the lamellar lipid structures might occur and would be more pronounced in the case of the ME which contains significantly more PG than the amphiphilic cream. Nevertheless, in an in vitro permeation study of Chen et al. MEs with a lower amount of propylene glycol provided higher flux rates of the moderately lipophilic drug triptolide than MEs with higher PG concentrations [38].

As shown in Fig. 2, a significantly deeper part of the horny layer could be removed by 20 tapes in the case of the ME. The colloidal formulation penetrates the skin better, and has less influence on the adhesiveness of the tapes than the constituents of the amphiphilic cream, e.g., soft paraffin, which evidently remain preferably in the superficial layers. These findings correspond to the work of Jacobi et al. on the differences in the amount of removed corneocytes using "vehicle-extrema" [2]. Applying the dehydrating and lipid-extracting agent ethanol, double the amount of corneocytes were stuck to the Tesa® compared to a w/o emulsion. In consequence, the comparison of penetration profiles would be misleading, if they refer only to the tape number and not to the SC-depth.

Up to now, no information about the specific localization of curcumin within the skin, mainly the SC, has been delivered. Therefore, LSM studies were employed.

The major localization of curcumin around the corneocytes, when it was applied in the ME (see Fig. 3a), indicates the distribution and accumulation of the dye inside the lipid layers. Indeed, permeation across intercellular lipids was discussed as the main pathway for topically applied substances [39,40]. In particular, this route was found to be dominant for curcumin [3]. In a corresponding study, Jacobi et al. showed a fluorescence image of a tape with a similar curcumin distribution as received by the ME using paraffin oil as vehicle. Although the ME contains only 5% of lipophilic ingredients, curcumin shows a similar distribution as with the pure, non-polar medium. The comparative consideration of the image taken after administration of the amphiphilic cream (Fig. 3b) clearly demonstrates the vehicle's influence on the localization of the dye. In this case, the substance was detected associated with the corneocytes. Even a crude o/w emulsion as used by Jacobi et al. predominantly shows an association of curcumin with the cells rather than an accumulation between them [3].

The reasons for the differences might be variations in the partitioning behavior and interaction potential of the vehicles (or single components) with the SC and of the curcumin within the vehicles, respectively, as described before. In the case of the ME, the affinity of curcumin to Pelemol® BIP could have an impact on the observed intercellular accumulation.

Additionally, the application of the dye in the ME resulted in a localization of curcumin inside the follicular infundibula (see Figs. 4a and b). In contrast, it was only detected in the orifices (see Figs. 4c and d) using the amphiphilic cream. The result indicates an increase in follicular penetration by the ME. This might be due to their typical very low interfacial tension realizing an outstanding surface contact. Hence, the formulation might also be capable of reaching the interior of the follicles more easily. Similar to the cream, the application of curcumin in oil only afforded localization in the follicular orifices [15]. Biju et al. achieved similar results when they compared the follicular concentration of tea tree oil applied in different vehicles, e.g., an ME and a multiple emulsion, on a perfused bovine udder [18]. Further reports exist regarding differences in the follicular penetration of topically applied substances dependent on the physicochemical properties of both the vehicle and the dye. Up to now, a follicular penetration of hydrophilic fluorescent dyes has been reported for the application in emulsions [20] or solvents such as propylene glycol [15,19]. Moreover, Changez et al. observed a participation of the appendages in the transdermal permeation process of a hydrophilic model fluorescent incorporated into an ME in vitro and in vivo in hairless mice skin. [21,22]. Otberg et al. reported on a dependence of the follicular reservoir on the body-site [41]. In the case of the ME, the apparently increased follicular penetration may be one reason for the observed lower amount of dye in the horny layer.

5. Conclusion

The investigations demonstrated that the penetration of curcumin applied in the o/w microemulsion, into both the stratum corneum and the hair follicles, is improved compared to the application in the amphiphilic cream. Lipid layers and, presumably, the follicles seem to be the preferred pathways for the lipophilic dye applied in the ME as indicated by microscopic observations.

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