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Research paper

Dermal targeting using colloidal carrier systems with linoleic acid

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ABSTRACT

In the basic therapy of chronic skin diseases characterized by xerosis, the local treatment is an essential strategy to reach ideal therapeutic effects. Suitable active ingredients for this aim are fatty acids, in particular linoleic acid, which is an essential component for the organization and perpetuation of the skin barrier. In the present work, the development of a well-tolerated colloidal carrier system (microemulsion) containing linoleic acid as active ingredient is described. A comprehensive physiochemical characterization of the novel microemulsion system was performed using different techniques. The potential of the developed microemulsion system compared to a cream as suitable carrier for the dermal delivery of linoleic acid was determined. Penetration studies showed higher linoleic acids concentrations after administration of the colloidal carrier system in all skin layers independent of the time of incubation. Up to 23% of applied dose reached the skin from the colloidal carrier system whereas at most 8% of the active ingredient could be detected after applying the cream. Particularly, the percentage of the linoleic acids penetrated through the microemulsion in the stratum corneum and the viable epidermis differed significantly ($p < 0.01$) when compared to that through a standard cream. Furthermore, linoleic acids accumulated in the epidermis at longer incubation times. Using the microemulsion, the penetration of linoleic acids was enhanced significantly ($p < 0.01$). Hence, the microemulsion might be an innovative vehicle for the delivery of linoleic acids to the epidermis improving its use as their barrier regeneration and providing possible anti-inflammatory effects.

1. Introduction

Since the skin is the largest organ of the body, it is an important target site for the application of drugs aside from its physiological functions. In the treatment of local diseases, topical drug delivery is a well-suited strategy to restrict the therapeutic effect to the affected area and to reduce systemic side effects. Additionally, transdermal delivery of drugs is an appropriate possibility to minimize the first-pass-effect or avoid gastrointestinal side effects. However, in order to reach therapeutic concentrations in the epidermis and/or in the dermis or even in the systemic circulation, the skin barrier has to be overcome.

In the basic therapy of chronic skin diseases characterized by xerosis, the local treatment with emollients is essential to prevent exacerbation of the disease. Hereby, the preservation and regeneration of the skin barrier is an important factor to increase hydration, to reduce transepidermal water loss and to avoid penetration of pathogens, e.g. bacteria or allergens. Well-established active pharmaceutical ingredients to achieve this are unsaturated fatty acids, components of the intercellular lipid matrix of the stratum corneum and thus contributing to the arrangement of its bilayers. Moreover, in particular, linoleic acid is an essential component for the organization and perpetuation of the skin barrier [1–3]. Aside from the possible integration of free linoleic acid in the lamellar bilayers, it is an essential part of the synthesis of ceramide [EOH], ceramide [EOP] and ceramide [EOS]. Ceramide [EOS] is known to play a key role in the organization of the bilayer structure and barrier function of the stratum corneum [4–7]. The synthesis of linoleic acid containing ceramides takes place in the odland bodies, located in the viable epidermis [8]. In addition to the simple substitution of linoleic acid, due to its chemical structure linoleic acid exerts multiple effects on cell membranes, epidermal barrier and even inflammatory processes [9–11]. Regarding the anti-inflammatory effects, evidence suggests that linoleic acid interferes with the prostaglandin and leucotriene synthesis and also binds to peroxisome proliferator activator receptors by unsaturated fatty acids or its metabolites [9,12,13].

The physicochemical properties of linoleic acid are not optimal for epidermal administration. It represents a highly lipophilic molecule with a very high partition coefficient ($\log P = 7.180 \pm 0.256$). The molar mass is 280.45, and its calculated p*K*a value is

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4.78 ± 0.10 [14]. A novel approach to deliver high contents of linoleic acid into the stratum corneum as well as into the viable epidermis is the application of colloidal carrier systems such as microemulsions (ME). These colloidal carrier systems are well described in literature as suitable vehicle for topical administration of drugs. Microemulsions are thermodynamically stable colloidal carriers of oil and water, stabilized by surfactants and in some cases additionally by co-surfactants. They are single, optically isotropic, transparent or slightly opalescent solutions of low viscosity with Newtonian behavior and can almost form spontaneously without any energy input. Microemulsion systems provide small particle sizes of the colloidal phase (5–100 nm) and due to their fluctuating interface various resulting microstructures [15–17]. The excellent drug delivery potential as well as the solubilization capacity is attributable to a variety of factors depending on the composition and the resulting microstructure, but in general, continuously and spontaneously fluctuating interfaces of microemulsions enable high drug mobility and might enhance the drug diffusion process [18,19]. Hereby, the application of microemulsion systems that feature several advantages like renewable raw materials, high skin tolerability and environmental compatibility is an innovative concept for the development of customized drug carrier especially in the treatment of diseased skin [20].

In the present work, the development of a well-tolerated colloidal carrier system containing linoleic acids as the active ingredient, alkylpolyglucosides (APG) acting as very mild surfactant system and 1,2-pentylene glycol as co-solvent is described. A comprehensive characterization of the novel microemulsion system was performed by constructing the pseudoternary phase diagram and regarding dynamic viscosity, electric conductivity as well as performing differential scanning calorimetry investigations. Additionally, the size of the colloidal phase was determined by dynamic light scattering. HET CAM was carried out to evaluate the irritating potential of the system. With regard to the linoleic acids, their stability was evaluated in the microemulsion. In this study, the potential of the developed microemulsion system as suitable vehicle for the dermal delivery of linoleic acid and thereby for an effective and cosmetically improved therapy of dry skin was determined. Therefore, penetration profiles on full thickness human skin of linoleic acids were achieved after applying the microemulsion compared to the penetration profile following the application of a standard vehicle containing the same active pharmaceutical ingredient.

2. Experimental

2.1. Materials

Linoleic acids, containing 25% 9,12-octadecadienoic acid and 65% 9,11-octadecadienoic acid, with a purity of 90% were purchased from Oelon GmbH (Emmerich am Rhein, Germany). Cognis GmbH (Duesseldorf, Germany) donated Plantacare 1200 UP, and Evonik Goldschmidt GmbH (Essen, Germany) kindly offered Tegocare CG 90. 1,2-Pentylene glycol was obtained by courtesy of Symrise GmbH & Co. KG (Holzminden, Germany). Dr. Straetmans GmbH (Hamburg, Germany) kindly offered the antioxidant ascorbyl palmitate. Butylated hydroxytoluene and trifluoroacetic acid were purchased from Sigma–Aldrich Laborchemikalien GmbH (Seelze, Germany). The standard vehicle containing the same ingredient as the microemulsion was a water-in-oil emulsion. The internal standard mometasone furoate was purchased from Almirall Hermal GmbH (Reinbek, Germany). HPLC grade acetonitrile and methanol were obtained from J.T. Baker, Mallinckrodt Baker B.V. (Deventer, The Netherlands). Merck KgaA (Darmstadt, Germany) supplied buffer substances. Octanol was supplied by Gruessing GmbH (Filsum, Germany). Water was of bidistilled quality. Human breast skin was kindly offered by Department of Dermatology with approval of the independent ethics committee of Faculty of Medicine at the Martin Luther University Halle-Wittenberg.

2.2. Construction of the phase diagram

The phase diagram was constructed at a fixed surfactant/co-surfactant mass ratio of Plantacare 1200 UP and Tegocare CG 90 of 3:1. Since Plantacare 1200 UP contains 51.5% active substance and 48.5% water, the mass ratio was corrected only considering the active substance to 1.545:1. Different systems were prepared by shaking the mixtures of 1,2-pentylene glycol in a fixed concentration of 20%, the surfactant system, the linoleic acids and water in appropriate ratios. Increments of 2.5–5% were chosen to determine the phase borders. All mixtures were stored at room temperature for 6 months for equilibration. Afterwards, the samples were investigated visually and by polarization light microscopy (Zeiss Axiolab Pol, Carl Zeiss MicroImaging GmbH, Jena, Germany). Microemulsions were identified as transparent, low-viscous and optical isotropic systems.

2.3. Viscosity measurements

Dynamic viscosity of the microemulsions was investigated using a rotational rheometer equipped with a cylindrical measuring cell and double slit (Anton Paar GmbH, Graz, Austria). Equalization of temperature was performed with a peltier cell (Anton Paar GmbH, Graz, Austria). Measurements were accomplished at a temperature of 25 °C ± 0.2 °C and shear rates ranging from 0.1 s^{-1} to 100 s^{-1}.

2.4. Conductivity measurements

Electrical conductivity measurements were performed at room temperature using Cyberscan CON 11 instrument (Eutech Instruments Europe B.V., Nijkerk, The Netherlands) with a cell constant of 1.0 cm^{-1}. Values, which remained stable for 1 min, were noted.

2.5. Differential scanning calorimetry

DSC investigations were carried out using a DSC 200 (Netzsch-Gerätebau GmbH, Selb, Germany). The samples (approx. 15 mg) were accurately weighed in and filled in aluminum pans. These were hermetically sealed to avoid water evaporation. DSC curves were achieved by cooling the samples from 40 °C to −60 °C with a cooling rate of 10 K min^{-1}. After equilibration of 5 min at −60 °C, the samples were heated to 120 °C with a heating rate of 10 K min^{-1}. An empty pan was used as reference. Nitrogen with a flow of 10 ml min^{-1} was applied as purge gas.

2.6. Dynamic light scattering

In advance of the DLS investigations, the samples were filtrated through a syringe filter (pore size 0.45 μm) (Rotilabo Nylon-Spritzenfilter, Carl Roth GmbH & Co. KG Karlsruhe, Germany). The measurements were realized at a temperature of 25.0 °C using a compact-goniometer ALV/SP 86 (ALV-Laser Vertriebsgesellschaft mbH, Langen, Germany) equipped with a Nd:YAG-Laser (ADLAS GmbH, Weil im Schönbuch, Germany) and a wavelength of 532 nm and an output of 140 mW.

All measurements were made at thirteen different scattering angles between 30° and 140°. The cylindrical sample cells consisted of Suprasil quartz glass by Hellma (Muellheim, Germany) and had a diameter of 10 mm. The necessary refractive index of the main phase for size calculation of the colloidal phase was ob-

tained using an Abbé-refractometer (Abbemat, Dr. Kernchen, Seelze, Germany).

2.7. Hen's Egg Test (HET)-Chorio allantoic membrane (CAM)

Naturally fertilized hen's eggs of New Hampshire breed were used to investigate irritative potential of the microemulsion. Only eggs providing well-developed vascular systems and a living embryo were chosen. The eggs were stored at a temperature of 37 °C and a humidity of 55% for 8 days and turned every 12 h except during the last 24 h. Afterwards, the eggs were dissected under a Laminar Air Flow Box. A circular hole with a diameter of 1.5 cm was cut into the weaker convex pole of the egg, and the amnion was wetted with saline solution. Afterwards, the CAM was exposed by removing of the amnion. A sample volume of 100 μl was applied on the CAM followed by its illumination with monochromatic laser light (helium–neon laser light 632.8 nm – red). After 5 min, a visual examination was performed. Lyses and transparency were estimated as membrane discoloration (MD) classified in three different severities (weak, medium, serious). Hemorrhage (HR) was also categorized in three different severity levels. Additionally, the vascular perfusion was determined using laser Doppler fluxmetry over 30 min [21].

2.8. Stability tests

Microemulsion a_2 containing 2% linoleic acids was stored at room temperature and protected from sunlight. Furthermore, the same system containing additionally 0.1% ascorbyl palmitate or 0.05% butylated hydroxytoluene acting as antioxidants was stored. After appropriate time intervals, 20 mg of all systems was weighed in, and the linoleic acids were extracted using 5 ml methanol. Analytics were performed with HPLC. The determination was carried out in triplicate.

2.9. Ex vivo penetration assay

The penetration experiments were performed under finite-dose conditions using Franz diffusion cells (Crown Glass Company, Somerville, NJ, USA) [22]. The investigations were made on excised human skin from reduction mammoplasty. The tissue sections were postoperatively cleaned with mull pads and isotonic NaCl solution. The subcutaneous adipose tissue was mechanically dissected and discarded. Circular pieces of skin (20 mm in diameter) were punched, hermetically sealed in tin foil, packed in an occlusive polyethylene bag and stored at −20 °C until use. At the time of the experiment, the pieces of skin were completely defrosted at room temperature, and the surface was dried using cotton pads. Phosphate-buffered saline pH 7.4 was used as acceptor phase (20.0 ml). Then, the specimens were stretched on the diffusion cell that was preheated to 32 °C, enabling the underside of the skin, with the filter gauze, to directly hit the acceptor medium that was continuously stirred. Skin specimens of three donors with three pieces of skin each per test preparation and for each of the application periods of 30 min, 300 min and 1000 min were investigated. A defined amount (20 μl) of the formulation was directly applied onto the skin surface (3.14 cm^2). After the incubation times, the skin specimens were taken out of the diffusion cell, and the remaining preparations (residues) were carefully removed from the surface using a cotton wool tip. Three punch biopsies (Kromayer punch, Stiefel-Laboratorium, Offenbach, Germany) were taken from each skin sample (0.2827 cm^2), and the individual skin layers were separated horizontally using a cryo-microtome (Jung, Heidelberg, Germany). A histological analysis of mammalian skin was performed before the penetration experiments for reliable separation of the different skin layers. The stratum corneum (SC) was dissected by one 10-μm cut. The viable epidermis (EP) was removed by four sections of 20 μm. All dermis layers (DR 1–5) were each dissected by five cuts of 40 μm. The remaining corium was also separated. The collected different skin layer sections were extracted using methanol containing 5 μg ml^{-1} mometasone furoate acting as internal standard with a volume between 300 μl and 500 μl depending on the expected amount of linoleic acids in the skin sections. Furthermore, the tubes were mixed with a vortex mixer for about 1 h, kept refrigerated over night and mixed again the next day for another 1 h before analysis. The amount of the linoleic acids that was removed from the skin surface with the swap was extracted for 12 hrs with 5 ml of methanol containing 5 μg ml^{-1} mometasone furoate. The quantification of the linoleic acids in all skin samples, the cotton wool tip and the undiluted acceptor fluid was carried out by HPLC.

2.10. Analytics

HPLC analysis was carried out using Agilent 1100 LC System (Agilent, Waldbronn, Germany) equipped with a vacuum degasser, a binary pump, an autosampler and a diode array detector. The LC column used was Nucleosil C18 125 × 2 (Macherey–Nagel GmbH & Co. KG, Dueren, Germany) maintained at a temperature of 40 °C. The isocratic mobile phase consisted of 0.1% trifluoroacetic acid in a mixture of acetonitrile and water (70:30, v/v) at a flow rate of 0.2 ml min^{-1}. The injection volume of each sample was 5 μl. Detection of 9,11-linoleic acid and mometasone furoate occurred at a wavelength of 234 nm. The limit of quantification was 0.5 μg ml^{-1}.

2.11. Statistics

Statistical significance was determined by one-way ANOVA followed by Dunnett's test as post hoc analysis. Differences were considered as significant at $p < 0.05$.

3. Results and discussion

3.1. Development and characterization of the colloidal carrier system

In the present work, sugar surfactants were used to form new microemulsion systems. The application of sucrose ester has already been studied by Lehman et al. and Thevenin et al. [23,24]. Because of the solid state and poor water-solubility of sucrose ester, the development of stable, skin-tolerable microemulsion systems is unpractical. Hence, alkylpolyglucosides (APG), a group of biodegradable, non-ionic and non-ethoxylated surfactants with excellent skin- and environmental-tolerability that are already used in several cleaning and skincare products [18,25,26] were chosen. Microemulsions containing only APG acting as surfactant system are already described in literature [27–30]. However, the potential of these microemulsions as colloidal carrier for the dermal administration of highly lipophilic components has not been investigated so far. The enhancing properties of APG for a hydrophilic drug were shown by Neubert et al. [31], whereby a contribution to an enhanced delivery of lipophilic components is also possible. Aside from that a co-solvent is necessary to create microemulsions containing APG. Usually, short chain alcohols have been used because they are able to influence the curvature and fluidity of the interfacial film, which leads to a decreased interfacial tension [32]. 1,2-Alkanediols, which show good co-solvent properties incorporated in microemulsions, are less toxic compared to their corresponding alkanols [33,34], were used alternatively to prepare skin-compatible microemulsions in the present work. Former studies showed that 1,2-alkanediols especially 1,2-pentylene glycol

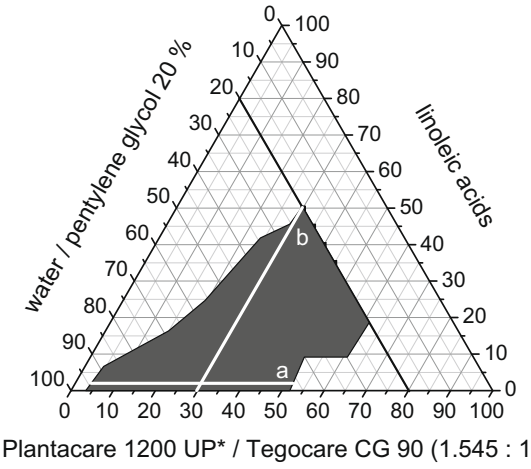

Fig. 1. Pseudoternary phase diagram of the developed microemulsion system. The isotropic area is marked in dark grey. Lines a and b indicate the dilution lines used for the physicochemical characterization.

incorporated in standard vehicles for dermal application resulted in good penetration-enhancing effects [35–37].

Because of these several advantages of APG, lauryl glucoside (Plantacare 1200 UP) and cetearyl glucoside (Tegocare CG 90) were selected as surfactant system for generating the microemulsion. 1,2-Pentylene glycol was chosen as co-surfactant, because it is able to decrease the interfacial tension and lead to an increased curvature of the interfacial film as well as bulk effects inside the system.

3.1.1. Phase diagrams

To determine the microemulsion area in the pseudoternary phase diagram, different mixtures were prepared of both surfactants in a corrected ratio of lauryl glucoside and cetearyl glucoside of 1.545:1, the lipophilic phase linoleic acids, the co-solvent 1,2-pentylene glycol and water. Clear, low-viscous, optically isotropic systems were identified as microemulsions. The area in the pseudoternary phase diagram consisting of these systems is shown in Fig. 1.

Based on the diagram, the phase borders of the developed microemulsion system could be determined. A microemulsion system that contains only APG acting as surfactant system and linoleic acids representing the lipophilic phase could be realized. A detailed characterization of different systems along the dilution lines a and b (Fig. 1) is shown below. The exact compositions of these tested microemulsions are listed in Table 1.

3.1.2. Viscosity measurements

A simple method to obtain information about the microstructure and stability of microemulsions is the determination of the dynamic viscosity, the parameter of the internal friction. Due to their low viscosity, a differentiation to liquid crystalline structures can be accomplished for microemulsions. O/w and w/o microemulsions generally show Newtonian behavior. However, non-Newtonian behavior could be observed for bicontinuous systems [16]. Since the quality and quantity of the components influence the viscosity of microemulsions varying the quantitative composition of a microemulsion system, structural changes can be observed. Phase transition of spherical particles to bicontinuous structures is accompanied with a viscosity maximum [16,38]. The measurements of the dynamic viscosity along both dilution lines a and b were taken to evaluate information about the microstructure of the different systems. The results are shown in Fig. 2.

Fig. 2 reveals that the dynamic viscosity apparently depends on the composition of the microemulsion. By increasing the water concentration along dilution line b (Fig. 2B), the viscosity of the microemulsions decreased. A corresponding decrease in the pentylene glycol and linoleic acids concentration probably causes this effect, because both components offer viscosities of 68–78 mPa s [39] and 42 mPa s [40] and, therefore, higher values than that of water (1 mPa s). On the other hand, unlike the variation of the water fraction in dilution line b, the influence of the surfactant concentration in line a (Fig. 2A) was only slight. A reason could be that by increasing the Plantacare 1200 UP concentration, the water concentration also increased. Therefore, in the microemulsion, no additional water had to be involved in the hydration of the head groups of the surfactant molecules. The amount of unbound water in the monocontinuous phase stayed nearly constant whereby the viscosity of the system remained low. Structural changes could not be observed in both dilution lines. Hence, further investigations were necessary to obtain more information.

3.1.3. Electric conductivity

Another approach to get insights into the structure is electrical conductivity measurements. Thereby, changes in the microstructure along dilution lines are detectable. The transition of isolated spherical water particles dispersed in a monocontinuous lipophilic phase to bicontinuous microemulsions with clustered droplets at the so-called percolation threshold generates conductive paths and typically leads to an increase in the electrical conductivity [41–43]. An addition of electrolytes providing charges to be transported was unnecessary in this work, because the APG surfactant Plantacare 1200 UP contains electrolytes derived from the production process [44].

However, the variation of the Plantacare 1200 UP concentration along the dilution lines causes a change in the number of charge carriers and adulterates the electric conductivity values of the microemulsions. Therefore, the values had to be corrected by dividing the electric conductivity κ by the surfactant fraction resulting in κ_a [20]. The corrected value κ_a plotted versus water fraction φ_w of dilution lines a and b is depicted in Fig. 3A.

An inflection point combined with a large conductivity increase in the diagram indicating the percolation threshold could be observed in dilution line a at water concentration of 59%. The assumption of a phase transition at this point could be confirmed by plotting the first derivative of κ_a ($d \log \kappa_a / d \varphi_w$) versus the water fraction φ_w (Fig. 3B). According to Mehta and Bala, the appearance of a maximum in the curve represents the percolation threshold (see Fig. 3B) [45].

Table 1
Composition of the studied microemulsion systems along dilution lines a and b.

	a_1	a_2	a_3	a_4	a_5	b_1	b_2	b_3	b_4	b_5
Linoleic acids	2	2	2	2	2	10	20	30	40	49
Pentylene glycol	20	20	20	20	20	20	20	20	20	20
Plantacare 1200 UP[a]	1.93	7.72	11.59	15.45	21.24	11.59	11.59	11.59	11.59	11.59
Tegocare CG 90	1.25	5	7.5	10	13.75	7.5	7.5	7.5	7.5	7.5
Water	74.82	65.28	58.91	52.55	43.01	50.91	40.91	30.91	20.91	11.91

[a] Only active substance regarded (%-m/m).

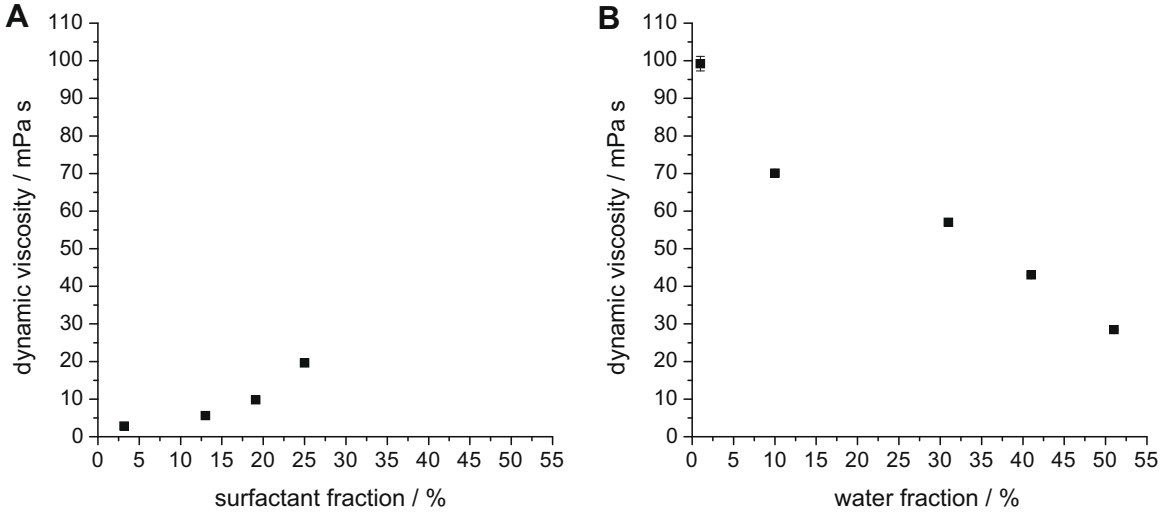

Fig. 2. Dynamic viscosity of the microemulsion (A) as function of the surfactant fraction along dilution line a and (B) as function of the water fraction along dilution line b.

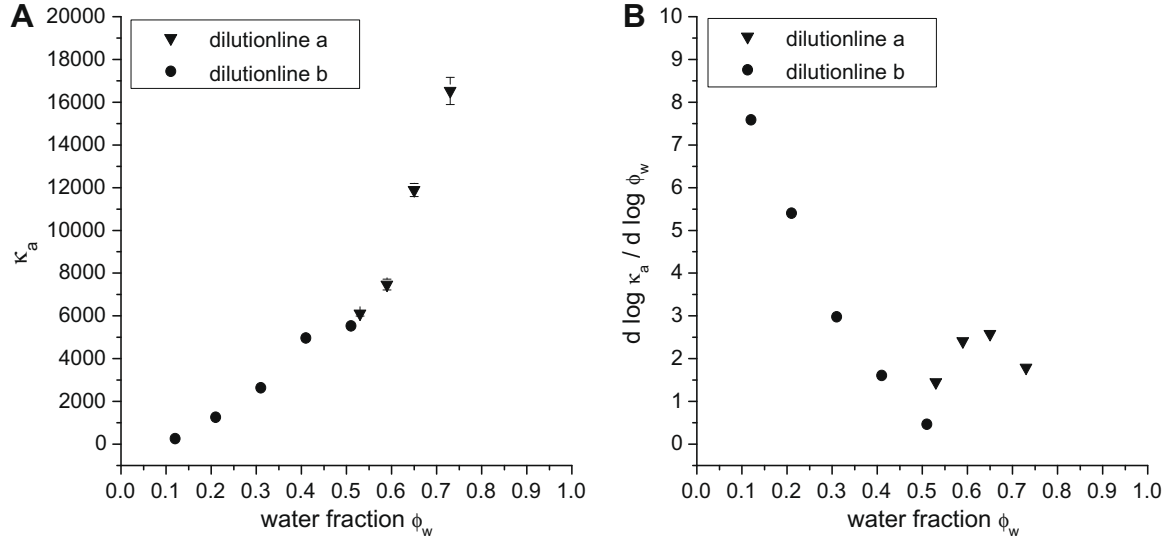

Fig. 3. (A) Corrected electric conductivity κ_a as function of the water fraction φ_w of the microemulsions along dilution lines a and b, and (B) Plot of κ_a ($d \log \kappa_a / d \varphi_w$) versus the water fraction φ_w along dilution lines a and b.

Dilution line b showed an increasing electric conductivity at higher water concentrations, but in both diagrams, there was no indication for phase transitions. Hence, due to their high conductivity values, systems with higher water concentration can be characterized as hydrophilic probably o/w character. Dilution line a additionally resulted at lower water concentrations in a phase transition of supposable hydrophilic spherical particles dispersed in a lipophilic phase to bicontinuous microemulsion systems.

3.1.4. Differential scanning calorimetry

To confirm these results, further investigations using differential scanning calorimetry (DSC) were performed in order to get information about phase transitions by investigating the water behavior in the microemulsions along dilution lines a and b. In the respective cooling curves, the size and position of the water peak refer to its physical state inside the microemulsion. Because of strong interactions of water and the surfactant molecules, the freezing peak appears at lower temperature compared to weak interacting or free water [46,47]. Therefore, a differentiation between o/w microemulsion, which is bicontinuous and contains amounts of free water, as well as w/o systems with a stronger bonded aqueous phase is accomplishable. Aside from the measurements along both dilution lines, DSC scans of system components were taken for an efficient interpretation. The results are demonstrated in Fig. 4.

The cooling curves of the pure linoleic acids and the surfactant system dispersed in pentylene glycol resulted in no freezing peak. Although the surfactant mixture contained water concentration of 22.3% brought by Plantacare 1200 UP, no peak occurred. According to Podlogar et al., an explanation is that strong interactions of the water and the surfactant molecules proceeded a shift of the water solidification towards lower temperatures than −30 °C and furthermore a reduced freezing enthalpy [47]. On the other hand, the absence of an exothermic water peak could also be influenced by the co-solvent pentylene glycol, which leads to a cryo-protective effect [20,48]. The measurement of pure water reference showed a freezing peak at −22.5 °C, indicating the presence of super-cooled water [46].

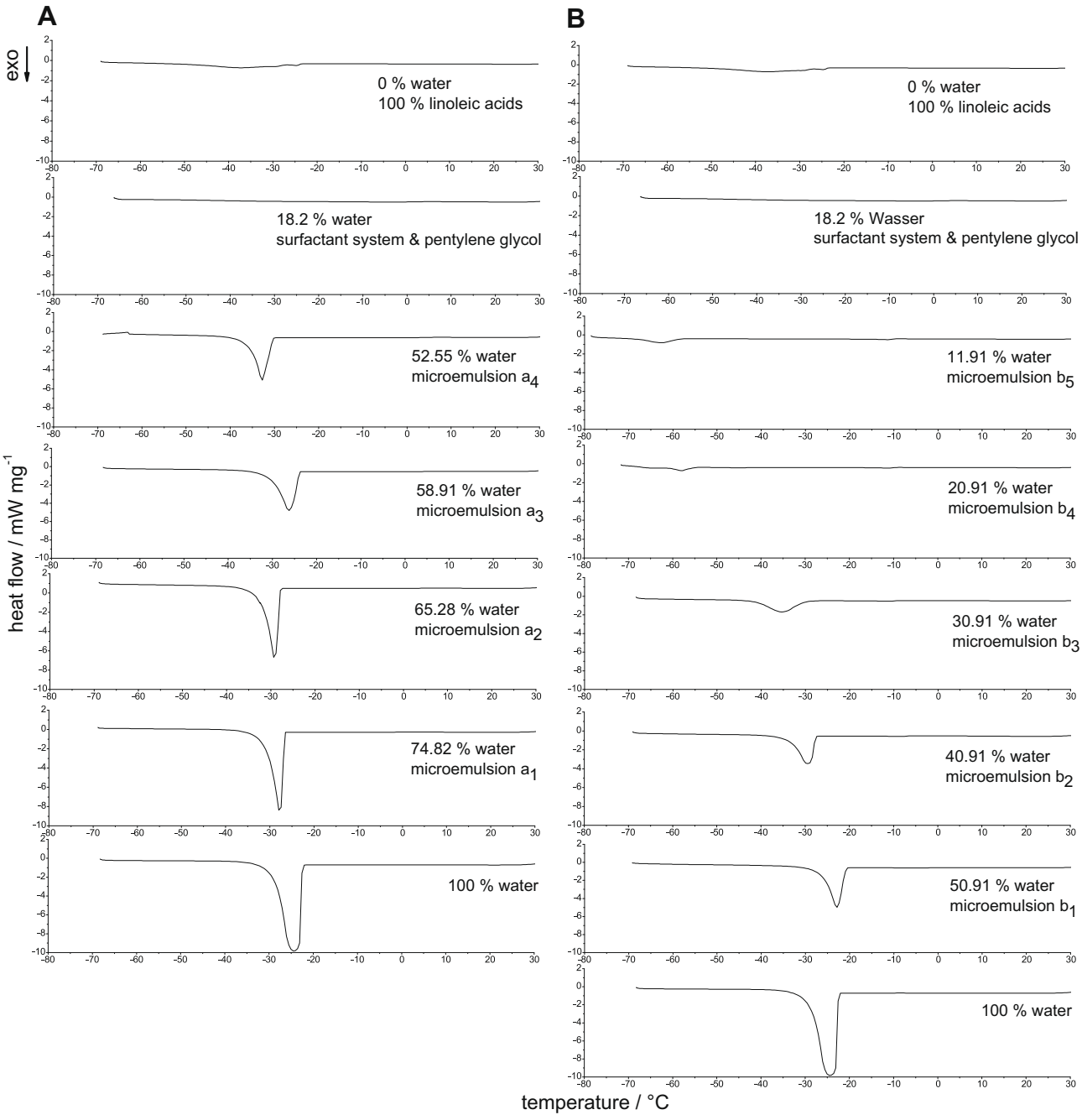

Fig. 4. DSC cooling curves of the microemulsions along dilution lines (A) a and (B) b, pure water, pure linoleic acids and the surfactant system dispersed in pentylene glycol.

The DSC curves of the investigated microemulsions showed exothermic water peaks from concentrations of 30.91%. At lower water amounts, no peak representing solidification was observable. With increasing water concentrations, the peak shifted to higher temperatures and the freezing enthalpy decreased. From concentrations of 40.91% in line b and 52.55% in line a, the peaks indicated a freezing behavior of free water. At that water concentration, phase transitions from bicontinuous systems to o/w microemulsions are suggested. The existence of free water at higher concentrations probably located in the monocontinuous phase could be confirmed. Whereby, o/w microemulsions, characterized by lipophilic droplets separated by surfactant rich domains located in a hydrophilic monocontinuous phase, could be presumed. The water in systems with lower amounts was involved in hydrating surfactant molecules and according to this bicontinuous or w/o microemulsion systems are presumable.

3.1.5. Dynamic light scattering

As a result of the above-described different characterization investigations, a microemulsion was chosen for the penetration assay. The aim of this work was to develop a system where the active ingredients, linoleic acids, generate the colloidal phase of the system. And since, for the most part, o/w microemulsions provide better penetration enhancement properties than bicontinuous or w/o systems [18], an o/w character was required of the selected colloidal carrier system. Therefore, microemulsion a_2, which offers according to the conductivity and DSC measurements spherical lipophilic particles dispersed in a hydrophilic main phase as well

as the required therapeutically appropriate linoleic acid concentration, was chosen. In advance of the penetration experiment, the colloidal size of the linoleic acids in the system was determined.

In literature, light scattering methods are frequently described [49–51]. In the present study, dynamic light scattering (DLS) was applied [52]. The DLS measurements of microemulsion a_2 at different scattering angles resulted in a hydrodynamic radius (R_h) of 2.87 ± 0.19 nm. The average radius of the R_h ranges at lower level of the colloidal size of microemulsions and could correspond to micelles formed by the surfactant system. This result is probably caused by the concentration of the linoleic acids of 2%. As already described in the literature, the oil concentration influences the extension of the swollen micelles in microemulsions and thereby the size of the colloidal phase [51,53]. Due to this, an increased colloidal size is expectable by adding higher linoleic acids concentrations. Another explanation of the small particle size of the microemulsion could be the presence of APG acting as surfactant system because radii in the same range of microemulsions containing sugar ester were already published [54,55]. The reason for the resulting small particle size is not clear yet, but probably an interpenetration of the surfactant molecules in adjacent micelles occurs, whereby the size of each micelle decreases [54]. Nonetheless, further experiments systematic regarding colloidal size changes along the dilution lines have to be carried out in the future. However, according to investigations of Shukla et al., the results of DLS support the existence of spherical particles consisting of the lipophilic phase and bounded surfactant molecules dispersed in a hydrophilic main phase [52].

3.1.6. HET CAM

Aside from the physicochemical characterization of the microemulsion system, further investigations are necessary to demonstrate the suitability of the colloidal carrier for its dermal application. Due to this, the irritant potential was determined using Hen's Egg Test (HET) Chorio allantoic membrane (CAM). HET CAM represents a suitable method to evaluate the tissue toxicity for dermal application of substances in vivo. Irritating effects appear in alteration of the vascular system like membrane discoloration and hemorrhage as well as in increased perfusion [21]. The evaluation of the perfusion is carried out by measuring Laser Doppler flux on defined time intervals. Followed by calculation of LDF-index that contains the perfusion differences divided by baseline values before application and thereby including differences caused by vascular diameter and number and blood pressure. HET CAM was performed on microemulsion a_2 compared with a negative control represented by NaCl 0.9% and the positive reference sodium lauryl sulphate (SLS) solution 0.5%. The visual examination after 5 min (Table 2) resulted in numerously membrane discolorations (MD) as well as weak hemorrhage (HR) for the positive control, whereas microemulsion a_2 and the negative control showed nearly no effects. Due to the visual examination, no irritant effects could be found for the microemulsion.

As the results in Fig. 5 show, LDF-index of the microemulsion was similar to the negative control and differed from the SLS solution, which showed a different biological reaction pattern due to increased LDF-index and perfusion, respectively. Due to this, no indication for an irritating or vascular active effect of microemulsion a_2 could be found. Hence, the developed microemulsion might be a skin-tolerated vehicle for dermal administration.

Table 2
HET CAM – visual examination of microemulsion a_2, the negative control NaCl 0.9% and the positive control SLS 0.5% (MD = membrane discoloration, HR = hemorrhage).

Preparation	Vital	HR weak	MD		
			Weak	Medium	Serious
NaCl 0.9 %	12	0	0	0	0
SLS 0.5 %	12	3	9	1	2
Microemulsion	12	0	3	0	0

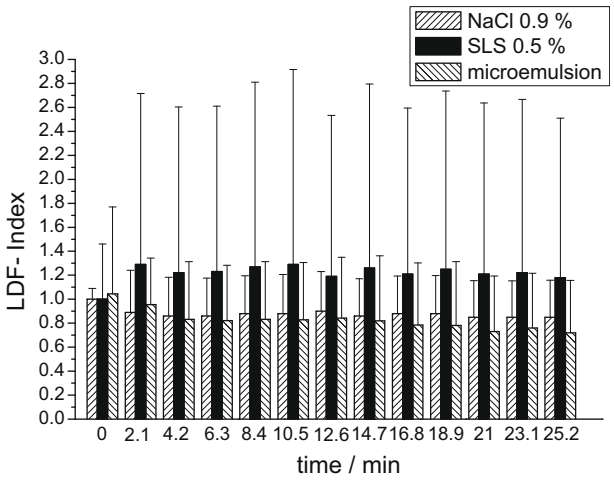

Fig. 5. LDF-index of microemulsion a_2, the negative control NaCl 0.9% and the positive control SLS 0.5% (data given as mean ± standard deviation, $n = 12$).

3.1.7. Stability investigations

The stability of linoleic acids in the microemulsion was determined because they are susceptible to oxidative stress caused by their unsaturated molecule structure. Due to this, to achieve optimal penetration results in high active ingredient concentrations in the skin, the stability of the linoleic acids in the system is an essential factor. The storage of microemulsion a_2 with and without antioxidants was tested over 9 months at room temperature. The quantification of the linoleic acids was realized by HPLC. The results are shown in Fig. 6.

The data in Fig. 6 reveals that linoleic acids were stable in all three tested formulations over 4 weeks. However, after 3 months, a decreased linoleic acids concentration was observable in the microemulsion without oxidation protection. While the concentration in this system was 82%, the amount of the linoleic acids in both microemulsions with butylated hydroxyl toluene or with ascorbyl palmitate remained stable. Furthermore, after 9 months, there were only 25% of linoleic acids in the microemulsion without

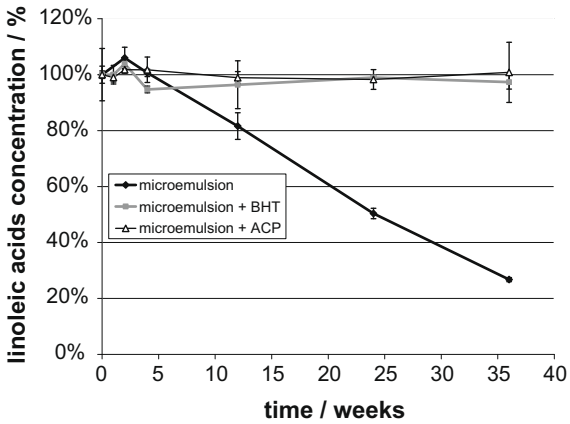

Fig. 6. Linoleic acids concentration in microemulsion a_2, microemulsion a_2 containing 0.05% butylated hydroxytoluene (BHT) and microemulsion a_2 containing 0.1% ascorbyl palmitate (ACP) stored for 36 weeks (data given as mean ± standard deviation, $n = 3$).

antioxidants whereas the concentration in the microemulsion with oxidation protection stayed stable with concentration over 95%. The obtained results agree with the study by an Italian group [56]. They investigated the stability of linoleic acid in microemulsion, an emulsion and a micellar solution and found the best stability of the fatty acid in the microemulsion compared to both other formulations.

Consequently, the microemulsion a_2 is a suitable vehicle for the dermal administration of linoleic acids, but an antioxidant is required for the stability of the fatty acids in the microemulsion.

3.2. Penetration studies ex vivo

Penetration was performed on full thickness human skin, testing the developed microemulsion a_2 and the standard vehicle represented by a cream drug product. For better comparability of the penetration profiles, the amount of the linoleic acids was increased from the commercially available concentration of 0.815% to 2%. Both formulations were tested at three different incubation times to obtain detailed penetration profiles at initial phase, medium incubation time and a long-term period. The results plotted in percentage of applied dose are presented in Figs. 7 and 8. Furthermore, depth profiles were constructed and are shown in Fig. 9.

The results generally demonstrate that higher linoleic acids concentrations were penetrated into the skin after applying the microemulsion compared to the cream.

Regarding the outermost skin layer stratum corneum (Fig. 7), representing a target site of linoleic acids, about 1% of applied dose reached the compartment after 30 min from the cream. At longer incubation times, no increase in the concentration was observable. Linoleic acids concentration stayed constant after applying the standard vehicle. Compared to the colloidal carrier system, the concentrations did not differ after the initial phase of 30 min. However, at longer incubation times, the amount increased up to 3.6%. Hence, significantly higher amounts of the active ingredient penetrated into the target site stratum corneum after applying the microemulsion compared to the standard vehicle.

Likewise, with a view on the depth profiles in Fig. 9, the improved penetration of linoleic acids into stratum corneum is evident. The molar concentrations of linoleic acids in skin depth of 10 μm were up to 2.5-fold higher from the microemulsion compared to the cream. Accordingly, higher amounts of linoleic acids are available for the regeneration and perpetuation of the skin barrier by integrating the fatty acids in the lipid bilayers of the stratum corneum. The penetration results of the viable epidermis, the second target site of linoleic acids, show a similar trend compared to the stratum corneum data. Both formulations resulted in different penetration characteristics of the active ingredient. After applying the microemulsion, 1.84% of the applied dose reached the viable epidermis after 30 min. In comparison with the cream, which delivered only 0.74%, a 2.5-fold higher concentration of linoleic acids was found. After 1000-min incubation time, a more considerable difference occurred. Only 1.73% linoleic acids were detectable in the viable epidermis from the cream whereas 7.37% of applied dose penetrated the viable epidermis after applying the microemulsion. Hence, linoleic acids concentration increased at longer incubation time in the viable epidermis and the upper dermis layer and significant higher concentrations occurred compared to the cream using the colloidal carrier. The standard vehicle delivered nearly constant amounts at all periods. According to their anti-phlogistic effect, the penetration of the fatty acids in the viable epidermis is essential. In that skin layer, enzymatic catalyzed reactions of linoleic acid in its oxidation products takes place, whereby anti-inflammatory processes are induced.

The linoleic acids concentration in the deeper dermis layer (Fig. 8) resulted in low amounts for both formulations at all incubation times and furthermore, the amounts stayed on a constant level. In skin depth from 700 μm, only small amounts, namely less than 250 μM, were detectable (Fig. 9).

The results indicate for both formulations an accumulation of linoleic acids in the epidermis, the target site of the active ingredient. With regard to the acceptor compartment (Fig. 8), apparently no linoleic acids permeated all skin layers from the cream. But the penetration enhancement properties of the colloidal carrier system probably created a higher concentration gradient and thereby linoleic acids were able to reach the acceptor compartment.

In consideration for the total penetrated linoleic acids concentration, significantly higher amounts of the active ingredient could be found in the skin from the microemulsion compared to cream. After 30 min, 7.3% of linoleic acids penetrated into the skin followed application of the microemulsion whereas only 3.1% was detectable from the standard vehicle. This difference increased with longer incubation times. After 300 min, threefold higher concentrations of the active ingredient reached the skin from the microemulsion compared to the cream, and after 1000 min, 23.3% linoleic acids could be quantified in contrast to only 8.4% after applying the cream. Acceptor fluid concentrations are not involved in the calculation. The data reveals a significant higher penetration of linoleic acids using the colloidal carrier system. The penetration rate of linoleic acids from the standard formulation increased only slightly, probably inter alia caused by the lipophilic vehicle characteristics, which generate a disadvantageous partition

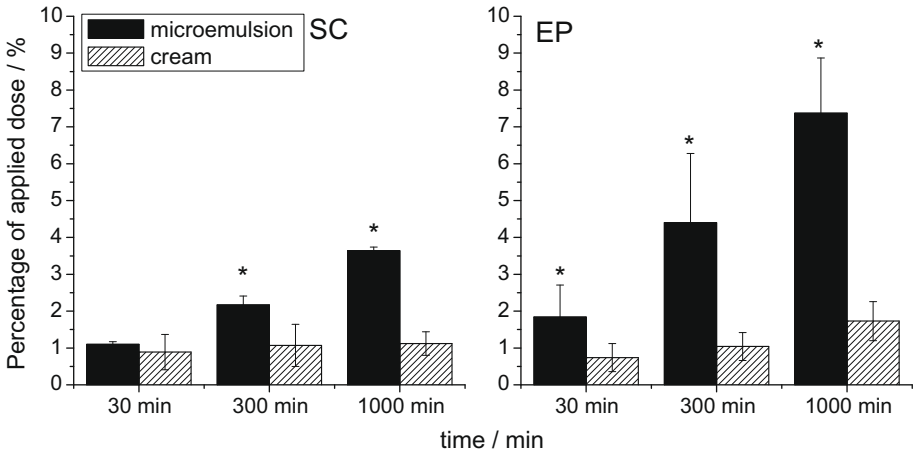

Fig. 7. Comparison of relative amounts of linoleic acids in the stratum corneum (SC) and the viable epidermis (EP) following application of microemulsion a_2 and the cream at different incubation times (data given as mean ± standard deviation, $n = 9$; $^*p < 0.01$ versus cream).

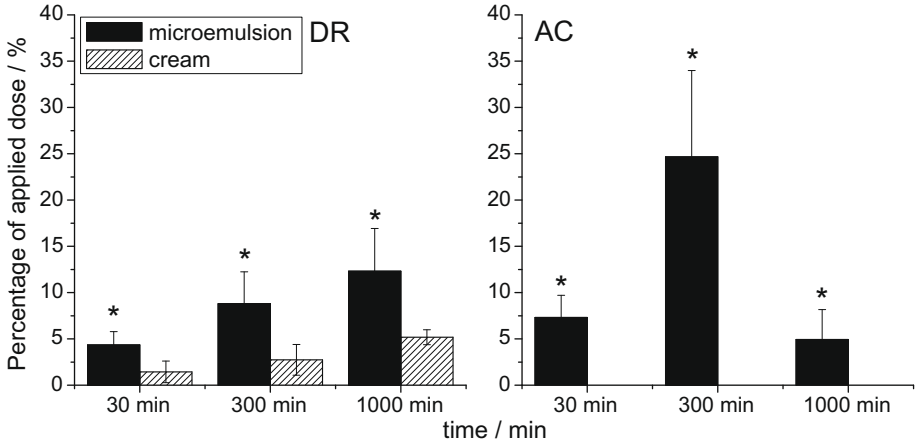

Fig. 8. Comparison of relative amounts of linoleic acids in the dermis (DR) and the acceptor compartment (AC) following application of microemulsion a_2 and the cream at different incubation times (data given as mean ± standard deviation, $n = 9$; $^{*}p < 0.01$ versus cream).

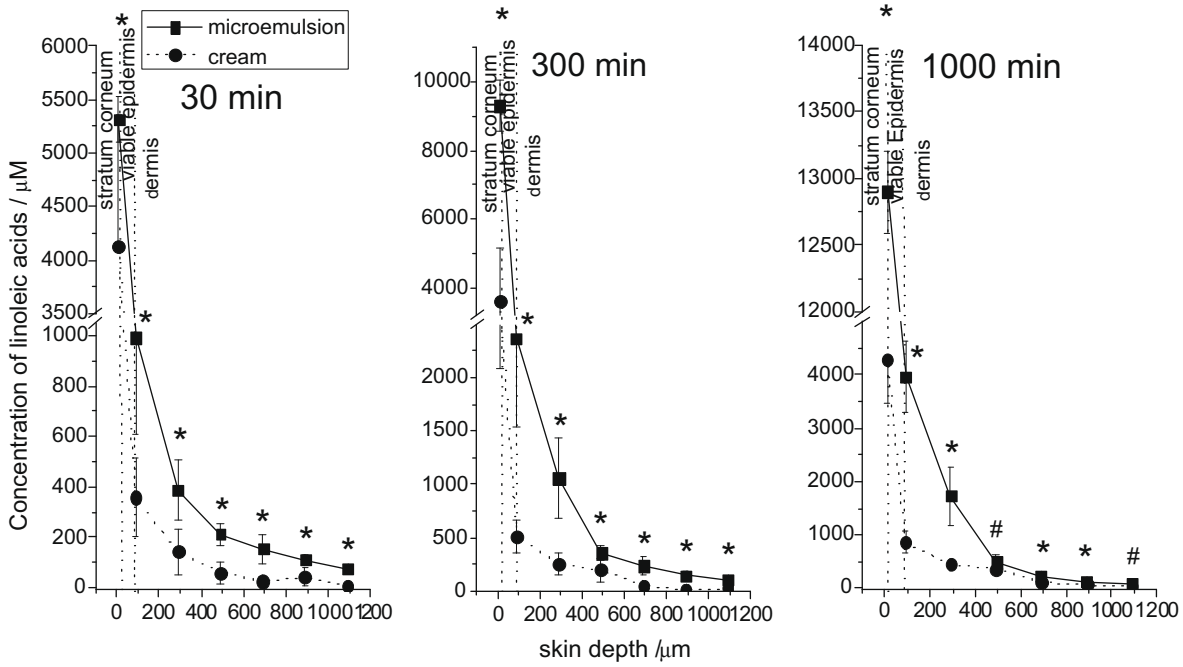

Fig. 9. Depth profiles of linoleic acids concentration within epidermis and dermis at different incubation times following application of microemulsion a_2 and the cream (data given as mean ± standard deviation, $n = 9$; $^{\#}p < 0.05$, $^{*}p < 0.01$ versus cream).

coefficient of the highly lipophilic active ingredient between the lipophilic cream and the lipophilic stratum corneum. Due to this, a low liberation rate is expected. In order to that, the application of the microemulsion system as colloidal carrier provided beneficial vehicle properties for the liberation and penetration of linoleic acids. The hydrophilic vehicle characteristics caused by the main water phase generate an excellent partition of the lipophilic linoleic acid into the stratum corneum. Furthermore, the continuous and spontaneous fluctuations inside the system enable high active ingredient mobility and accelerate the diffusion process [57–59].

In the literature, different studies are available concerning the penetration of linoleic acid. A British group investigated the influence of occlusive conditions on the penetration of linoleic acid incorporated in an ethanolic solution or in cyclomethicone [60]. The results revealed higher penetrations rates from both vehicles under non-occlusive conditions. The authors suggested as explanation, the evaporation of the vehicles and thereby increasing of the thermodynamic activity and concentration gradients in the systems. Nonetheless, the total amounts of applied dose of less than 5% into the skin stayed under the linoleic acids concentration in the skin using the microemulsion in the present study.

Roguet et al. investigated the in vivo distribution of linoleic acid in hairless rat skin [61]. The authors noticed uncommonly high concentrations of the fatty acid in skin depth between 160 µm and 400 µm. Due to this, an accumulation of the active ingredient in the sebaceous glands was assumed. Nevertheless, the measured concentrations at a lower nanomol level ranged under the linoleic acids amounts after applying the microemulsion ex vivo.

4. Conclusion

As shown in the results, a well-tolerated stable colloidal carrier system was developed. The system showed bicontinuous character

as well as spherical particles dispersed in a monocontinuous phase within the phase borders confirmed by different characterization techniques. The developed microemulsion system can be classified as well tolerated according to HET CAM. The stability of linoleic acids in the microemulsion was demonstrated up to 9 months.

The penetration studies showed that using the o/w-microemulsion system resulted in higher concentrations of linoleic acids in all skin layers independent of the time of incubation. Up to 23% of applied dose reached the epidermis and dermis from the microemulsion whereas at most 8% of the active ingredient could be detected after applying the lipophilic cream. Particularly, the percentage of the linoleic acids penetrated through the microemulsion in the stratum corneum (3.64 ± 0.10%) and the viable epidermis (7.37 ± 1.50%) differed significantly ($p < 0.01$), when compared to that through the cream which yielded concentrations of 1.12 ± 0.32% and 1.73 ± 0.53%, respectively. Furthermore, linoleic acids accumulated in the epidermis at longer incubation times with amounts of 2.94 ± 0.94% at 30 min, 6.57 ± 2.12% at 300-min and 11.01 ± 1.60% at 1000-min incubation time. In contrast, linoleic acids from the cream did only reach the essential skin compartments represented by the stratum corneum and the viable epidermis in small amounts at most 2.85 ± 0.85% after the longest incubation time of 1000 min. No increased concentrations were measured extending the experimental period up to 1000 min. As a result of these experiments, using a colloidal carrier system, the penetration profile of linoleic acids was enhanced significantly ($p < 0.01$). Hence, the microemulsion with its beneficial vehicle properties for the liberation and penetration process might be an innovative vehicle for the delivery of linoleic acids to the epidermis improving its use as their barrier regeneration and providing possible anti-inflammatory effects.

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