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Dermal targeting of tacrolimus using colloidal carrier systems

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ABSTRACT

In the therapy of chronic inflammatory skin diseases, the epicutaneous application of anti-inflammatory drugs in combination with maintenance therapy leads to ideal therapeutic long term effects. In this work, the development of well-tolerated colloidal carrier systems (ME) containing tacrolimus is described. A comprehensive physico-chemical characterization of the novel systems was performed using different techniques. The potential of three ME compared to an ointment as suitable carrier for dermal delivery of tacrolimus was determined. The penetration studies demonstrated that in comparison to the standard vehicle ointment, all three ME resulted in higher concentrations of tacrolimus in the deeper skin layers independent of the time of incubation. Particularly, the percentage of the bioavailable amount of tacrolimus (sum of the amount found in the dermis and acceptor compartment) from the ME with concentrations up to 20.95 ± 12.03% after 1000 min incubation time differed significantly ($p < 0.01$), when compared to the ointment which yielded a concentration of 6.41 ± 0.57%. As a result of these experiments, using colloidal carrier systems, the penetration profile of tacrolimus was enhanced significantly ($p < 0.01$). High drug amounts penetrated the target site in a short period of time after applying the ME.

1. Introduction

In therapy of chronic diseases especially atopic dermatitis and psoriasis, the treatment and prevention of acute inflammatory processes is essential to avoid exacerbation of the diseases. Site specific cutaneous drug delivery is gaining ground because it has advantages, compared to other administration routes, in several aspects, i.e. to minimize side effects as well as first pass metabolism (Heuschkel et al., 2008a). Classically established drugs for the therapy are glucocorticoids which offer various therapeutic effects but unfortunately also several side effects mainly skin atrophy. For that reason an improvement of the therapy using different drug classes is still required (Bornhovd et al., 2000). In this case the topical application of immunosuppressants such as calcineurin inhibitors (e.g. tacrolimus) is of great interest.

Tacrolimus, a macrocyclic lactone, fermented by *Streptomyces tsukubaensis* is a strong immunosuppressant. Compared to cyclosporine A, its immunosuppressive activity is 50–100 fold higher in vitro and 10–20 fold higher in vivo, respectively (Honbo et al., 1987). Even though it is structurally unrelated to cyclosporine A, its mode of action is similar. The target sites of both drugs are the T-lymphocytes. There tacrolimus inhibits the enzyme calcineurin phosphatase which causes an inhibition of transcription of the IL-2 gene, resulting in suppression of the T-lymphocyte response (Thomson et al., 1995; Wohlrab, 2006). Based on this mechanism of action, there are drug products of tacrolimus available which prevent organ rejection after liver and kidney transplantation (Rupprecht, 2005). Furthermore, tacrolimus has proven to be effective for the therapy of atopic dermatitis (Carroll and Fleischer, 2006; Fleischer et al., 2007; Singalavanija et al., 2006) or psoriasis (Freeman et al., 2003; Kroft et al., 2005; Lebwohl et al., 2005; Steele et al., 2005). In 2002 Protopic®, a tacrolimus containing ointment was launched for the therapy of atopic dermatitis.

Although it has not yet been approved for the local therapy of psoriasis, tacrolimus can be classified as an effective anti-inflammatory agent for psoriasis because studies on facial or inverse lesions (Freeman et al., 2003; Kroft et al., 2005; Lebwohl et al., 2005; Steele et al., 2005) as well as the use of modified vehicles or occlusion showed promising results (Erdogan et al., 2002; Pople and Singh, 2010; Remitz et al., 1999).

However, the standard vehicles currently available do not insure adequate topical delivery of the substance into deeper skin layers when applied on plaque psoriatic lesions. The main target for tacrolimus is the dermis with its lymphocytes. Therefore, it is highly challenging to overcome the skin barrier stratum corneum (SC), which is extended at psoriasis, and reach the dermis in sufficient active drug concentrations because the physico-chemical proper-

Abbreviations: APG, alkyl polyglucoside; DLS, dynamic light scattering; DSC, differential scanning calorimetry; HET CAM, hen's egg test-chorio allantoic membrane; HLB, hydrophilic-lipophilic-balance; LDF, Laser-Doppler-fluxmetry; ME, microemulsion; SC, stratum corneum; TCL, tacrolimus.

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ties with respect to size (822.05 g mol^{-1}), structure and lipophilicity (log P 3.96 ± 0.83 (SciFinderScholar, TM 2007)) of the macrolide lactone are very disadvantageous for dermal delivery.

A novel approach to improve the penetration of highly molecular and lipophilic substances into the SC and deeper skin layers is the application of colloidal carrier systems such as ME. These systems are thermodynamically stable, optically isotropic colloidal systems of water and oil, stabilized by surfactants and in some cases additionally by co-surfactants. They are transparent or slightly opalescent solutions of low viscosity with Newtonian behavior and can almost form spontaneously without any energy input. ME obtain small particle sizes of the colloidal phase (10–100 nm) and due to their fluctuating interfaces various resulting microstructures (Eccleston, 1994; Goebel and Neubert, 2008; Moulik and Paul, 1998). Their excellent drug delivery potential as well as the solubilization capacity is caused by a variety of factors depending on the composition and the resulting microstructures but mainly, the continuously and spontaneously fluctuating interfaces of ME enable high drug mobility and might enhance the drug diffusion process (Delgado-Charro et al., 1997; Heuschkel et al., 2008a). Hereby, the application of ME systems which feature several advantages like highly skin-tolerable surfactants and oils as well as low tenside concentration is an innovative concept for the development of customized drug carrier especially in the treatment of diseased skin.

The objective of the present work was the development of different well-tolerated colloidal carrier systems for the enhanced dermal delivery of tacrolimus containing lecithin, alkylpolyglucosides (APG) and mainly non-ethoxylated tensides as mild surfactants, diols such as 1,2-pentylene glycol and 1,2-propylene glycol acting as co-solvent and the emollient dibutyladipate (Cetiol B) representing the lipophilic phase. Different physico-chemical parameters of tacrolimus were determined to select appropriate systems for cutaneous application. Additionally, a comprehensive characterization of the novel ME systems was performed by constructing pseudoternary phase diagrams and regarding dynamic viscosity, electric conductivity as well as performing differential scanning calorimetry (DSC). Furthermore, the size of the colloidal phase was determined by dynamic light scattering (DLS) and hen's egg test-chorio allantoic membrane (HET CAM) was carried out to evaluate the irritating potential of the systems. The main purpose of the study was to examine the potential of the developed ME systems as innovative vehicles for the dermal delivery of tacrolimus and thereby for a more effective and therapeutically improved therapy of atopic dermatitis and especially psoriasis. Penetration profiles on full thickness human skin of tacrolimus were obtained after applying three different ME compared to the penetration profile following the application of a commercially available standard vehicle.

2. Materials and methods

2.1. Substances

Tacrolimus was purchased from Haorui Pharma-Chem Inc. (Edison, USA). Plantacare 2000 UP and Cetiol B were kindly offered by Cognis GmbH (Duesseldorf, Germany) and Phospholipid GmbH (Koeln, Germany) donated Phospholipon 90G. 1,2-Pentylene glycol was obtained by courtesy of Symrise GmbH & Co. KG (Holzminden, Germany). Span 80, Tagat S2, 1,2-propylene glycol were purchased from Caesar & Loretz GmbH (Hilden, Germany). Formic acid was supplied by Sigma–Aldrich Laborchemikalien GmbH (Seelze, Germany). The standard vehicle Protopic® was purchased from Astellas Pharma GmbH (Munich, 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. Water was of bidistilled quality. Human breast skin had the approval of the independent ethics committee of faculty of medicine at the Martin Luther University Halle-Wittenberg.

2.2. Saturation solubility

Tacrolimus was added in excess to the different test-media and shaken for 48 h at room temperature. Afterwards samples were filtrated using a 0.45 μm pore size filter, diluted and analyzed by HPLC. All experiments were performed in triplicate.

2.3. Partition coefficient

Equal volumes of phosphate buffer-saturated octanol and octanol-saturated phosphate buffer were mixed. Tacrolimus was added in an appropriate amount meeting the saturation solubility in both media. The partition coefficient was tested at three different pH-values 5, 7.4, and 9. Samples were shaken for 48 h at room temperature. After phase separation both media were diluted and analyzed by HPLC. All experiments were carried out in triplicate.

2.4. Construction of phase diagrams

Phase diagrams were constructed at fixed surfactant/co-surfactant mass ratio of Phospholipon 90G and Tagat S2 of 1:3 for ME system B, Phospholipon 90G and Span 80 of 3:2 for ME system C and Phospholipon 90G and Plantacare 2000 UP of 1:2 for ME system A. Since Plantacare 2000 UP contains 53% active substance and 47% water the mass ratio was corrected only considering the active substance to 1.06:1.

The different ME were prepared by shaking mixtures of 1,2-pentylene glycol or 1,2-propylene glycol, the surfactant system, Cetiol B and water in appropriate ratios. 2.5–5% increments were chosen to determine the phase borders. All mixtures were stored at room temperature for six months for equilibration. After that time the samples were investigated visually and by polarization light microscopy (Zeiss Axiolab Pol, Carl Zeiss MicroImaging GmbH, Jena, Germany). ME were identified as transparent, low viscous and optically isotropic systems.

2.5. Viscosity measurements

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

2.6. Conductivity measurements

Electrical conductivity measurements were accomplished 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 considered.

2.7. Differential scanning calorimetry

DSC investigations were performed using a DSC 200 (Netzsch-Gerätebau GmbH, Selb, Germany). All samples (approx. 15 mg) were accurately weighed in and filled into aluminum pans and hermetically sealed to avoid water evaporation. DSC-curves were generated 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 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.

2.8. Dynamic light scattering

All samples were filtrated through a filter (pore size 0.45 μm) (Rotilabo Nylon-Spritzenfilter, Carl Roth GmbH & Co. KG Karlsruhe, Germany) before the DLS-investigations. Measurements were performed 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.

Thirteen different scattering angles between 30° and 140° were chosen for all measurements. The cylindrical sample cells consisted of Suprasil quartz glass by Hellma (Muellheim, Germany) and had a diameter of 10 mm. Refractive index of the main phase for size calculation of the colloidal phase was obtained using an Abbé-refractometer (Abbemat, Dr. Kernchen, Seelze, Germany).

2.9. Hen's egg test-chorio allantoic membrane

Naturally fertilized hens eggs of New Hampshire breed were used to investigate acute irritative potential of the ME. Only eggs providing a living embryo and well-developed vascular systems were chosen. Storage conditions were a temperature of 37 °C and a humidity of 55% for eight days. The eggs were 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. The CAM was exposed by removing the amnion. Each sample had a volume of 100 μL and was applied on the CAM followed by its illumination with monochromatic laser light (helium–neon laser light 632.8 nm – red). After 5 min visual examination was performed. Additionally, the vascular perfusion was determined using laser Doppler fluxmetry over 25 min (Wohlrab et al., 2002).

2.10. Ex vivo penetration assay

Penetration experiments were performed using Franz diffusion cells (Crown Glass Company, Somerville, NJ, USA) under finite-dose conditions (Franz and Barker, 1977). Excised human breast skin from reduction mammoplasty was used. The tissue sections were postoperatively cleaned with mull pads and isotonic NaCl solution. Subcutaneous adipose tissue was mechanically dissected and discarded. Circular pieces of skin (20 mm in diameter) were punched and stored for maximum six months 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. In the following, 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 (20.0 mL phosphate buffered saline pH 7.4) that was continuously stirred. Skin specimens of three donors, each with three pieces of skin per test preparation and experimental periods of 30 min, 300 min and 1000 min were investigated. A defined amount (20 μL or 20 mg) 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 cells. 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). 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 4 sections à 20 μm. All dermis layers (DR 1–5) were dissected by 5 cuts à 40 μm each. The remaining corium was also separated. The collected different skin layer sections were extracted using methanol with a volume between 300 μL and 500 μL depending on the expected amount of tacrolimus in the skin sections. Furthermore, the tubes were mixed with a vortex mixer for about 1 h, kept refrigerated overnight and mixed again the next day for another 1 h before analysis. The amount of tacrolimus that was removed from the skin surface with the swap following application of the ME was extracted for 12 h with 3 mL of methanol. The extraction procedure of the swab containing the residue of Protopic® was more complex and is described in previous work (Goebel et al., 2009b). Quantification of tacrolimus in all skin samples, the cotton wool tip and the undiluted acceptor fluid was carried out by HPLC. The average previously accomplished recovery rate was 97.61 ± 7.68%.

2.11. Analytics

HPLC–MS 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 and coupled with Finnigan LCQ ion-trap mass spectrometer (Thermo Fisher, Egelsbach, Germany). The HPLC column used was YMC-ODS AQ 150 × 2.1 S-3 μm, 200 Å (YMC Europe, Dinslaken, Germany) maintained at a temperature of 60 °C. The isocratic mobile phase consisted of 0.005% formic acid in a mixture of acetonitrile and water (65:35, v/v) at a flow rate of 0.2 mL min^{-1}. The injection volume of each sample was 5 μL.

Mass spectrometric detection was performed with electrospray ionization in a positive ion mode applying an electrospray voltage of 4.5 kV and a capillary temperature of 250 °C. For analysis, the peak area of tacrolimus, with a mass to charge ratio of 826.3 for the $[M+Na]^+$ ion, was detected in selected ion monitoring mode. Lower limit of quantification was 5 ng mL^{-1} (Goebel et al., 2009b).

2.12. 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 of the microemulsion systems

The development of a suitable vehicle for dermal administration of tacrolimus is highly challenging because of its lipophilic properties. Available drug products are mainly based on lipophilic components. Due to this, the liberation of the drug from those lipophilic vehicles into the lipophilic SC is limited. The creation of a more hydrophilic carrier for tacrolimus with solubilization capacity and penetration enhanced properties could increase the bioavailability of the drug in the skin. ME provide both features. Therefore, the aim of the present work was the development of hydrophilic oil-in-water ME for the incorporation of tacrolimus. Hereby, the concentration of the surfactant systems should be not more than 30% and the amount of the lipophilic phase should be at least 5% to achieve a sufficient solubilization of the drug but also a skin-tolerated system. The main focus was on the choice of surfactants like lecithin, alkylpolyglucosides, macrogol glycerol fatty acid esters and non-ethoxylated tensides, e.g. Span 80. The use of lecithin as surfactant in topical products is well established because

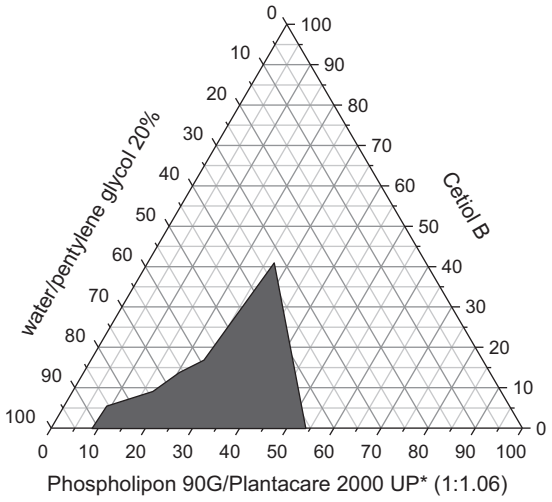

Fig. 1. Pseudoternary phase diagram of the developed microemulsion system A. Isotropic area is marked in dark grey.

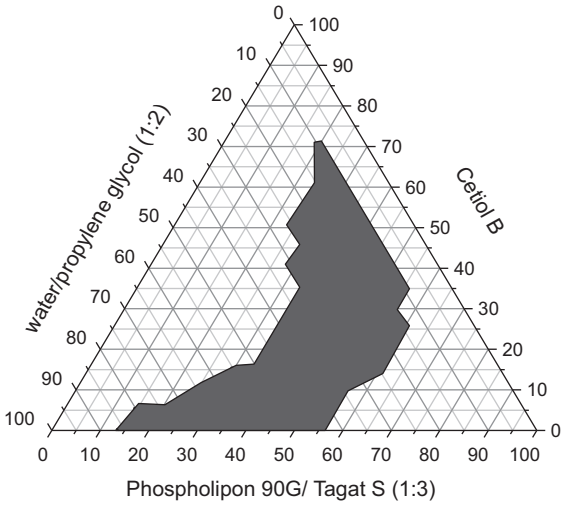

Fig. 2. Pseudoternary phase diagram of the developed microemulsion system B. Isotropic area is marked in dark grey.

of its good tolerability and enhancer properties (Bonina et al., 1995). Particularly, phospholipid-containing ME has been proven to be advantageous for dermal penetration enhancement (Changez et al., 2006; Heuschkel et al., 2008a). Furthermore, 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 (Andree and Middelhauve, 1991; Heuschkel et al., 2008a) were chosen. The potential of APG-containing ME as colloidal carrier for the dermal administration of highly lipophilic and hydrophilic components has already been shown (Goebel et al., 2010; Neubert et al., 2005).

Regarding the co-surfactants, usually short chain alcohols have been used to influence the curvature and fluidity of the interfacial film, which leads to a decreased interfacial tension (Attwood, 1994). Since 1,2-alkanediols, which show good co-solvent properties, are less toxic compared to their corresponding alkanols (Kahlweit et al., 1995, 1996), they were used alternatively to create ME in this study. Furthermore, former studies showed that 1,2-alkanediols especially 1,2-pentylene glycol incorporated in standard vehicles and ME for dermal application resulted in good penetration enhancing effects (Duracher et al., 2009; Goebel et al., 2009a, 2010; Heuschkel et al., 2008b).

In summary, very mild and penetration enhancing substances were selected for generating an excellent ME system for the dermal administration of tacrolimus. Three different ME systems (ME A–C) were developed. The pseudoternary phase diagrams are shown in Figs. 1–3.

Only clear, low-viscous and optically isotropic systems were identified as ME. Based on those diagrams the phase borders of the ME systems could be estimated. All three systems showed ME areas over a wide range. The formation was probably positively influenced by the co-solvents which contribute to the fluidity of the surfactant rich interfaces. Particularly, phospholipid-containing ME which tend to form rigid films require the addition of co-solvents to increase interface-flexibility (Aboofazeli et al., 1994). ME C resulted interestingly in a different phase position compared to ME A and ME B probably affected by the low HLB-value of the co-surfactant Span 80. Due to this, mostly ME with a lipophilic main phase could be realized. Nonetheless, it was possible to create ME providing a hydrophilic main-phase for all systems. The use of these oil-in-water ME should improve the liberation of lipophilic tacrolimus from the hydrophilic vehicle into the lipophilic SC. One ME was chosen from each diagram for further characterization and

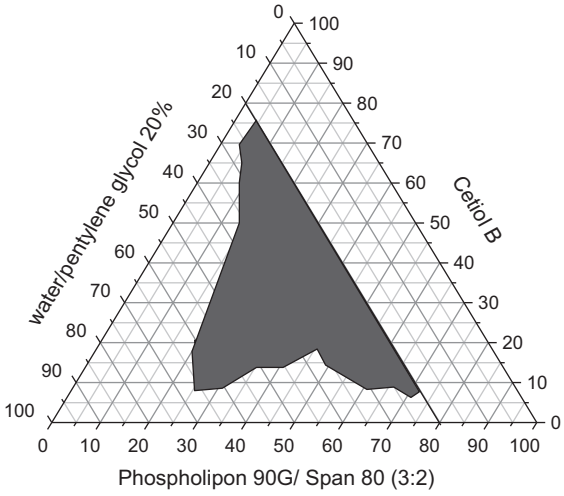

Fig. 3. Pseudoternary phase diagram of the developed microemulsion system C. Isotropic area is marked in dark grey.

penetration studies. Hereby, the surfactant concentration of the selected ME should not be more than 30% and the amount of the lipophilic phase was 10% to achieve a well-tolerated ME with sufficient solubilization capacity and penetration enhanced properties. The exact compositions of these microemulsions (ME TCL A–C) are listed in Table 1.

Table 1
Composition of the studied ME systems (%-m/m).

	ME TCL A	ME TCL B	ME TCL C
1,2-Pentylene glycol	20%		20%
1,2-Propylene glycol		43.3%	
Cetiol B	10%	10%	10%
Phospholipon 90 G	10%	6.25%	15%
Plantacare 2000 UP[a]	10.6%		
Span 80			10%
Tagat S2		18.75%	
Water	49.4%	21.7%	45%

[a] Only active substance regarded.

Table 2
Saturation solubility of tacrolimus in different media ($n = 3$, data given as mean ± standard deviation).

Medium	Saturation solubility, c_s (mg mL^{-1})	Solubility according to Ph. Eur.
1,2-Pentylene glycol	35.61 ± 2.56	Soluble
1,2-Propylene glycol	27.29 ± 1.39	Sparingly soluble
Buffer pH 5	$62.42 \times 10^{-3} \pm 34.51 \times 10^{-3}$	Practically insoluble
Buffer pH 7.4	$11.88 \times 10^{-3} \pm 16.45 \times 10^{-3}$	Practically insoluble
Buffer pH 9	$0.25 \times 10^{-3} \pm 0.35 \times 10^{-3}$	Practically insoluble
Cetiol B	14.13 ± 2.38	Sparingly soluble
Octanol	36.20 ± 3.21	Soluble
Unbuffered water	$2.15 \times 10^{-3} \pm 1.12 \times 10^{-3}$	Practically insoluble
ME TCL A	11.84 ± 0.38	Sparingly soluble
ME TCL B	6.93 ± 0.61	Slightly soluble
ME TCL C	10.93 ± 0.23	Sparingly soluble

3.2. Tacrolimus – determination of physico-chemical parameters

Tacrolimus was physico-chemically characterized by determination of its saturation solubility in different media (Table 2) as well as partition coefficients at different pH-values (Table 3).

The poor water solubility of the drug which can be found in literature (SciFinderScholar, TM 2007) could be approved. Tacrolimus was practically insoluble at the chosen pH-values and unbuffered water. Nonetheless, solubility differed at the three pH-values. Tacrolimus dissolved in lower amounts at higher pH-values probably not caused by a decreased solubility but rather by drug instability at basic pH-values as described in literature (Askin et al., 1989a,b; Tanaka et al., 1987). Due to this, the pH-value in all three ME was adjusted to pH 4.8 to assure drug stability and solubility. With respect to the other tested media, the drug was soluble in octanol which approves its lipophilic character. However, the solubility in the oily phase of Cetiol B (log P 4.22) was lower than in 1,2-pentylene glycol (log P −0.28) and propylene glycol (log P −1.34) (SciFinderScholar, TM 2007). These hydrophilic diols probably lead to higher tacrolimus solubility because of their amphiphilic structure and thus co-solvent properties. Solubility of the drug in the ME was quite similar. Only ME TCL B resulted in lower amounts probably due to the use of 1,2-propylene glycol instead of 1,2-pentylene glycol.

For better assessment of the penetration properties of tacrolimus, partition coefficients in octanol and buffer at different pH-values were generated because correlation between the partition coefficient of this two phases and the coefficient between human SC and water could be found in literature (Ohman and Vahlquist, 1994). Hereby, an acidic pH which is present on skin surface, the physiological pH of 7.4 and one basic pH-value was chosen. The achieved log D (Table 3) showed only minor differences.

In summary, it is not possible to change partition behavior of tacrolimus by adjusting pH-value in the ME system. But it can be assumed that the obtained log D values reveal an affinity of tacrolimus to the lipophilic uppermost layer and thereby disadvantageous properties to overcome the SC and penetrate into deeper, more hydrophilic skin layers.

Table 3
Octanol–buffer partition coefficients of tacrolimus ($n = 3$, data given as mean ± standard deviation).

pH	log D
5	3.56 ± 0.20
7.4	3.56 ± 0.1
9	4.12 ± 0.57

Table 4
Physico-chemical parameters of ME TCL A–C (data given as mean ± standard deviation).

	ME TCL A	ME TCL B	ME TCL C
Dynamic viscosity (mPas)	13.76 ± 0.02	100.75 ± 0.14	23.47 ± 0.06
Electric conductivity (μS cm^{-1})	2530 ± 60.28	11.17 ± 0.05	84.08 ± 0.88
DSC/existence of free water	Yes	No	Yes
Hydrodynamic radius (nm)	3.82 ± 0.25	10.77 ± 0.61	8.20 ± 0.48

3.3. Microemulsions – physico-chemical characterization

Based on the chosen three ME, different physico-chemical characterization methods were applied to obtain different information about the microstructure or the ME type.

A simple method to obtain information about the microstructure and stability of ME is the determination of the dynamic viscosity, the parameter of the internal friction. Due to the low viscosity of ME, a differentiation to liquid crystalline structures can be accomplished. Hereby, o/w and w/o ME systems generally show Newtonian behavior. However, non-ideal viscosity was partially observed for bicontinuous systems (Moulik and Paul, 1998). All tested ME resulted in Newtonian behavior with a very low viscosity, according to their definition, less than 110 mPas (Table 4) and no crystalline structures were observed. In addition, conductivity measurements were performed to achieve information about conductivity values of the ME. An increased conductivity can be observed if bicontinuous or o/w ME are present because here conductive paths are available which typically lead to a high mobility of charges (Alany et al., 2001; Bennett et al., 1982; Georges and Chen, 1986). The measurements resulted in values (Table 4) which indicate conductivity paths caused by a bi- or monocontinuous hydrophilic phase, but ME TCL A showed very high conductivity because of the APG surfactant Plantacare 2000 UP which contains electrolytes derived from the production process (Hensen, 2009).

Further investigations using differential scanning calorimetry (DSC) were performed in order to get information about the microstructure by investigating the water behavior in the ME. In the respective cooling curves, the size and position of the water peak refer to its physical state inside the ME. Because in case of strong interactions of water and the surfactant molecules the freezing peak appears at lower temperature compared to peaks of weak interacting or free water (Podlogar et al., 2004, 2005). Therefore, a differentiation between o/w ME, which contains amounts of free water and bicontinuous and w/o systems with a stronger bonded aqueous phase is accomplishable. Aside from the measurements of the three ME, DSC-scans of systems components were performed for an efficient interpretation. The results are presented in Fig. 4.

The cooling curves of the surfactant system dispersed in pentylene glycol resulted in no freezing peak (not shown). Although the surfactant mixture of ME TCL A contained a water concentration of 22.3% brought by Plantacare 2000 UP, no peak occurred. One explanation for that observation is that strong interactions of water and the surfactant molecules proceeded a shift of the water solidification towards lower temperatures than −60 °C and furthermore a reduced freezing enthalpy (Podlogar et al., 2005). In addition, solidification could also be influenced by the co-solvents pentylene glycol and propylene glycol which lead to a cryo-protective effect (Boutron and Kaufmann, 1979; Heuschkel et al., 2009). Cetiol B accomplished an exothermic peak at temperature of −60 °C. Measurement of pure water reference showed a freezing peak at −22.5 °C, indicating the presence of super-cooled water (Podlogar et al., 2004).

The DSC-curves of the ME showed exothermic water peaks for ME TCL A and ME TCL C with water concentrations of 49.5% and 45%, respectively. With decreasing water concentration the peak

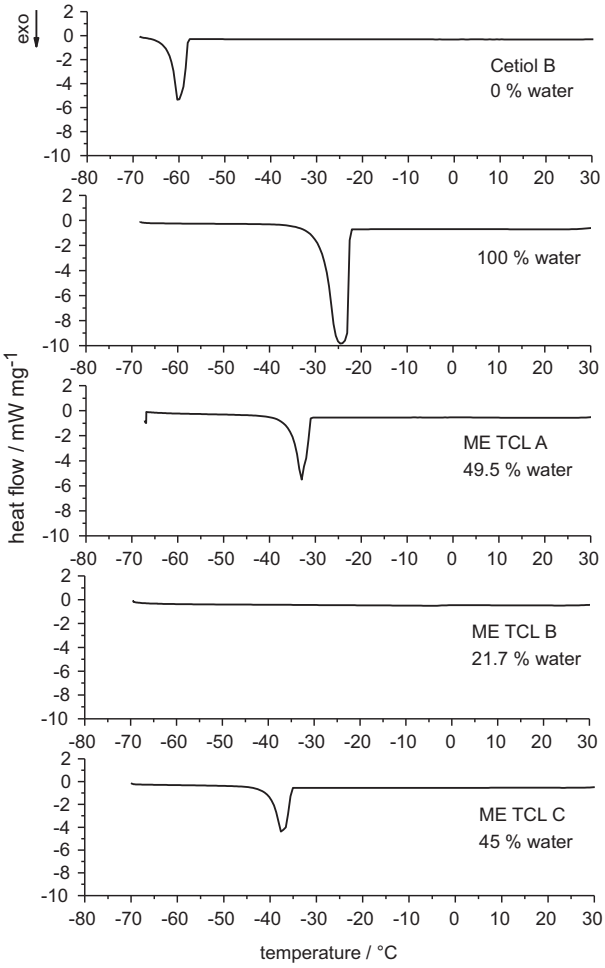

Fig. 4. DSC cooling curves of the microemulsions TCL A–C, pure water and pure Cetiol B.

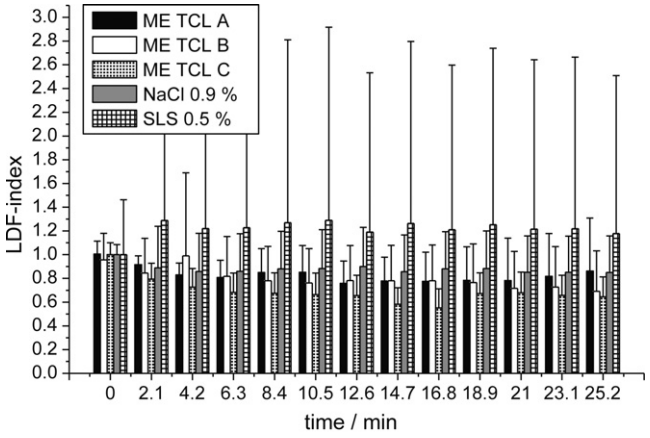

Fig. 5. LDF-index of the microemulsions ME TCL A–C, the negative control NaCl 0.9% and the positive control SLS 0.5% (data given as mean ± standard deviation, $n = 12$).

shifted to lower temperatures and the freezing enthalpy decreased. However, the cooling curves of both ME indicate the existence of free water at higher concentrations probably located in the monocontinuous phase. O/w ME, characterized by lipophilic droplets separated by surfactant rich domains located in a hydrophilic monocontinuous phase, could be presumed for ME TCL A and ME TCL C. For ME TCL B containing a water concentration of 21.7% no peak representing solidification was observable. Here the water was possibly involved in hydrating surfactant molecules and according to this, bicontinuous or w/o ME systems are presumable or the cryo-protective effect of the high content of propylene glycol adulterated the results of ME TCL B. Since for the most part o/w ME provide better penetration enhancement properties than bicontinuous or w/o systems (Heuschkel et al., 2008a) especially for lipophilic drugs, an o/w character was required for the selected colloidal carrier systems. The results of the conductivity and DSC-investigations suggest a monocontinuous hydrophilic phase and viscosity measurements offer Newtonian behavior with low viscosity. Therefore, o/w systems with spherical lipophilic particles can be assumed.

Determinations regarding the size of the colloidal dispersed phase were performed using dynamic light scattering (DLS) (Shukla et al., 2002). In literature light scattering methods are frequently described (Aboofazeli et al., 2000; Attwood and Ktistis, 1989; Saint Ruth et al., 1995). DLS investigations of the ME TCL A–C at different scattering angles resulted in dissimilar hydrodynamic radii (Table 4). ME TCL B and ME TCL C revealed an usual colloidal size diameter about 20 nm whereas the average radius of ME TCL A with 3.82 nm ranged at lower level of the colloidal size of ME and could correspond to micelles formed by the surfactant system. An explanation of the small particle size of the ME could be the presence of APG acting as surfactant, because radii in the same range of ME containing sugar ester were already published (Fanun, 2009; Glatter et al., 2001; Goebel et al., 2010). Another approach could be the occurrence of an inter-penetration of the surfactant molecules in adjacent micelles, whereby the size of each micelle decreases (Fanun, 2009). In summary, 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 (Shukla et al., 2002).

3.4. Microemulsions – tolerability test

In addition to physico-chemical characterization of the ME systems their acute 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. Hereby, irritating effects become visible in alteration of the vascular system like membrane discoloration and hemorrhage and in increased perfusion (Wohlrab et al., 2002). The evaluation of the perfusion can be measured by Laser-Doppler-fluxmetry (LDF) in 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, number and blood pressure.

In this study HET CAM was performed on ME TCL A–C compared with a negative and a positive control represented by NaCl 0.9% and sodium lauryl sulphate (SLS) solution 0.5%, respectively. Visual inspection after 5 min showed numerous membrane discolorations as well as weak hemorrhage for the positive control, whereas ME TCL A–C and the negative control resulted in nearly no effects. Furthermore, LDF-measurements (Fig. 5) demonstrate that LDF-index of the ME 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. In conclusion, with regard to visual inspection and LDF-index, no indication for an irritating or vascular active effect of all three ME TCL A–C could be found. Hence, the developed ME might be a suitable skin-tolerated vehicle for dermal administration.

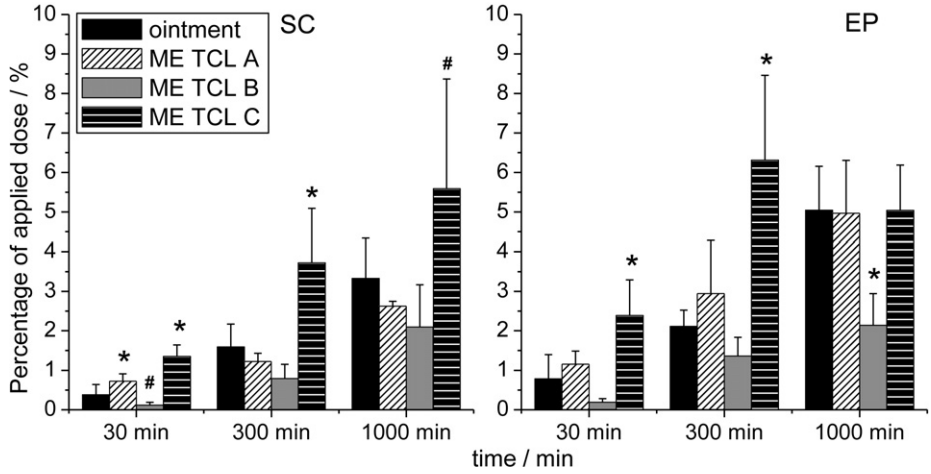

Fig. 6. Comparison of relative amounts of tacrolimus in the stratum corneum (SC) and the viable epidermis (EP) following application of the microemulsion ME TCL A–C and ointment at different incubation times (data given as mean ± standard deviation, $n = 9$; $^{\#}p < 0.05$, $^{*}p < 0.01$ versus ointment).

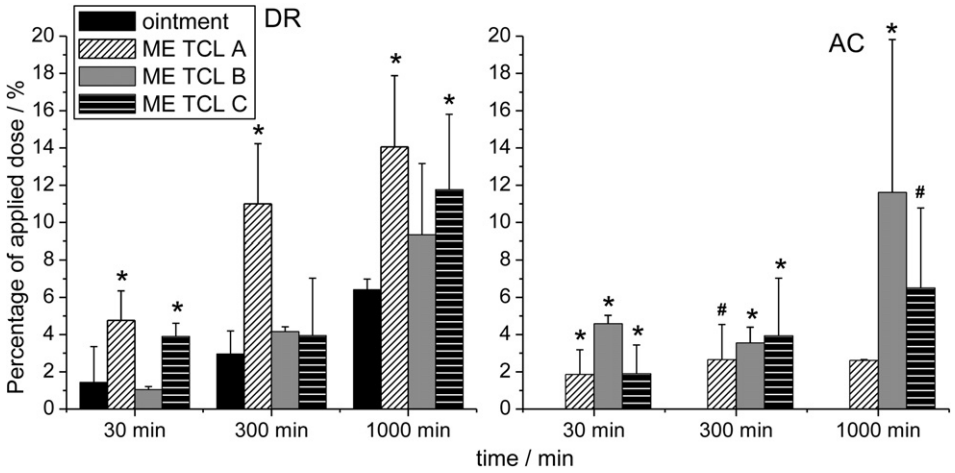

Fig. 7. Comparison of relative amounts of tacrolimus in the dermis (DR) and the acceptor compartment (AC) following application of the microemulsions ME TCL A–C and ointment at different incubation times (data given as mean ± standard deviation, $n = 9$; $^{\#}p < 0.05$, $^{*}p < 0.01$ versus ointment).

3.5. Penetration studies ex vivo

Penetration profiles of tacrolimus were generated using full thickness human skin. The different developed microemulsion systems ME TCL A–C were tested. For better assessment of the penetration behavior also a standard vehicle was investigated represented by an ointment drug product. Drug concentration in all vehicles was 0.1%. The formulations were tested at three different incubation times (30 min, 300 min, and 1000 min) 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. 6–8. Furthermore, depth profiles were compiled and are visualized in Fig. 9.

The obtained results of the three ME systems reveal that ME TCL A–C lead to a fast penetration of the drug into the skin. TCL reached the dermis with its lymphocytes which represents the target site in high amounts especially after time periods of 30 min and 300 min (Fig. 7). Penetration behavior of TCL after applying ME TCL C was comparable to ME TCL A but lower drug amounts reached the dermis and rather stayed in the viable epidermis. The concentrations in the acceptor compartment were similar for both ME. The penetration results of TCL following application of ME TCL B differed from the other systems. At shorter incubation times lower amounts of the drug were found in the skin but at longer experimental periods of 1000 min equal concentrations were measured for all three systems. However, with regard to the acceptor compartment (Fig. 7) ME TCL B resulted in the highest drug amounts compared to ME TCL A and ME TCL C. One explanation for the different penetration profile could be that as a result of the saturation solubilities

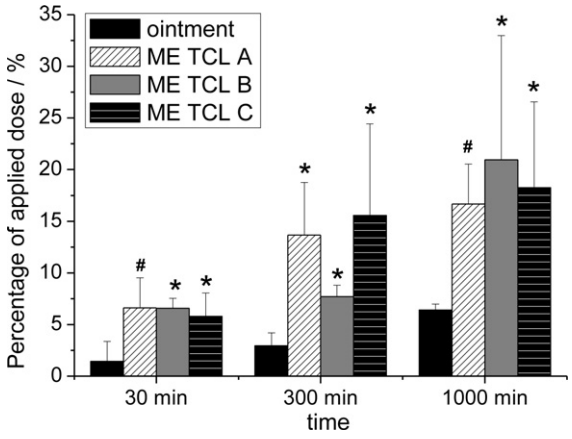

Fig. 8. Bioavailable amount of tacrolimus (sum of the amount found in the dermis and acceptor compartment) in percentage of applied dose following application of the microemulsions ME TCL A–C and ointment at different incubation times (data given as mean ± standard deviation, $n = 9$; $^{\#}p < 0.05$, $^{*}p < 0.01$ versus ointment).

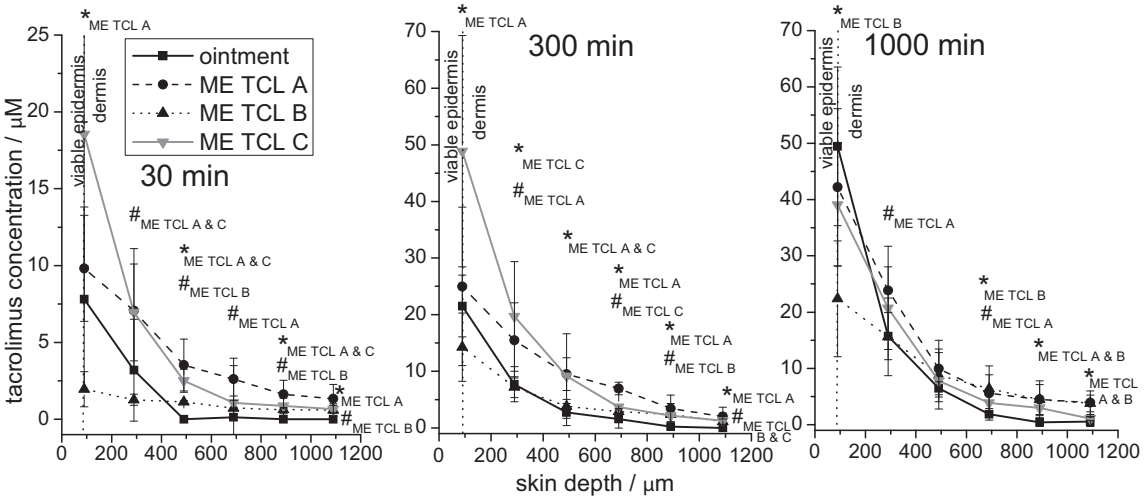

Fig. 9. Depth profiles of tacrolimus concentration in the viable skin layers (viable epidermis and dermis) at different incubation times following application of the microemulsions ME TCL A–C and ointment at different incubation times (data given as mean ± standard deviation, $n = 9$; $^{\#}p < 0.05$, $^{*}p < 0.01$ versus ointment).

(Table 2) and thereby a lower solubilization capacity for TCL of ME TCL B the thermodynamic activity of TCL in that system was higher compared to ME TCL A and C. Due to this and the increased concentration gradient the drug did not remain in the skin but rather permeate through all skin layers into the acceptor compartment. Nonetheless, all developed ME showed high concentrations in the target site and therefore good properties for the therapy of chronic skin diseases.

In comparison to the standard vehicle represented by an ointment, all ME resulted in higher penetration rates at all incubation times especially in the deep skin layer compartment dermis (exception ME TCL B at 30 min). Furthermore, acceptor compartment concentrations of TCL after applying the ME were significant higher than after applying the ointment. The drug did not reach the acceptor compartment at any incubation time following administration of the standard vehicle (Fig. 7). Possible transdermal effects could not be measured with the performed penetration experimental design.

However, in the upper skin layers SC and viable epidermis the ointment resulted in similar concentrations compared to the ME because TCL was not able to penetrate deeper skin layers and rather accumulated in the epidermis (Fig. 6).

Nevertheless, the bioavailable amount of TCL (Fig. 8) is represented by the sum of the amount found in the dermis and the acceptor compartment because here the lymphocytes are located, which is the target of the calcineurin phosphatase inhibition. Drug amounts which permeated through or accumulated in the target site constitute the bioavailable amount. After 30 min incubation time, 6% of the applied drug dose was bioavailable from the ME whereas only 1.4% TCL reached the deeper skin layers after applying the ointment. That difference increased with longer incubation times. After 300 min ME TCL C showed concentrations of 15.6% and after 1000 min ME TCL B resulted in bioavailable amounts of 21% while following application of the standard vehicle only 2.95% and 6.41% TCL concentrations were measured, respectively.

Furthermore, the generated depth profiles of TCL (Fig. 9) reveal that the standard vehicle was able to reach the dermis, but the drug penetrated only into a skin depth up to 700 µm and did not reach deeper dermis layers. The same trend was evident with longer incubation times. Nonetheless, all ME demonstrated drug quantities in the deeper skin layers as well as in the acceptor compartment.

Improved tacrolimus concentrations are required in the target site for a successful chronic skin diseases especially psoriasis therapy. In contrast to atopic dermatitis, here thickened skin barrier occurs because of a keratinocyte hyper-proliferation which leads to plaque formation. That means a larger barrier for the penetration of drugs. The hyper-proliferation is caused by an immune reaction pattern which has already to be suppressed in the dermis by inhibition of the calcineurin phosphatase. The obtained penetration results expose that the penetration behavior of tacrolimus was significantly increased using colloidal carrier systems. The skin barrier SC was permeated to a greater extend compared to the standard vehicle. The significantly improved bioavailability was achieved by that novel carrier systems without any physical enhancement e.g. ultrasound or occlusion whereby the patient compliance especially in the treatment of larger body regions could be enhanced. Erdogan et al. already successfully investigated the penetration improvement of tacrolimus by using an innovative vehicle named liposomal lotion. Liposomes were effective in the treatment of immune-mediated skin diseases tested in murine models. Unfortunately, the comparison to a standard vehicle is missing in this study (Erdogan et al., 2002). Additionally, Pople and Singh (2010) studied the effect of tacrolimus loaded nanoparticles for targeted drug delivery into deeper skin layers. The authors found a significantly improved penetration to the deeper skin layers compared to the standard vehicle Protopic® confirmed in vivo in albino rats by skin localization studies. In vitro diffusion cell studies indicated the same effect of the innovative drug carrier. Unfortunately, the results of this study cannot be compared to the results of the microemulsions because pig ear skin was used and the results are plotted in µg without any further information.

However, the developed ME showed significantly improved bioavailability compared to the ointment and furthermore differed in their penetration profiles of tacrolimus. Due to this, the influence of the used excipients is obvious whereby further modulation and optimization of tacrolimus penetration profiles is possible.

4. Conclusions

Three different well-tolerated stable colloidal carrier systems for the dermal administration of tacrolimus were developed. The generated pseudoternary phase diagrams showed isotropic areas over a wide range. All three chosen ME revealed spherical colloidal particles dispersed in a hydrophilic main phase with a colloidal size between 8 nm and 22 nm confirmed by different characterization techniques. The favored o/w character can be assumed. Furthermore, according to HET CAM, the developed ME systems can be classified as well-tolerated.

The penetration studies demonstrated that applying o/w ME systems resulted in different penetration profiles of tacrolimus using diverse ME particularly confirmed by the penetration behavior of the drug after administration of ME TCL B. Due to this, a penetration modulation by varying excipients of the ME is possible. In comparison to the standard vehicle ointment all three o/w ME resulted in higher concentrations of tacrolimus in the deeper skin layers independent of the time of incubation. 9–14% of applied dose reached the dermis and 3–12% the acceptor compartment from the ME whereas 6.5% at most of the active ingredient could be detected in the dermis after applying the ointment. Tacrolimus did not reach the acceptor compartment after applying the standard vehicle at any incubation time. Particularly, the percentage of the bioavailable amount of tacrolimus (sum of amount found in the dermis and acceptor compartment) from the ME with concentrations up to $16.67 \pm 3.88\%$ (ME TCL A), $20.95 \pm 12.03\%$ (ME TCL B) and $18.25 \pm 8.32\%$ (ME TCL C) after 1000 min incubation time differed significantly ($p < 0.01$), when compared to the ointment which yielded a concentration of $6.41 \pm 0.57\%$. Furthermore, penetration profiles revealed that tacrolimus only penetrated the upper dermis compartment and did not reach the deeper skin layers whereas the drug penetrated all dermis layers after a short period of time of 30 min following application of the ME. As a result of these experiments, using a colloidal carrier system, the penetration profile of tacrolimus was enhanced significantly ($p < 0.01$). High drug amounts penetrated the target site dermis in a short period of time after applying the ME. Hence, the ME with its beneficial vehicle properties for the liberation and penetration process might be an innovative vehicle for the delivery of tacrolimus to the dermis. The galenical conditions for an improved therapy of atopic dermatitis and particularly psoriasis might be generated. All ME showed promising data but clinical studies especially on diseased skin are necessary to confirm the results.

Conflicts of interests

Johannes Wohlrab was investigator in clinical trials and got a project grant sponsored by Fujisawa Deutschland GmbH as well as received lecture fees from Astellas Pharma GmbH. Nonetheless, there is no conflict of interest. The present work is independent and neutral.

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References

Aboofazeli, R., Barlow, D.J., Lawrence, M.J., 2000. Particle size analysis of concentrated phospholipid microemulsions: II. Photon correlation spectroscopy. AAPS Pharmsci. 2, U175–U188.

Aboofazeli, R., Lawrence, C.B., Wicks, S.R., Lawrence, M.J., 1994. Investigations into the formation and characterization of phospholipid microemulsions. 3. Pseudo-ternary phase-diagrams of systems containing water-lecithin-isopropyl myristate and either an alkanoic acid, amine, alkanediol, polyethylene-glycol alkyl ether or alcohol as cosurfactant. Int. J. Pharm. 111, 63–72.

Alany, R.G., Tucker, I.G., Davies, N.M., Rades, T., 2001. Characterizing colloidal structures of pseudoternary phase diagrams formed by oil/water/amphiphile systems. Drug Dev. Ind. Pharm. 27, 31–38.

Andree, H., Middelhauve, B., 1991. Possibilities of the use of alkylpolyglucosides in detergents and cleaning liquids. Tenside Surfact. Det. 28, 413–418.

Askin, D., Reamer, R.A., Joe, D., Volante, R.P., Shinkai, I., 1989a. A mechanistic study of the Fk-506 tricarbonyl system rearrangement – synthesis of C.9 labeled Fk-506. Tetrahedron Lett. 30, 6121–6124.

Askin, D., Reamer, R.A., Jones, T.K., Volante, R.P., Shinkai, I., 1989b. Chemistry of Fk-506 – benzilic acid rearrangement of the tricarbonyl system. Tetrahedron Lett. 30, 671–674.

Attwood, D., 1994. Microemulsions. In: Kreuter, J. (Ed.), Colloidal Drug Carrier Systems. Marcel Dekker, New York.

Attwood, D., Ktistis, G., 1989. A light scattering study on oil-in-water microemulsions. Int. J. Pharm. 52, 165–171.

Bennett, K.E., Hatfield, J.C., Davis, H.T., Macosko, C.W., Scriven, L.E., 1982. Viscosity and conductivity of microemulsions. Microemulsions [Proc. Conf. Phys. Chem. Microemulsions], 65–84.

Bonina, F.P., Montenegro, L., Scrofani, N., Esposito, E., Cortesi, R., Menegatti, E., Nastruzzi, C., 1995. Effects of phospholipid based formulations on in-vitro and in-vivo percutaneous-absorption of methyl nicotinate. J. Control. Release 34, 53–63.

Bornhovd, E.C., Schuller, E., Bieber, T., Wollenberg, A., 2000. The use of immunosuppressive macrolides in clinical dermatology. Hautarzt 51, 646–654.

Boutron, P., Kaufmann, A., 1979. Stability of the amorphous state in the system water–glycerol–ethylene glycol. Cryobiology 16, 83–89.

Carroll, C.L., Fleischer Jr., A.B., 2006. Tacrolimus: focusing on atopic dermatitis. Drugs Today 42, 431–439.

Changez, M., Varshney, M., Chander, J., Dinda, A.K., 2006. Effect of the composition of lecithin/n-propanol/isopropyl myristate/water microemulsions on barrier properties of mice skin for transdermal permeation of tetracaine hydrochloride: in vitro. Colloid Surf. B 50, 18–25.

Delgado-Charro, M.B., Iglesias-Vilas, G., Blanco-Mendez, J., Arturo Lopez-Quintela, M., Marty, J.P., Guy, R.H., 1997. Delivery of a hydrophilic solute through the skin from novel microemulsion systems. Eur. J. Pharm. Biopharm. 43, 37–42.

Duracher, L., Blasco, L., Hubaud, J.C., Vian, L., Marti-Mestres, G., 2009. The influence of alcohol, propylene glycol and 1,2-pentanediol on the permeability of hydrophilic model drug through excised pig skin. Int. J. Pharm. 374, 39–45.

Eccleston, G., 1994. Microemulsions. In: Swarbrick, J., Boylan, J. (Eds.), Encyclopedia of Pharmaceutical Technology. Marcel Dekker, New York.

Erdogan, M., Wright, J.R., McAlister, V.C., 2002. Liposomal tacrolimus lotion as a novel topical agent for treatment of immune-mediated skin disorders: experimental studies in a murine model. Brit. J. Dermatol. 146, 964–967.

Fanun, M., 2009. Properties of microemulsions with sugar surfactants and peppermint oil. Colloid Polym. Sci. 287, 899–910.

Fleischer Jr., A.B., Abramovits, W., Breneman, D., Jaracz, E., 2007. Tacrolimus ointment is more effective than pimecrolimus cream in adult patients with moderate to very severe atopic dermatitis. J. Dermatol. Treat. 18, 151–157.

Franz, T.J., Barker, E.A., 1977. Finite dose technique as a valid invitro model for study of percutaneous absorption in man. Clin. Res. 25, A198–A1198.

Freeman, A.K., Linowski, G.J., Brady, C., Lind, L., VanVeldhuisen, P., Singer, G., Lebwohl, M., 2003. Tacrolimus ointment for the treatment of psoriasis on the face and intertriginous areas. J. Am. Acad. Dermatol. 48, 564–568.

Georges, J., Chen, J.W., 1986. Microemulsions studies – correlation between viscosity, electrical-conductivity and electrochemical and fluorescent-probe measurements. Colloid Polym. Sci. 264, 896–902.

Glatter, O., Orthaber, D., Stradner, A., Scherf, G., Fanun, M., Garti, N., Clement, V., Leser, M.E., 2001. Sugar-ester nonionic microemulsion: structural characterization. J. Colloid Interface Sci. 241, 215–225.

Goebel, A., Neubert, R.H.H., 2008. Dermal peptide delivery using colloidal carrier systems. Skin Pharmacol. Phys. 21, 3–9.

Goebel, A., Schmaus, G., Wohlrab, J., Pillai, R., Neubert, R.H.H., 2009a. 1,2-pentanediol enhances cutaneous penetration and bioavailability of active ingredients. J. Am. Acad. Dermat. Poster Abstr. Am. Acad. Dermatol. 67th Annu. Meet. 60, AB82.

Goebel, A., Wohlrab, J., Neubert, R., 2009b. Quantification of tacrolimus in human skin samples and ointment by LC–MS. Chromatographia 70, 581–584.

Goebel, A.S.B., Knie, U., Abels, C., Wohlrab, J., Neubert, R.H.H., 2010. Dermal targeting using colloidal carrier systems with linoleic acid. Eur. J. Pharm. Biopharm. 75, 162–172.

Hensen, H., 2009. e-mail correspondence. Cognis GmbH, Application Technology Hair Body Oral Care, Düsseldorf.

Heuschkel, S., Goebel, A., Neubert, R.H.H., 2008a. Microemulsions – modern colloidal carrier for dermal and transdermal drug delivery. J. Pharm. Sci. 97, 603–631.

Heuschkel, S., Wohlrab, J., Neubert, R.H.H., 2009. Dermal and transdermal targeting of dihydroavenanthramide D using enhancer molecules and novel microemulsions. Eur. J. Pharm. Biopharm. 72, 552–560.

Heuschkel, S., Wohlrab, J., Schmaus, G., Neubert, R.H.H., 2008b. Modulation of dihydroavenanthramide D release and skin penetration by 1,2-alkanediols. Eur. J. Pharm. Biopharm. 70, 239–247.

Honbo, T., Kobayashi, M., Hane, K., Hata, T., Ueda, Y., 1987. The oral dosage form of FK-506. Transplant P 19, 17–22.

Kahlweit, M., Busse, G., Faulhaber, B., 1996. Preparing nontoxic microemulsions with alkyl monoglucosides and the role of alkanediols as cosolvents. Langmuir 12, 861–862.

Kahlweit, M., Busse, G., Faulhaber, B., Eibl, H., 1995. Preparing nontoxic microemulsions. Langmuir 11, 4185–4187.

Kroft, E.B.M., Erceg, A., Maimets, K., Vissers, W., van der Valk, P.G.M., van de Kerkhof, P.C.M., 2005. Tacrolimus ointment for the treatment of severe facial plaque psoriasis. J. Eur. Acad. Dermatol. 19, 249–251.

Lebwohl, M., Freeman, A., Chapman, M.S., Feldman, S., Hartle, J., Henning, A., 2005. Proven efficacy of tacrolimus for facial and intertriginous psoriasis. Arch. Dermatol. 141, 1154–11154.

Moulik, S.P., Paul, B.K., 1998. Structure, dynamics and transport properties of microemulsions. Adv. Colloid Interface Sci. 78, 99–195.

Neubert, R.H.H., Schmalfuss, U., Wolf, R., Wohlrab, W.A., 2005. Microemulsions as colloidal vehicle systems for dermal drug delivery. Part V: microemulsions without and with glycolipid as penetration enhancer. J. Pharm. Sci. 94, 821–827.

Ohman, H., Vahlquist, A., 1994. In-vivo studies concerning a pH gradient in human stratum–corneum and upper epidermis. Acta Derm. Venereol. 74, 375–379.
Podlogar, F., Gasperlin, M., Tomsic, M., Jamnik, A., Rogac, M.B., 2004. Structural characterization of water-Tween 40/Imwitor 308-isopropyl myristate microemulsions using different experimental methods. Int. J. Pharm. 276, 115–128.
Podlogar, F., Rogac, M.B., Gagperlin, M., 2005. The effect of internal structure of selected water-Tween 40 (R)-Imwitor 308 (R)-IPM microemulsions on ketoprofene release. Int. J. Pharm. 302, 68–77.
Pople, P.V., Singh, K.K., 2010. Targeting tacrolimus to deeper layers of skin with improved safety for treatment of atopic dermatitis. Int. J. Pharm., 165–178.
Remitz, A., Reitamo, S., Erkko, P., Granlund, H., Lauerma, A.I., 1999. Tacrolimus ointment improves psoriasis in a microplaque assay. Brit. J. Dermatol. 141, 103–107.
Rupprecht, H., 2005. Immunsuppression. Elsevier, Urban & Fischer, München [u.a.].
Saint Ruth, H., Attwood, D., Ktistis, G., Taylor, C.J., 1995. Phase studies and particle size analysis of oil-in-water phospholipid microemulsions. Int. J. Pharm. 116, 253–261.
SciFinderScholar, TM 2007. Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris (1994–2008 ACD/Labs) ed.
Shukla, A., Janich, M., Jahn, K., Krause, A., Kiselev, M.A., Neubert, R.H., 2002. Investigation of pharmaceutical oil/water microemulsions by small-angle scattering. Pharm. Res. 19, 881–886.
Singalavanija, S., Noppakun, N., Limpongsanuruk, W., Wisuthsarewong, W., Aunhachoke, K., Chunharas, A., Wananukul, S., Akaraphanth, R., 2006. Efficacy and safety of tacrolimus ointment in pediatric patients with moderate to severe atopic dermatitis. J. Med. Assoc Thai. = Chotmaihet thangphaet 89, 1915–1922.
Steele, J.A., Choi, C., Kwong, P.C., 2005. Topical tacrolimus in the treatment of inverse psoriasis in children. J. Am. Acad. Dermatol. 53, 713–716.
Tanaka, H., Kuroda, A., Marusawa, H., Hatanaka, H., Kino, T., Goto, T., Hashimoto, M., Taga, T., 1987. Structure of Fk506 – a novel immunosuppressant isolated from Streptomyces. J. Am. Chem. Soc. 109, 5031–5033.
Thomson, A.W., Bonham, C.A., Zeevi, A., 1995. Mode of action of tacrolimus (FK506): molecular and cellular mechanisms. Ther. Drug Monit. 17, 584–591.
Wohlrab, J., 2006. Calcineurin inhibitors for topical therapy in psoriasis. Hautarzt 57, 685–689.
Wohlrab, J., Siemes, C., Marsch, W.C., 2002. The influence of L-arginine on the regulation of epidermal arginase. Skin Pharmacol. Appl. Skin Physiol., 44–54.