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Retarder action of isosorbide in a microemulsion for a targeted delivery of ceramide NP into the *stratum corneum*

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Ceramide [NP] is an integral component of the *stratum corneum* (SC) lipid matrix and is capable of forming tough and stable lamellar structures. It was proven, that in skin diseases as psoriasis or atopic dermatitis different ceramide (CER) classes, including [NP], are degraded. It is obvious that topically application of CER on impaired skin is useful for repairing the skin barrier but a tendency for low penetration due to its poor solubility in conventional dosage forms was observed. Therefore, a stable and physiologic compatible colloidal carrier system, a microemulsion (ME), was developed and characterized. The increasing knowledge of the new colloidal systems in this last decade shows their benefits in dermal application. Isosorbide (Polysorb P) was incorporated into the ME developed. It was expected that Polysorb P has a retarder potential in order to accumulate the CER in the SC, the outermost layer of the skin. Thereby the CER [NP] would be able to interact with the affected skin layers to strengthen the skin barrier. The release and penetration behavior of the CER [NP] from the ME was assessed *ex vivo* in a Franz diffusion cell. The results of the study showed that CER [NP] penetrate largely in the upper layers of the skin (from SC to *stratum basale*), which was the desired region. A recovery in the acceptor could not be detected that underlines an accumulation in upper layers. Furthermore, significantly increased values for the SC for the ME with retarder were not received. No differences in the concentrations of CER [NP] were observed. However, the toxicity of MEs was investigated using hen´s egg test chorioallantoic membrane (HET-CAM). For the isosorbide-containing ME no difference was obtained in comparison to the non-containing. The results showed that both MEs are safe to be used on the skin for the controlled penetration of CER [NP] into the skin. The isosorbide had no effect on the irritating effect as well as on the penetration of the used CER.

1. Introduction

The human skin is the largest organ of the body (Sand et al. 2009). The primary barrier function of our skin against environmental influences and transpiration is located in the outermost layer of the skin, the 10 till 20 µm thick *stratum corneum* (SC) (Lampe et al. 1983; Wartewig and Neubert 2007). The SC consists of dead cells (corneocytes), which are embedded in a lipid matrix. This matrix is arranged inform of bilayers (Bonte et al. 1995). Its main components are ceramides (CER), cholesterol and free fatty acids in almost equimolar amounts (Ponec et al. 2003).

The CER are crucial for an unimpaired barrier, because many skin diseases like psoriasis (Cho et al. 2004) and atopic dermatitis (Proksch et al. 2003) are associated with depletion of the levels of CER in SC especially with depletion of the CER [NP] subclass which is present in high percentage in the SC (Farwanah et al. 2005; Sahle et al. 2015). CER play a key role in the water-retaining properties of the epidermis, they are rapidly increasing the hydration level of the SC and are helpful for anti- aging (Holleran et al. 2006; Mizutani et al. 2009). Hence, a direct replacement of the missing lipids should be investigated. For this, a physiological and effective formulation concerning CER [NP] is necessary and had to be developed. However, the effectiveness of CER [NP], like other CER subclasses, is limited due to its inherent hydrophobicity and precipitation as fine lipid micellar suspensions when administered in hydrophilic formulations (Stover et al. 2005).

In combination with the premises of supplying an adequate amount of the depleted lipids, ME could regenerate irritated skin. MEs are transparent, optically isotropic, low-viscous and thermodynamically stable colloidal dispersions of oil, water, surfactant and mostly in a combination with a co- surfactant (Lawrence and Rees 2012; Zhao et al. 2006). In recent years, MEs have proved themselves as promising vehicles for dermal and transdermal drug delivery systems (Hathout et al. 2010; Heuschkel and Neubert 2005; Yuan et al. 2009). They significantly enhance the penetration of hydrophilic, lipophilic and amphiphilic substances (Yuan et al. 2010). ME are easy to prepare, because they form themselves spontaneously after combining all ingredients. Nevertheless, formulation of MEs might need high levels of surfactant and cosurfactant that might irritate the skin.

Therefore, the objective of this work was to develop CER [NP] containing MEs using mild surfactants that can enhance penetration of the CER into the SC. Isosorbide (Polysorb P) was incorporated into the ME in order to accumulate the CER in the SC. The isosorbide (Polysorb P) should improve the skin barrier. The knowledge about exact mechanism of skin penetration retardation is quite limited and there are only few studies in this area (Yuan et al. 2009). The retarder effect could arise from the inability of formulation's component to hydrate the SC or due to its relative osmotic effect, as in case of polyethylene glycol (Freemann et al. 1986).

The penetration of xenobiotics through human skin into the systemic circulation can be avoided or retarded. This is relevant for toxic substances like pesticides. A reduction of the penetration trough the human skin is also required for topically administered formulations where the active ingredients should act only locally. The physical barrier function can be improved by substances that are called retarders or reducers, like isosorbide.

Previous penetration studies have shown a high affinity of MEs for deeper skin layers and the acceptor of the Franz diffusion cell plus a high penetration capability (Heuschkel et al. 2008; Sahle et al. 2012a). This should be prevented with this new formulation containing including isosorbide due to the reasons mentioned above.
Furthermore, a test was applied to ascertain toxicity and skin irritation. The HET-Cam Test was established to evaluate the irritative potential of pharmaceutical preparations on the skin (Goebel et al. 2010).
For identification and quantification of small quantities of CER [NP] from the ME in different skin layers a highly sensitive and robust method, high performance liquid chromatography-atmospheric pressure-chemical ionization- mass spectrometry (HPLC-APCI-MS) was applied to differentiate between endogenous and exogenous components that have the same chemical structure and masses. Threefold deuterated CER [NP] was used in the topical formulations like in the studies before (Sahle et al. 2012b). A sufficient selectivity, accuracy and precision of this method were ensured.

2. Investigations, results and discussion

2.1. HPLC-APCI-MS measurements

Prior use of isotopic forms of lipids, exemplified by deuterated analogue of CER [NP], could illustrate their usefulness for analytical quantification. They come closest to the endogenous SC lipids and can be easily differentiated by mass spectrometry (Sahle et al. 2012b). The development of a sensitive method for quantification of low amounts of compounds in biological samples demands maximum separation from interfering matrix components.
Extensive optimizations were made for further development of a previously reported method (Sahle et al. 2012b). The aggressive eluent system consisting of tetrahydrofuran and acetic acid was substituted by a gentle gradient mixture of methanol and water. This mobile phase preserves column, seals and other functional devices at the HPLC and ensures a longer column durability.
In addition to the new gradient a ionization mode with better intensity was chosen. In contrary to the ESI (electron spray ionization) APCI makes sure to detect low amounts of deuterated CER [NP]. The added internal standard prevents errors, e.g. fluctuation of the injection volume.
The limit of detection (LoD) and limit of quantification (LoQ) could be reduced due to the excellent ionization until 0.07 µg/mL and until 0.2 µg/mL, respectively. A good peak separation and increased peak intensity led to a long gradient program. This prolonged retention time of the CER [NP] has a limiting effect on the method, but method development was focused on avoiding a matrix effect. The investigated sample was quantified and the mean and the RSD of the MF were calculated to be 107.1 % and 4.7 %, respectively.

2.2. Preparation and characterization of MEs

In this study, the permeability of model SC lipid into the SC from two different MEs was investigated, one of it with 5 % retarder, and a conventional creme (Basiscreme DAC). Besides, the toxicity profile of the MEs was investigated *ex vivo*. CER [NP] was chosen as a model SC lipid. To distinguish the endogenous CER [NP] from the externally administered species, MEs containing deuterated CER [NP] were developed.
First of all the method of Sahle et al. (2012a,b) was used to produce the ME, but mixing all components followed by sonification was not successful. Therefore it was, decided to firstly solubilize the Cer [NP] in the oil phase. Various oil phases were tested. It is widely reported that short chain oils are preferred because they can penetrate better through the interfacial film and provide an optimal film curvature. On the other hand, long chain oils are preferred for the solubilisation of hydrophobic drugs, because there is a direct relationship between the solubilisation capacity and the chain of the oil (Vandamme 2002). Therefore the choice of the oil is a compromise between the drug solubilisation capacity and the desired characteristics of the ME. In some cases it can depend on the penetration enhancer capacity (Heuschkel et al. 2008).
Increasing viscosity of the formulation reduces penetration through the human skin. Since the target compartment is the outermost skin layer, an oil with increased viscosity was chosen (Lawrence and Rees 2012; Mizutani et al. 2015). Another important point that should be underlined is the adoption of the CER [AP]. It was experimentally observed, that the combination especially of CER [AP] and [NP] is able to increase the stability of the entire colloidal system (Engelbrecht et al. 2012).
For both MEs, two viscosity measurements in steady shear over a range of 1 to 100 or 1 to 1000 1/s were performed. For ME 1 (without retarder) 0.14 ± 0.07 Pa*s and for ME 2 0.17 ± 0.00 Pa*s were received. In both cases the viscosity is independent of the shear rate meaning that the samples behave like Newtonian fluids (Sahle et al. 2014). Since the differences were very small, there was no influence of the retarder with respect to viscosity. Furthermore, both formulations have a viscosity twice as high as the formulations investigated by Sahle et al. (2012a). This could account to the enrichment of the MEs in the SC. Furthermore no amount of CER [NP] was detected in the acceptor compartment of our penetration studies.
The isotropy was determined by a clear system that appears as dark background was categorized as ME (Hathout et al. 2011).
The size distribution of the ME droplets was determined using PCS technique, which involved the measurement of the diffusion coefficient of the droplets and calculation of the corresponding diameter using Stokes-Einstein equation. Further details of the calculation are described elsewhere (Sahle et al. 2014). The hydrodynamic diameter of ME1 with 0.1 % of Cer [NP] was 130.4±40.82 nm. It is believed that the diameter is not influenced by the higher concentration of 0.3 % of CER [NP] which was used in the penetration study.
Even though, the obtained diameter was larger than the usually reported ME droplet diameter of 10 - 100 nm (Safavi et al. 2010), but is smaller than that obtained by Sahle et al. (2012, 2014). According to these studies, the diameter was dependent on emulsifier and composition, and ranged from around 200 up to over 400 nm. The smaller droplet size obtained in this work could influence the penetration behavior; it can enhance the effective of the ME on the SC.
Probably, the droplet size differences arise through the diverse oil concentrations. In previous ME the content of the oil phase was 2- 5 %, actually in ME1 and ME2 the proportion of the oil phase was 10 %. This was necessary for a sufficient solubilization of CER [NP].
After the addition of isosorbid in ME2, a measurement of the droplet size was not possible. It is conceivable, that the structure of ME2 turns into bicontinuous phase behavior. It is known, that the viscosity of bicontinuous microemulsions is increased in comparison to oil-in-water or water-in-oil microemulsions. Consequently, the increased viscosity in ME2 implies also a bicontinuous phase. Usually the viscosity of microemulsion is in the range of 10-150 mPas*s. In ME1 and ME2 an increased surfactant amount of 40 % was incorporated. This could lead to an increased water binding rate in the emulsifier film, which would make less free water available. This may results in a higher viscosity (0.17 Pa*s for ME2).

2.3. Ex vivo skin permeability study

The objective of this penetration study was to incorporate high levels of CER [NP] into the SC, because an interaction of topical formulations with the outermost layers is preferable with regard to many skin diseases. The incorporation of a potential retarder, in this case isosorbide, should increase the level of CER [NP] in this favored skin layers. Two MEs were compared, one without isosorbide (ME 1) and one with 5 % isosorbide (ME 2). Additionally, both were compared with an established standard crème from the German Pharmaceutical Codex (DAC) (Basiscreme).
The degree of the penetration of deuterated CER [NP] into the different layers of the skin was determined after 30, 100 and 300

min from the formulation. In this case, all formulations were applied on three different skin parts – forearm, shin and back.

As shown in Figs. 2, 3 and 4 penetrations from both ME accumulate as might be desired in the two outermost layers – the SC of the upper arm. Furthermore, low concentrations of the CER were detected in deeper skin layers (dermis). No detectable amount of the CER was measured in the acceptor of the Franz cell also when the Basiscreme DAC was used.

As shown in the barplot figure the pathway through all layers of skin is visible. The Basiscreme DAC achieved higher levels of CER [NP] in nearly all three skin types than the ME but not significantly higher concentrations. For further investigations maybe the water content should be raised up, because of high tensid concentration it could be possible, that all water molecules are needed for the hydration of the polar surfactant headgroups, instead of transportation of CER [NP]. Investigations showed that in the presence of free water the penetration-enhancing properties of ME could be increased.

In addition, it has frequently been observed that, as the concentration of emulsifier increases, the flux of the active ingredient does not necessarily increase, too. Presumably, the thermodynamic activity of the active substance in the system decreases with high surfactant amount, which could result in a poorer rate of liberation. Nonetheless CER [NP] was successfully solubilised in the dermal formulation. Possibly the flux of CER [NP] stopped because of the high tensid concentration which could inhibit the thermodynamic activity in the MEs.

The impact of isosorbide on the permeability of CER [NP] in the outermost layers was calculated with the Kruskal-Wallis test (Table 1) for all three different types of skin. These tests were performed for each time point and each skin layer, comparing the impact of the different types of treatments (with retarder, without retarder, and with Basiscreme) to find significant changes. After performing the Benjamini-Hochberg correction on the resulting p values, no significant differences between the treatments (P values > 0.05) could be detected for Skin A- forearm, B- shin and C- back.

Table 1: P-values of the formulations in the Kruskal-Wallis Test

	30 min	100 min	300 min
A- SC1	0.45	0.48	0.17
A- SC2	0.13	0.13	0.29
A- EP1	0.13	0.13	0.28
A- EP2	0.13	0.13	0.69
A- DR1	0.69	0.23	0.48
A- DR2	0.13	0.13	0.85
A- DR3	0.13	0.13	0.32
B- SC1	0.13	0.13	0.18
B- SC2	0.60	0.13	0.60
B- EP1	0.13	0.13	0.27
B- EP2	0.13	0.13	0.40
B- DR1	0.46	0.13	0.28
B- DR2	0.36	0.13	0.60
B- DR3	0.27	0.13	0.13

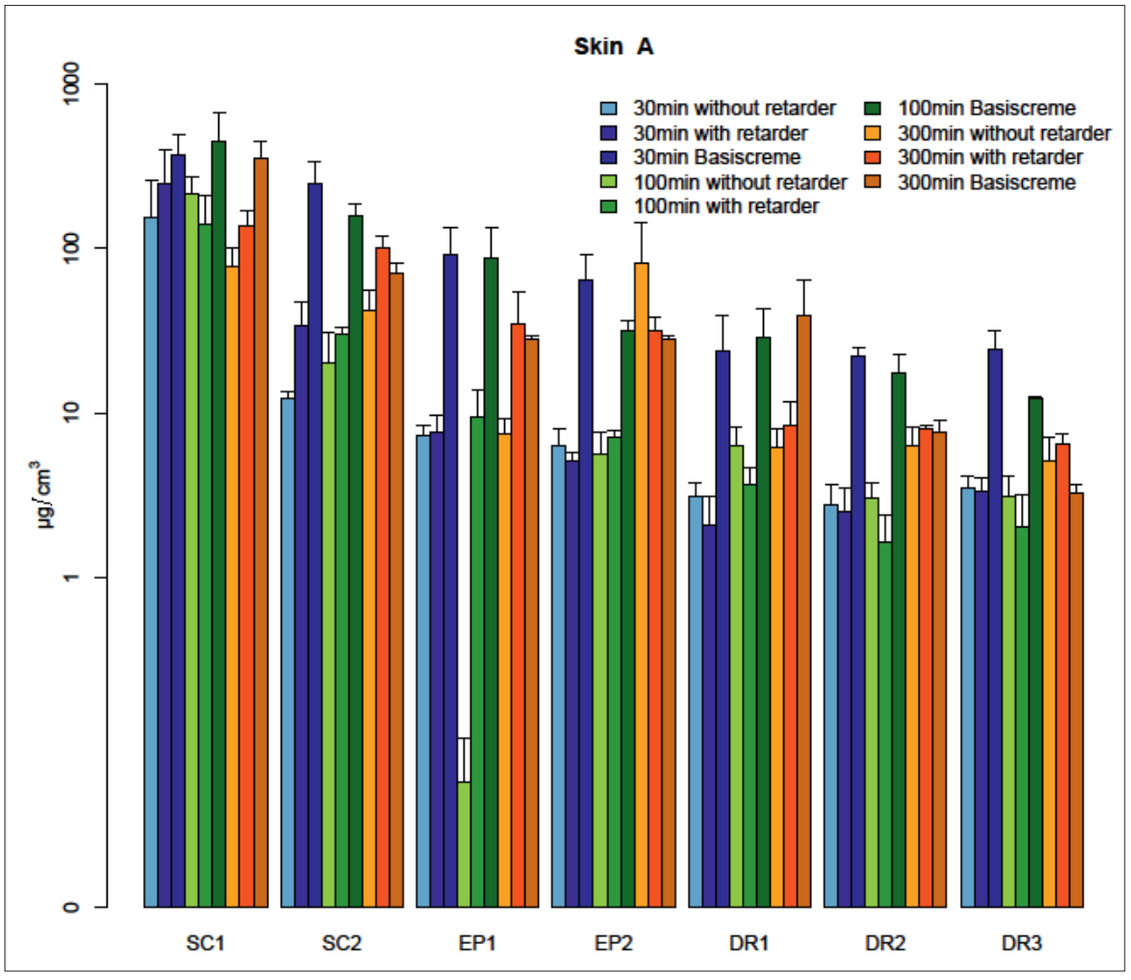

Fig. 1: Barplot of the skin A (forearm) for all investigated times and in comparison of the ME1 (non-isosorbide containing) and ME2 (isosorbide containing): SC1 outer stratum corneum 1, SC2 inner stratum corneum 2, EP epidermis, DR dermis)

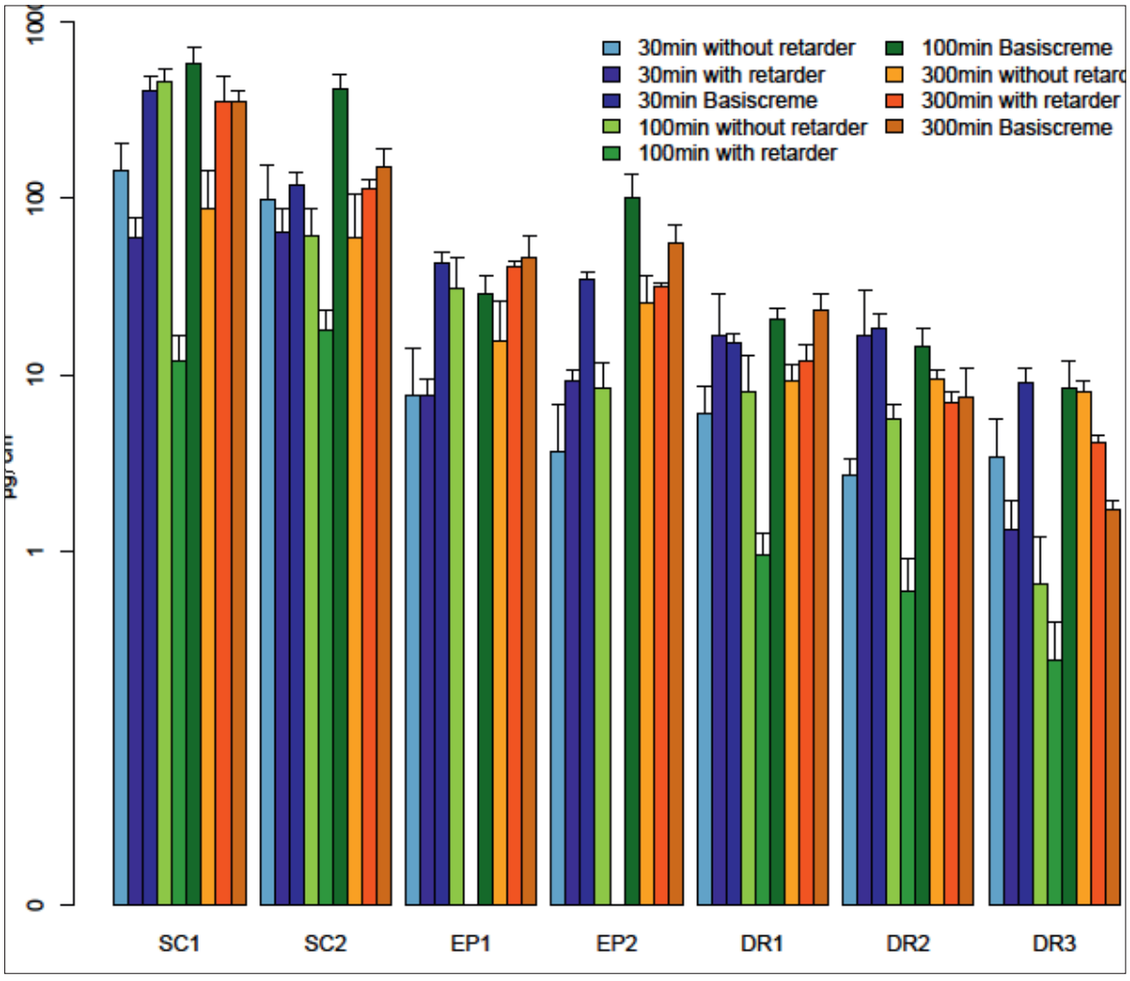

Fig. 2: Barplot of the skin B (shin) for all investigated times and in comparison of the ME1 (non-isosorbide containing) and ME2 (isosorbide containing): SC1 outer stratum corneum 1, SC2 inner stratum corneum 2, EP epidermis, DR dermis)

	30 min	100 min	300 min
C- SC1	0.13	0.13	0.13
C- SC2	0.13	0.17	0.13
C- EP1	0.13	0.27	0.13
C- EP2	0.13	0.13	0.32
C- DR1	0.13	0.36	0.32
C- DR2	0.13	0.32	0.88
C- DR3	0.13	0.18	0.17

The three formulations (ME1, ME2 and Basiscreme DAC) were tested against each other in different pieces of human skin (A – forearm, B - shin and C - back). No significant differences were detected (all P- values > 0.05)

2.4. Skin irritation study

This used oil-in-water formulation has the advantage of a pleasant skin feeling and moderate penetration, but a reversal of the phases could influence a higher penetration and less residues of CER [NP] in the swap or formulation. However, the penetration into deeper layers in a water-in-oil-ME is probably increased. Moreover, the irritating effect of the water soluble emulsifier Tegocare PL4 (polyglyceryl-4-oleate) and Hydriol PGMO.4 (polyglyceryl-4-laurate) should be decreased when they are solubilized in the inner oil phase.

The isosorbide-based ME has an IS value of 8.44 and the ME without isosorbide of 7.35. The marginal influence of the isosorbide can be neglected; both were classified as moderately irritant. Nonetheless, the composition should be modified in view of reducing skin irritation.

The results show that both MEs are safe to be used for a controlled penetration of CER [NP] into the skin. Isosorbide has neither an effect on the irritating potential nor on the penetration of the used CER. The results of the penetration study show that the rate and the extent of CER [NP] released and penetrated from ME 1 and ME 2 were surprisingly not preferable to the penetration of the CER from the standard Basiscreme (Araujo 2010). Isosorbide in a concentration of 5 % in the ME does not significantly influence the accumulation of poorly water soluble CER [NP] in the SC. But a sensible and reliable method for quantification of CER [NP] was evaluated. Also a formulation for solubilisation of CER [NP] was developed and extensively characterized. On the basis of three different types of human skin the impact of different regions on the permeation could considered.

3. Experimental

3.1. Materials

Carbamazepine was purchased from Sigma- Aldrich (St. Louis, USA). Absolute ethanol was supplied by Bundesmonopolverwaltung für Branntwein (Offenbach, Germany). Isosorbide was generously donated by Roquette (Lestrem, France). HPLC-grade methanol and HPLC- grade n-hexane were obtained from NeoLabMigge GmbH (Heidelberg, Germany). C12-15 Alkyl Benzoate, Ceramid [AP] and polyglyceryl-4-lauratewere from Evonik Industries AG (Essen, Germany). Polyglycer-in-4-oleate was supplied by Hydrior (Wettingen, Switzerland). 1,2- Pentyleneglycole was supplied by Symrise AG (Holzminden, Germany)

Distilled water was used throughout the experiment. Human back skin was kindly donated by the Department of Dermatology and Venereology of the Faculty of Medi-

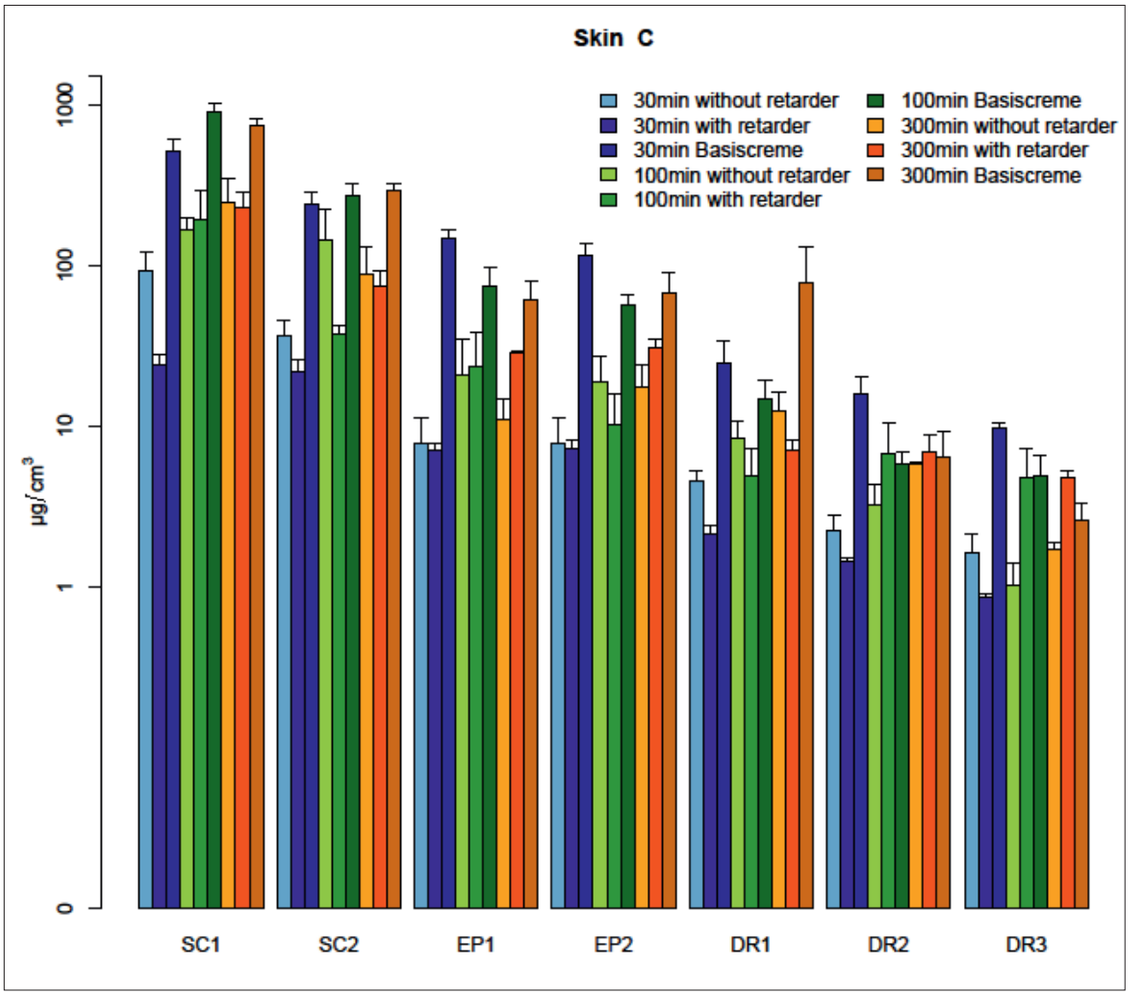

Fig. 3: Barplot of the skin C (back) for all investigated times and in comparison of the ME1 (non-isosorbide containing) and ME2 (isosorbide containing): SC1 outer stratum corneum 1, SC2 inner stratum corneum 2, EP epidermis, DR dermis)

cine, Martin Luther University Halle-Wittenberg after approval by the independent ethics committee of the Faculty.

3.2. Synthesis and purification of deuterated CER [NP]

A terminally threefold deuterated CER [NP] was synthesized and purified as described by Sahle et al. (2012b). In short, terminally deuterated stearic acid and phytosphingosine were brought to condensation in ethanol using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinolin. The reaction product was purified using column chromatography.

3.3. HPLC- APCI- MS

Separation and quantification of deuterated CER [NP] in the samples was performed using Agilent Technologies 1200 series HPCL (Agilent Technologies, Waldbronn, Germany). As detector an ion trap mass spectrometer Finnigan LCQ classic (Thermo Electron, San Jose, CA, USA) equipped with an APCI source was used throughout. A reversed phase YMC ODS-AQ C-18 HPLC column, 150 mm x 2 mm (YMC America Inc., Allentown, PA, USA) was used as stationary phase. Samples for injection were prepared in methanol and the injection volume was set at 5 µl. The elutions were carried out at a flow rate of 0.2 ml/min with a gradient comprising two components A and B, where A was a mixture consisting of methanol/water 93:7 (v/v) and B was pure methanol. Starting with A 100 % until 0 % in 20 min. Hold B 100 % for 5 min until 25 min and switch to A 100 % until 26 min. Concentration was hold until 35 min. APCI-MS was used as a detector with positive ion mode and the heater was set to 500 °C. Nitrogen was used as both auxiliary and sheath gas at flow rates of 0.15 and 1.05 L/min, respectively.

For quantification, a calibration curve was constructed using six concentrations of CER [NP] (0.04; 0.06; 0.08; 0.1; 0.2 and 0.5 µg/mL). The linearity of each plot, concentration *versus* peak area, was tested using linear regression analysis. Carbamazepine was used as internal standard for all measurements in a concentration of 1.0 µg/mL. It was chosen on the basis of its good ionizability, adequate selectivity and it's natural absence in human skin.

Limit of quantification (LoQ) and limit of detection (LoD) was calculated using the signal to noise ratio. The peak height should be ten times higher than the baseline noise for quantification and three times higher for detection limit. The between-run precision and accuracy of the method were determined by analyzing four replicates containing 0.05; 1.0; 1.5 and 2.0 µg/mL CER [NP] in methanol. Five determinants of same concentrations were conducted over three runs on three different days. Deviations at the LOQ are allowed in a range of +/- 20 % and for higher concentrations +/- 15 %. The carry over effect of the method was analyzed by observing the occurrence of MS peaks within the retention window of deuterated CER [NP] after injecting blank samples preceded by running a high concentration of CER[NP] (2,5 µg/mL). The matrix effect was investigated through analysis of extract from human skin spiked with deuterated CER [NP]. For each analyte, the matrix factor (MF = peak are in the presence of matrix/ peak are in the absence of matrix) was calculated. This was investigated to avoid interfering peaks which can occur in the analyte range and influence the peak areas. Skin samples were treated like in the penetration study and spiked with deuterated CER [NP].

3.4. Preparation of microemulsions

Since CER [NP] was poorly soluble in all of the solvents, the water and oil phases were prepared separately. The MEs were prepared by weighing CER [NP] and [AP], the oil phase (Tegosoft TN) and the oil in water surfactant (Hydriol PGMO4) in a glass vial followed by heating at 90 °C (drying oven OV500, Memmert GmbH, Schwambach, Germany) for 3 h. The Hydrolite 5: water mixture (9:1, v/v) with the oil in water surfactant (Tegocare PL4) was also heated in a drying oven for 15 min. Contents of both glass vials were mixed and heated further 15 min. Additional to the basic ME a second formulation with 5 % isosorbide (Polysorb P) was produced. All components of both MEs are summarized in Table 2.

Table 2: Composition of the investigated microemulsions

	C12-C15 Alkyl Benzoate	Polyglycerin-4-oleat	Polyglycerin-4-laurat	1,2-Pentylene glycol/ Water (9:1)	Isosorbide	Cer [AP]	Cer [NP]
ME1	10%	20%	20%	50%	-	+0.3%	+0.3%
ME2	10%	20%	20%	45%	5%	+0.3%	+0.3%

3.5. Characterization of microemulsions

The physical stability of the MEs was routinely evaluated at ambient conditions by visual inspection of the samples over a period of time. Any physical change, like as turbidity, phase separation, flocculation or precipitation was taken as indicator for instability. The isotropy was verified using cross-polarized light microscope (Zeiss Axiolab Pol, Carl Zeiss MicroImaging GmbH, Jena, Germany) whereby a clear system appears as dark background was categorized as ME. The viscosity was measured at 25±0.2 °C using a rotational viscometer (Anton Paar GmbH, Graz, Austria) at 11 varying share rates (1-100 s^{-1}) and, since all MEs exhibited Newtonian type of flow, the average of viscosity and their RSD value were calculated.

Photon correlation spectroscopic (PCS) results were obtained using a light scattering hardware set-up (ALV-Laser, Langen, Germany) for the determination of droplet diameter of MEs. A green Nd: YAG DPSS-200 laser (532 nm, Coherent, Auburn, USA) with an output of 200 mW was used as a light source. The sample cell was placed on a motor- driven precision goniometer (±0.01°) which enabled the photo-multiplier detector to be moved from 20° to 150° scattering angles, and was allowed 10 min for temperature equilibration. The second- order intensity time correlation functions (g*2) were recorded with an ALV-5000E Multiple Tau Digital Correlator with fast option, with a sampling time of 60 s. Cyclindrical sample cell made of Suprasil quartz glass (10 mm dm: Hellma, Mühlheim, Germany) was used as a sample holder. Measurements were made at 25 °C at six different angles, and analysis of the results was made using ALV-5000 Multiple Tau Digital Correlator (ALV-Laser Vertriebsgesellschaft mbH., Langen, Germany)

Additionally, a pseudo-ternary phase diagram (PT-PD), where the surfactant concentration was plotted against the concentration of the lipophilic phase and against the concentration of the hydrophilic phase was constructed at room temperature. It is well suitable to estimate the phase boundaries of an optically isotropic ME region. For each sample 2.0 g of ME containing 0.3 % CER [NP] and 0.3 % CER [AP] were prepared. Both ME were compared to an established crème of the German Pharmaceutical Codex, the Basiscreme (DAC) (Table 3).

Table 3: Formulation of Basiscreme DAC

Glycerolmonostearate 60	4,0 g
Cetylalcohol	6,0 g
Medium-chain triglycerides	7,5 g
White vaseline	25,5 g
Macrogol-20-glycerolmonostearate	7,0 g
Propylene glycol	10,0 g
Purified water	40,0 g

Glycerol monostearate 60, cetylalcohol, medium-chain triglyceride and white vaseline were heated at 60 °C and a mixture of macrogol-20-glycerolmonostearate, propylene glycol and purified water, also heated at the same temperature, was added proportionally. The crème was stirred in a fanta cup till cooling and the evaporated water was compensated.

3.6. Hen´s egg test chorioallantoic membrane (HET- CAM)

Fertilized eggs of the New Hampshire hens were incubated for 8 days at 37 °C and relative humidity of 55 %. The eggs were turned around every 12 h, except of the last 24 h. After that period a circular hole with a diameter of about 1.5 cm was cut in the less convex pole of the eggs. The amnion was carefully removed to expose the CAM. Then 100 µL of the MEs, 1 % sodium lauryl sulfate (SLS) as positive control and normal saline (negative control) were applied on the CAM. Finally, the CAMs were observed for 300 s for occurrence of any haemorrhage (H. bleeding from vessels), vascular lyses (L. blood vessel disintegration) and coagulation (C. intra- and extra-vascular protein denaturation). Six replicates were examined by this method.

The irritation scores (ISs) of two different MEs were determined in order to evaluate the skin irritation or corrosion potential of ME without and with 5 % retarder isosorbide. According to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the IS, calculated using Eq., can be used to assess the degree of irritation caused by pharmaceutical preparations: 0-0.09 non- irritation potential; 1-4.9 slight irritation potential; 5-8.9 moderate and 9-21 severe irritation potential.

3.7. Ex vivo permeability study

Three pieces of excised human thigh skin (postoperatively cleaned and the subcutaneous tissue mechanically dissected) from forearm (Skin A), shin (Skin B) and back (Skin C) was mounted on a Franz diffusion cell (Crown Glass Company, Somerville; NJ, USA) the outer side of the skin facing the donor compartment. The acceptor compartment was filled with distilled water, and 20 mg of the formulation were applied on the skin surface (3.1416 cm^2) and allowed to permeate for 30,100 and 300 min. The temperature of the cell was maintained at 32 °C. At the given time, the apparatus was dismantled; the remaining formulation was thoroughly wiped with a cotton swab. The skin was then punched out in three discs (0.2827 cm*2) using a Kromayer punch (Stiefel-Laboratorium, Offenbach, Germany). All discs were cut uniformly into horizontal skin layers. Two 10 µm thick slices to represent the SC (SC 1 and SC2), subsequently, two 20 µm thick slices for the epidermis (EP1 and EP2) and three 40 µm thick slices were cut out to represent the dermis (DR1, DR2, DR3) using a cryo-microtome (Jung, Heidelberg, Germany). Each underlying layer is summarized under the definition stump.

Then, each layer, including, swab, acceptor and gaze were sonicated at 50 °C in hexane/ethanol (2:1, v/v) for 2 h in a sonication bath (Bandelin electronic, Berlin, Germany) and was left over night for maximum lipid extraction. Evaporated with N_2 stream at 50 °C and stored at -18 °C in the freezer until measurement took place the day after. The samples were resolved in 100 µL methanol containing the internal standard (IS) carbamazepine with a concentration of 1.0 µL/ml.

Other volumes were used for extraction of acceptor, swab and gauze. For the acceptor 4 mL were taken for dissolving and 0.4 mL for resolving in methanol with IS. For swab and gauze 1.5 mL of hexane/ethanol (2:1, v/v) were added for dissolving. According to the procedure described above, after sonication and evaporation, they were resolved in 5 mL. Prior to the experiment, an ethical clearance was obtained from the Independent Ethics Comittee of the Faculty of Medicine, Martin Luther University Halle-Wittenberg.

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