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Enhancement of stability and skin permeation by sucrose stearate and cyclodextrins in progesterone nanoemulsions

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ABSTRACT

Lecithin-based nanoemulsions are colloidal drug delivery systems which offer fundamental advantages in topical therapy, such as excellent skin permeation of lipophilic drugs; however, their physicochemical long-term stability is usually rather poor without the use of additional synthetic surfactants such as polysorbates. In a novel approach negatively and positively charged formulations were developed without the use of conventional synthetic surfactants. Natural substances such as sucrose esters and different cyclodextrins were additionally used as stabilising agents. Emphasis was laid on optimisation of the homogenisation process and formulation properties. The optimised formulations were tested for their potential as drug delivery systems for progesterone. Furthermore, crucial formulation parameters such as particle size and zeta potential were monitored for more than a year. In this context, the effect of the natural excipients sucrose stearate and cyclodextrins α, β and γ on in vitro skin permeation was investigated; the influence of the positive particle surface charge induced by incorporation of the cationic phytosphingosine was evaluated as well. The results showed that in particular the cyclodextrins seemed to induce fundamental changes in formulation microstructure as confirmed by cryo TEM, thus leading to remarkably increased skin permeation rates of progesterone compared to the control.

1. Introduction

Multicomponent systems with particle sizes in the submicron range play an important role as drug delivery systems today. In topical drug delivery, colloidal multiphase systems such as nanoemulsions offer fundamental advantages in terms of permeation enhancement for lipophilic drugs and skin-compatibility (Tadros et al., 2004). However, their widespread use is limited by their comparatively poor long-term stability when compared to thermodynamically stable systems such as microemulsions (Tadros et al., 2004).

Nanoemulsions are metastable systems characterised by a mean droplet diameter below 100 nm (Sonneville-Aubrun et al., 2004). However, the term is widely used to describe formulations with particle sizes in the submicron range below 500 nm (Porras et al., 2004). Hence for the sake of simplicity, the submicron-sized formulations developed in this study will be referred to as nanoemulsions.

A major amount of research has been devoted to the development of lecithin-based nanoemulsions with acceptable physicochemical stability (Yilmaz and Borchert, 2005; Hoeller et al., 2009). The small content of mostly eudermic surfactants in these formulations renders them highly suitable for dermal application.

Both negatively and positively charged nanoemulsions have been developed in order to overcome the barrier posed by the stratum corneum of the skin (Piemi et al., 1999; Yilmaz and Borchert, 2005; Hoeller et al., 2009). Literature suggests that formulations with positive droplet surface charge yield higher permeation rates for drugs because of better interaction with negatively charged residues of skin proteins (Piemi et al., 1999; Hoeller et al., 2009) or lipids (Michniak-Kohn et al., 2005). The cationic base phytosphingosine (PS) has already been incorporated into nanoemulsions successfully (Yilmaz and Borchert, 2005; Hoeller et al., 2009) and was therefore selected as additional compound to induce a positive particle surface charge.

So far, however, additional synthetic surfactants such as polysorbates (Tweens) were generally necessary to stabilise such nanoemulsions for a longer period of time (Yilmaz and Borchert, 2005; Hoeller et al., 2009). Polysorbates are ethoxylated tensides which supposedly cause skin irritation and contact dermatitis (Bergh et al., 1998a,b). They might therefore impair the skin-friendliness of the developed nanoemulsion systems.

In this context, natural emulsifiers offer an appropriate alternative to conventional synthetic surfactants. Apart from lecithin mixtures, various carbohydrates have been shown to possess interesting properties in terms of stabilising multiphase systems. In recent years, the so-called "sugar surfactants" have enjoyed increased attention in formulation development. This term not only refers to alkylpolyglucosides, but also to the less frequently investi-

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gated sucrose esters such as sucrose stearate (SS). These non-ionic surfactants are fatty acid esters of sucrose which exhibit different HLB values according to the type and number of their fatty acid residues (Csoka et al., 2007). Certain sucrose esters have been shown to increase skin permeation of drugs (Ayala-Bravo et al., 2003; Cazares-Delgadillo et al., 2005; Calderilla-Fajardo et al., 2006; Csoka et al., 2007). Therefore, these natural surfactants were chosen as co-emulsifying agents to be tested for their suitability in formulation development and their effect on skin.

Another class of carbohydrates which has recently (Duchene et al., 2003; Bochot et al., 2007; Inoue et al., 2008; Rother, 2009) been investigated in terms of emulsifying properties are the cyclic oligosaccharides called cyclodextrins (CDs). These well-known pharmaceutical compounds are able to form non-covalent inclusion complexes with suitable lipophilic molecule structures. They are therefore frequently used to stabilise or solubilise lipophilic drugs (Challa et al., 2005). Their rather poor water-solubility has led to the development of a large number of chemically modified derivatives with improved physicochemical properties. The present study, however, focuses on the use of the common natural CDs α, β and γ.

In drug delivery systems used on skin, CDs have been reported to improve the dispersion of drugs and influence their skin permeation rates (Loftsson et al., 2007). In addition, they can be used to stabilise emulsion systems by complexation of fatty acid residues of the oil phase. Thus, new surface active molecule complexes are formed which lower interfacial tension and stabilise the systems (Duchene et al., 2003; Inoue et al., 2008). These newly formed amphiphilic "supermolecules" (Rother, 2009) represent highly eudermic emulsifying agents as the CDs themselves cannot permeate the skin due to their large molecular weight (Loftsson and Masson, 2001).

The concept of using CDs as emulsifying agents in multiphase systems was first introduced by Shimada et al. (1991, 1992). However, it has only been investigated to a limited extent in simple emulsion or multiple emulsion systems (Yu et al., 2003; Hashizaki et al., 2007; Inoue et al., 2008). To date few approaches have been made to test the feasibility of using CDs as additional stabilising agents in complex multicomponent systems such as lecithin-based nanoemulsions. Hence, the natural CDs α, β and γ were chosen as respective co-stabilising excipients in order to investigate their effect on formulation properties and skin permeation of the model drug progesterone.

In conclusion, the primary aim of the present study was the development of stable nanoemulsions as delivery systems for progesterone without the use of possibly skin-irritating synthetic surfactants such as polysorbates. To this end, lecithin-based nanoemulsions with negative or positive particle surface charge were developed and optimised with the help of additional stabilising agents such as sucrose esters and CDs. The second objective of this study was the investigation of the effect of aforementioned excipients, in particular the CDs, on formulation properties and microstructure as well as skin permeation of the model drug progesterone *in vitro*. In this context, the hypothesis of superior skin permeation from positively charged formulations containing phytosphingosine in comparison to their negatively charged counterparts was evaluated as well.

2. Materials and methods

2.1. Materials

Cyclodextrin α (Cavamax® W6 Pharma) and γ (Cavamax® W8 Pharma) were obtained from Wacker Chemie AG (Munich, Germany); cyclodextrin β (Kleptose) was kindly donated by Roquette frères (Lestrem, France). Progesterone (CAS: 57-83-0) was purchased from Sigma–Aldrich (St. Louis, USA). Phytosphingosine was kindly provided by Degussa (Cosmoferm BV, NL). Lipoid E-80 was donated by Lipoid GmbH (Ludwigshafen, Germany), containing 81.8% phosphatidylcholine, 8.2% phosphatidylethanolamine and 2.0% lysophosphatidylcholine according to manufacturers' specifications. Sucrose stearate (Ryoto Sugar Ester® S-970) and sucrose laureate (Ryoto Sugar Esters® L-595 and L-1695) were supplied by Mitsubishi-Kasei Food Corporation (Tokyo, Japan). Propylene glycol (1,2-propanediol) and the antioxidant α-tocopherol were purchased from Pauli GmbH & Co. KG (Vienna, Austria). The preserving agent potassium sorbate was obtained from Herba Chemosan Apotheker-AG (Vienna, Austria). PCL-liquid (cetearyl ethylhexanoate, isopropyl myristate) was provided by Symrise GmbH & Co. KG (Holzminden, Germany). All other chemicals used were of analytical reagent grade and used as received without further purification.

2.2. Preliminary tests and solubility studies

2.2.1. Optimisation of formulation composition

Different sugar esters S-970, L-595 and L-1695 were tested as co-emulsifiers; to this end, they were incorporated (1%, w/w) into the basic formulations. Furthermore, different concentrations of CDs were incorporated into the basic mixtures both alone and in combination with sucrose esters; the most suitable concentration of CDs in terms of formulation properties was selected for further studies.

2.2.2. Solubility of progesterone

An important aspect of skin permeation studies *in vitro* is the choice of the receptor fluid. Progesterone is almost insoluble in water (3.79×10^{-5} M at 25 °C) (Zoppetti et al., 2007); since a wholly aqueous receptor medium like phosphate buffer is unsuitable for drugs with a water solubility lower than 10 μg/ml (Brain et al., 1998), propylene glycol/water (40 + 60, w/w) was chosen as receptor phase. The solubility of progesterone in this acceptor medium has already been established and has been found to be suitable for *in vitro* skin diffusion studies (Valenta et al., 2001; Biruss and Valenta, 2006).

2.3. Formulations

Nanoemulsions were prepared as previously described (Hoeller et al., 2009); process parameters were optimised. The aqueous and oily phases were prepared separately. The aqueous phase, consisting of freshly distilled water and potassium sorbate, was stirred at 50 °C. In the respective formulations, sucrose ester and CD α, β or γ were incorporated into the aqueous phase. The oil phase consisted of PCL-liquid, Lipoid E-80, propylene glycol and α-tocopherol; phytosphingosine and progesterone were dissolved in the oil phase as well in the respective formulations. The two phases were mixed and pre-homogenised for 4 min with an ultra-turrax (Omni 500) at 2500 rpm. Afterwards, the mixture was stirred and heated to 50 °C before it was further homogenised with a high-pressure homogeniser (EmulsiFlex C3, Avestin) for 16 homogenisation cycles at 750 bar. Table 1 shows the different formulations as well as their composition and abbreviations.

2.4. Nanoemulsion characterisation

2.4.1. Particle size

All formulations were analysed for their particle size and particle size distribution by photon correlation spectroscopy using a Zetasizer Nano ZS (Malvern, UK) at 25 °C. Samples were diluted with freshly distilled water 1:100 (v/v) to diminish opalescence.

Table 1
Composition of optimised nanoemulsion formulations with negative particle surface charge and abbreviations. The corresponding positively charged formulations were created by addition of the cationic phytosphingosine (PS, 0.1%, w/w) and marked with "+". Drug-loaded formulations were created by incorporation of progesterone (1%, w/w) and marked with "prog".

Excipients	Nanoemulsion composition (% w/w)							
	α	β	γ	Control	α-SS	β-SS	γ-SS	Control-SS
Lipid phase								
PCL-liquid	20	20	20	20	20	20	20	20
Lipoid E80	4	4	4	4	4	4	4	4
Propylene glycol (PG)	3	3	3	3	3	3	3	3
α-Tocopherol	1	1	1	1	1	1	1	1
Aqueous phase								
Sucrose stearate S-970 (SS)	–	–	–	–	1	1	1	1
Cyclodextrin α (α-CD)	0.5	–	–	–	0.5	–	–	–
Cyclodextrin β (β-CD)	–	0.5	–	–	–	0.5	–	–
Cyclodextrin γ (γ-CD)	–	–	0.5	–	–	–	0.5	–
Potassium sorbate	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Distilled water to	100	100	100	100	100	100	100	100

The obtained polydispersity index (PDI) values represent the particle size distribution within the formulations. PDI values below 0.2 indicate a narrow size distribution; this indicates good long-term stability due to reduction of degradation processes like Ostwald ripening (Yilmaz and Borchert, 2005). The parameters of interest were measured immediately after preparation of the formulations; the obtained nanoemulsions were stored at 4 °C and measurements were performed every fortnight over a period of more than 12 months. Thus, information about the long-term stability of the formulations was gained.

2.4.2. Particle surface charge (zeta potential)

The particle surface charge of the formulations was determined by laser Doppler electrophoresis using a Zetasizer Nano ZS (Malvern, UK). Zeta potential (ZP) values of the formulations were determined at 25 °C. Samples were diluted with distilled water (1:100, v/v) containing sodium chloride (0.01 mmol) in order to ensure constant conductivity below 0.05 mS/cm. As distilled water alone might lead to fluctuating conductivity as solvent, addition of electrolytes ensures reproducible measurement conditions (Mueller, 1996; Yilmaz and Borchert, 2005). The ZP roughly characterises the surface charge of the emulsion particles. High absolute values lead to repulsive forces between particles which might improve the stability of multiphase systems. Absolute values higher than 30 mV generally indicate good long-term stability (Mueller, 1996). Zeta potential values were measured immediately after preparation of the formulations and were continuously analysed every fortnight over the observation period of more than 12 months.

2.4.3. Cryo transmission electron microscopy (Cryo TEM)

Standard nanoemulsion samples containing β-CD were compared to a control nanoemulsion without CD in order to establish differences in particle formation and microstructure of the formulations. The samples were dissolved (1:10, v/v) in distilled water (pH 6.7); then a 4 μm drop of each solution was placed on a TEM copper grid covered with a perforated carbon film (Pelco International) and blotted with a filter paper to form a thin liquid film of the sample (thickness of 100–250 nm). The thinned sample was plunged into liquid ethane at its freezing temperature (−183 °C) to form a vitrified specimen, and then transferred to liquid nitrogen (−196 °C) for storage until examination. Vitrified specimens were examined in a Philips T12 transmission electron microscope (Philips) operating at an accelerating voltage of 120 kV using an Oxford CT3500 (Oxford Instruments) cryo holder that maintained the vitrified specimens at −160 °C during sample observation. Images were recorded digitally on a cooled Gatan BioScan CCD camera (Gatan) using the DigitalMicrograph 3.4 software (Gatan) in low-dose imaging mode to minimise beam exposure and electron beam radiation damage.

2.5. Chemical stability

In order to investigate a possible influence of the CDs on stability and distribution of progesterone, stability studies were performed for all drug-loaded formulations. The drug content was analysed immediately after preparation and set as 100%. The nanoemulsions were stored at 4 °C. Samples were taken every fortnight for 6 months. Briefly, 10 mg of nanoemulsion were dissolved in 1 ml of methanol, centrifuged for 6 min at 12,000 rpm (Hermle Z323K, MIDSCI, USA) and analysed by HPLC. Samples were taken at least in triplicate ($n \geq 3$).

2.6. Skin permeation experiments

In vitro skin permeation studies were performed using standard Franz-type diffusion cells (Permegear, USA). Porcine abdominal skin was chosen as model membrane because of its morphology and permeability, which are similar to those of human skin (Cazares-Delgadillo et al., 2005; Michniak-Kohn et al., 2005). The porcine abdominal skin was freed from hair and treated with a dermatome (GB 228R, Aesculap) set at 1.2 mm. The obtained skin was stored at −20 °C until use. The samples were defrosted 2 h prior to the experiment.

Appropriate skin patches were clamped between the donor and the receptor chamber of the diffusion cells having a permeation area of 1.13 cm^2. The receptor compartment was filled with 2 ml of propylene glycol/water (40 + 60, w/w) to provide sink conditions for progesterone, as previously reported (Valenta and Wedenig, 1997; Biruss and Valenta, 2006).

The diffusion cells were kept at skin surface temperature (32 °C) and stirred with magnetic bars for 48 h. The formulation (0.6 g) was placed on the excised skin in the donor chamber. Samples of 200 μl were removed at defined time intervals for analysis and replaced by fresh receptor medium. At least five parallel experiments were performed for each formulation ($n \geq 5$). The samples were analysed for their drug content by HPLC. Permeation profiles of progesterone were constructed by plotting time (hours) against the cumulative amount of the drug (μg/cm^2) measured in the receptor solution. In addition, the steady state flux (J, μg cm^{-2} h^{-1}) was calculated by linear regression after 8 h of lag-time.

2.7. HPLC analysis of progesterone

Samples were analysed for their drug content by HPLC (PerkinElmer, USA), consisting of an auto sampler (ISS-200), a

Table 2
Physicochemical properties of different nanoemulsions: comparison of blank versus drug-loaded formulations containing β-CD. Values are means ± SD of at least three formulations ($n \geq 3$). Measurements were performed in triplicate on a Zetasizer Nano ZS (Malvern, UK) at 25 °C. Samples were diluted with distilled water (1:100, v/v) containing sodium chloride (0.01 mmol) before the experiments to ensure constant conductivity around 0.025 mS/cm. Parameters shown are mean particle size (MPS), polydispersity index (PDI) and zeta potential (ZP).

	MPS (nm)	PDI	ZP (mV)	Conductivity (mS/cm)
β	149.58 ± 10.05	0.07 ± 0.01	−23.54 ± 3.38	0.023 ± 0.002
β prog	194.62 ± 13.18	0.07 ± 0.01	−20.82 ± 4.47	0.025 ± 0.003
β-SS	140.35 ± 6.40	0.08 ± 0.01	−41.96 ± 2.67	0.025 ± 0.002
β-SS prog	174.33 ± 13.86	0.08 ± 0.01	−37.51 ± 4.57	0.024 ± 0.003
β+	258.80 ± 56.56	0.19 ± 0.06	55.20 ± 3.238	0.028 ± 0.005
β + prog	316.59 ± 20.42	0.24 ± 0.04	52.90 ± 3.64	0.023 ± 0.005
β-SS+	175.37 ± 12.43	0.11 ± 0.02	46.33 ± 3.46	0.031 ± 0.006
β-SS + prog	266.67 ± 22.65	0.20 ± 0.03	44.75 ± 3.61	0.023 ± 0.002

pump (lc pump, ISS-200) and an UV-diode array detector (235C). A previously reported method was used with slight adaptations (Biruss and Valenta, 2006), using a Nucleosil 100-5 C18 column (250 mm × 4 mm, Macherey-Nagel, USA) plus pre-column (SS 8/4). The mobile phase consisted of methanol/water (70 + 30, w/w). The detection wavelength was set at 255 nm and the retention time was approximately 7 min at a flow rate of 1.0 ml/min. A calibration curve was calculated based on peak area measurements of diluted standard solutions ranging from 0.79 to 101.30 μg/ml. The obtained correlation coefficient was 0.9999.

2.8. Differential scanning calorimetry (DSC)

Skin samples were analysed with a differential scanning calorimeter (PerkinElmer DSC-7) according to an established method in order to investigate the thermal transitions of the porcine skin used for the experiments (Valenta et al., 2001; Hoeller et al., 2009). In short, about 25 mg of porcine skin were impregnated with 3 ml of nanoemulsion for 24 h. Control samples were treated with 3 ml of distilled water in the same fashion. The skin samples were then blotted dry, sealed within an aluminium holder and heated from 30 to 120 °C at a heating rate of 5 °C/min. The obtained DSC curves were compared to the control samples in terms of linear onset and transition temperature (peak maximum) of the obtained endothermic peak. Gravimetric analysis of the samples prior to DSC experiments showed an average water content of 55.61 ± 1.82% (w/w).

2.9. Statistical data analysis

Statistical data analysis was performed with the software program GraphPadPrism using the Student's *t*-test with $P < 0.05$ as minimum level of significance. Results are expressed as means of at least three experiments ± SD.

3. Results

3.1. Formulations

Based on previous studies, basic lecithin-based nanoemulsions were optimised in terms of composition and production conditions. Blank and drug-loaded nanoemulsions were created using an established method (Hoeller et al., 2009). Table 2 shows the physicochemical properties of the different formulations. It was also possible to introduce a positive surface charge by addition of PS; a concentration of 0.1% (w/w) proved to be most suitable.

Preliminary studies with different concentrations of CDs showed an amount of 0.5% (w/w) to be appropriate for creating stable nanoemulsions with acceptable solubilising capacity for other compounds.

Three saccharose esters were tested as co-surfactants. Sucrose laureate L-595 led to systems with high lipophilicity, thus rendering incorporation of lipophilic drugs impossible. Sucrose laureate L-1695 led to unstable formulations with quick increase in particle size and fluctuating zeta potential. Sucrose stearate S-970 (SS) led to satisfying results; it was therefore chosen as most suitable co-surfactant for all further studies.

3.2. Nanoemulsion characterisation

Visual inspection revealed whitish, homogenous formulations of low viscosity in all cases. As the droplet size is above 100 nm, the formulations appear white due to significant multiple scattering of light (Mason et al., 2006).

3.2.1. Particle size, polydispersity index and zeta potential

All formulations were analysed directly after production. Critical parameters such as particle size, PDI and zeta potential were determined. Since properties of corresponding formulations were similar irrespective of the type of CD used, nanoemulsion properties are demonstrated only on formulations containing β-CD as representative example. Table 2 demonstrates their physicochemical properties after 16 homogenisation cycles.

Negatively charged blank nanoemulsions showed particle sizes between 140 and 195 nm while particle sizes of drug-loaded formulations were slightly increased. Formulations containing additional SS exhibited lower particle sizes. As Table 2 clearly shows, PDI values of all negatively charged formulations were far below 0.2 which indicates high homogeneity within the formulations. Addition of PS led to positively charged nanoemulsions with increased particle sizes of at least 170 nm up to over 300 nm. As expected, the PDI was slightly deteriorated, thus affecting storage stability of the formulations. The additional incorporation of SS again decreased particle sizes in most cases. In contrast, the addition of progesterone led to strongly increased particle sizes.

The particle surface charge (zeta potential, ZP) values were determined for all formulations. Conductivity was kept constantly below 0.05 mS/cm, thus ensuring reproducible measurement conditions. Average conductivity of the diluted samples was around 0.025–0.030 mS/cm for all measurements. Negatively charged nanoemulsions showed average ZP values around −20 mV. Addition of SS led to an increase in absolute ZP values from around −20 to −40 mV, thereby increasing electrochemical stability. Addition of PS led to formulations with positive ZP values between +44 up to +55 mV. In these positively charged nanoemulsions, incorporation of SS only induced minor changes in surface charge; ZP values were not influenced or even decreased in some cases. Incorporation of progesterone generally led to a slight decrease of absolute ZP values.

The physicochemical long-term stability of the formulations was monitored over 12 months and investigations are still ongo-

Table 3
Influence of cyclodextrins on long-term stability: effect of β-cyclodextrin on positively charged drug-loaded formulations with (a) or without (b) sucrose stearate (SS). Parameters shown are mean particle size (MPS) in nm, polydispersity index (PDI) and zeta potential (ZP) in mV. Measurements were performed at least in triplicate ($n \geq 3$; Zetasizer Nano) every 2 weeks for 12 months or until phase separation occurred (*). Numbers are means ± SD.

(a)

Time (weeks)	β + prog			Control + prog		
	MPS ± SD	PDI ± SD	ZP ± SD	MPS ± SD	PDI ± SD	ZP ± SD
0	316.59 ± 20.42	0.24 ± 0.04	52.90 ± 3.64	465.47 ± 70.66	0.35 ± 0.04	54,45 ± 4.60
2	320.89 ± 41.49	0.25 ± 0.02	53.39 ± 2.16	472.32 ± 50.50	0.32 ± 0.04	50,02 ± 1.55
4	314.20 ± 28.87	0.24 ± 0.05	50.37 ± 4.70	524.64 ± 95.74	0.37 ± 0.05	44.78 ± 5.02
6	307.57 ± 13.54	0.21 ± 0.03	49.33 ± 3.42	*	*	*
8	325.35 ± 34.64	0.21 ± 0.01	49.65 ± 1.58	*	*	*
10	312.77 ± 7.78	0.24 ± 0.03	50.41 ± 2.51	*	*	*
12	334.74 ± 25.82	0.23 ± 0.09	46.60 ± 3.82	*	*	*
14	350.15 ± 52.30	0.22 ± 0.07	41.11 ± 1.80	*	*	*
16	362.41 ± 48.32	0.26 ± 0.08	40.72 ± 6.06	*	*	*
18	336.06 ± 17.83	0.26 ± 0.04	48.52 ± 1.80	*	*	*
20	352.01 ± 36.34	0.28 ± 0.06	38.08 ± 2.78	*	*	*
22	375.77 ± 55.26	0.23 ± 0.07	37.93 ± 4.74	*	*	*
24	326.45 ± 10.36	0.23 ± 0.05	33.97 ± 9.36	*	*	*
52	465.41 ± 56.76	0.34 ± 0.06	34.24 ± 6.79	*	*	*

(b)

Time (weeks)	β-SS + prog			Control-SS + prog		
	MPS ± SD	PDI ± SD	ZP ± SD	MPS ± SD	PDI ± SD	ZP ± SD
0	266.67 ± 22.65	0.20 ± 0.03	44.75 ± 3.61	504.36 ± 81.79	0.32 ± 0.04	56.54 ± 3.93
2	274.04 ± 21.78	0.18 ± 0.04	45.46 ± 1.75	487.41 ± 136.26	0.34 ± 0.12	46.80 ± 2.96
4	287.23 ± 49.09	0.162 ± 0.07	40.65 ± 1.33	511.18 ± 85.54	0.34 ± 0.02	46.79 ± 1.50
6	326.66 ± 57.14	0.21 ± 0.03	40.18 ± 1.21	*	*	*
8	353.21 ± 74.78	0.21 ± 0.05	38.15 ± 6.71	*	*	*
10	381.09 ± 102.43	0.19 ± 0.01	34.92 ± 3.83	*	*	*
12	474.67 ± 188.08	0.24 ± 0.01	35.35 ± 4.73	*	*	*
14	516.09 ± 199.21	0.25 ± 0.02	29.54 ± 8.13	*	*	*
16	630.00 ± 307.00	0.24 ± 0.03	31.63 ± 10.20	*	*	*
18	*	*	*	*	*	*

ing. For negatively charged nanoemulsions, particle sizes and ZP values remained almost constant for both blank and drug-loaded formulations. Fig. 1 clearly illustrates that incorporation of progesterone hardly influenced formulation properties of negatively charged formulations. However, its effect on the corresponding positively charged formulations was more pronounced. The droplet sizes of positively charged nanoemulsions showed a more rapid increase and ZP values generally showed a stronger decrease, indicating a more rapid destabilisation process (data not shown). These effects were aggravated in the presence of progesterone (Table 3). However, the degree of destabilisation caused by incorporation of progesterone was dependent on the CD incorporated. In this context, formulations containing α- or β-CD showed acceptable particle sizes between 250 and 350 nm, followed by γ-CD. Control formulations without any CD, however, were completely unstable with particle sizes soon deteriorating far beyond 500 nm. In addition, the respective PDI values were increased. Table 3a and b distinctly show the physical destabilisation of the control formulations caused by agglomeration of particles. As can be seen in Table 3b, no positive influence of the additional SS was noticeable here. This indicates that it is the presence of CD alone that contributes to the enhanced physical stability of the respective formulations.

3.2.2. Cryo TEM

As illustrated in Fig. 2, the nanoemulsion droplets appeared mostly spherical, if slightly deformed; this observation is in accordance with previous studies (Mason et al., 2006; Shakeel et al., 2007). The observed droplet size is in good correspondence with the results achieved by photon correlation spectroscopy measurements. However, the incorporated CD apparently leads to the formation of additional multilamellar structures, as can be seen

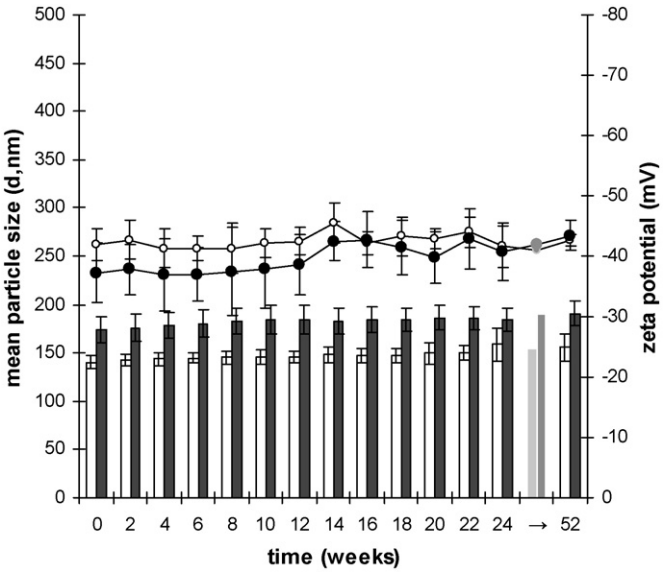

Fig. 1. Influence of progesterone incorporation on long-term stability of nanoemulsions: development of mean particle size (bars) and zeta potential (lines) over an observation period of 12 months as observed on negatively charged formulations, shown on systems containing β-cyclodextrin. Measurements were performed at least in triplicate ($n \geq 3$) every 2 weeks for over a year, again using a Zetasizer Nano. For reasons of clarity, the time frame is shortened in these graphs. Indicated values are means ± SD. β-SS (white bars (□) and white dots (○), respectively); β-SS prog (black bars (■) and black dots (●), respectively).

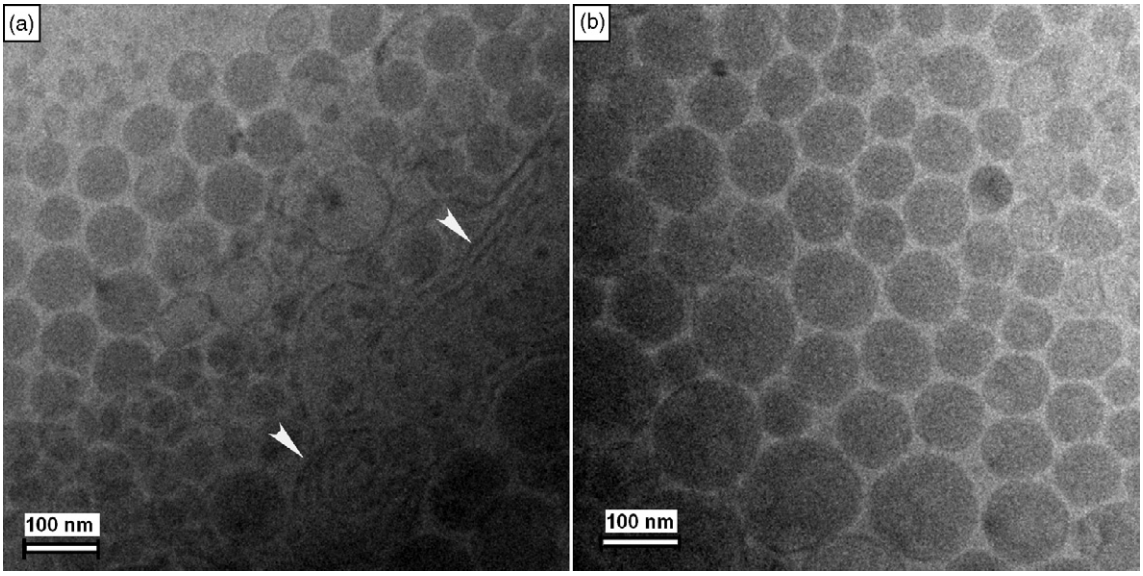

Fig. 2. Cryo TEM photographs of the microstructure of nanoemulsions (a) with β-cyclodextrin and (b) without cyclodextrin. The white arrows indicate the multilamellar structures detected in formulations containing cyclodextrin. The magnification is illustrated by the scale bars.

in Fig. 2a. None of these structures were observed in the control formulation without CD (Fig. 2b).

3.3. Chemical stability

The chemical stability of all drug-loaded formulations was analysed by HPLC every fortnight during 6 months or until decomposition. The average content of progesterone remained around 80% for most nanoemulsions during the observation period (data not shown). Variations in drug content were rather due to problems in analysis than to instability of the steroid hormone, as literature suggests (Kunze, 2006). No degradation products could be detected by HPLC. The incorporation of CDs apparently did not influence the recovered drug content.

3.4. Skin permeation experiments

Permeation profiles of progesterone from different nanoemulsions are shown in Fig. 3. Comparison of the cumulative amounts permeated after 48 h revealed a significant influence of the CDs on skin diffusion rates of progesterone compared to the control ($P < 0.05$). In negatively charged formulations, all CDs led to an increase in skin permeation of progesterone despite the different sizes of their lipophilic cavities. The highest permeation rates were achieved with γ-CD, which was up to 5-fold compared to the control (Table 4) in terms of the cumulative amount of progesterone. These permeation rates were followed by formulations containing β-CD and α-CD. Interestingly, the same ranking of permeation enhancement was observed both in presence or absence of SS, namely γ-CD > β-CD > α-CD > control. An additional effect of SS as permeation enhancer was noticed in all formulations irrespective of the type of CD used (Table 4). The sugar surfactant led to increased skin permeation from negatively charged formulations, acting in synergy with the CDs used. Furthermore, Table 4 clearly indicates that the drug fluxes obtained by linear regression are in good accordance with the conclusions derived from analysis of the cumulative amounts permeated. A comparison of the mean drug fluxes shows a significant if slightly smaller effect of the different CDs with a 3.5-fold increase for formulations with γ-CD and SS.

In positively charged formulations, similar tendencies were observed. The addition of PS led to a further increase of skin permeation, especially in formulations containing γ-CD (Fig. 3). In formulations containing β- and α-CD the effect was not as pronounced in all cases. Further studies are being conducted to achieve consistent results.

3.5. Differential scanning calorimetry (DSC)

DSC studies were performed using a single piece of porcine skin, which showed one endothermic peak at around 78 °C. This peak corresponds to intracellular lipid layers and is in good accordance with literature (Golden et al., 1987). All negatively charged formulations led to a significant change in peak maximum and linear onset of the peak compared to the control samples which were pre-impregnated with water ($P < 0.05$) (Table 5). Both of these parameters were shifted to lower values. This indicates changes

Table 4
Influence of cyclodextrin α, β and γ on cumulative amounts permeated and mean drug flux J of progesterone; indicated values are means ($n \geq 5$) ± SD. The calculated enhancement factor highlights the effect of the cyclodextrins alone or in synergy with sucrose stearate (SS) compared to the control formulation.

Formulation	Cumulative amount ± SD ($\mu g/cm^2$, after 48h)	Enhancement factor versus control	Mean drug flux ± SD (J, $\mu g\,cm^{-2}\,h^{-1}$)
Control prog	9.57 ± 3.56	1.00	0.35 ± 0.08
α prog	18.06 ± 2.22	1.89	0.58 ± 0.14
β prog	25.05 ± 2.29	2.62	0.63 ± 0.06
γ prog	41.86 ± 4.46	4.37	1.04 ± 0.10
α-SS prog	24.53 ± 5.37	2.56	0.72 ± 0.08
β-SS prog	34.16 ± 5.00	3.57	0.86 ± 0.12
γ-SS prog	48.63 ± 5.08	5.08	1.21 ± 0.11

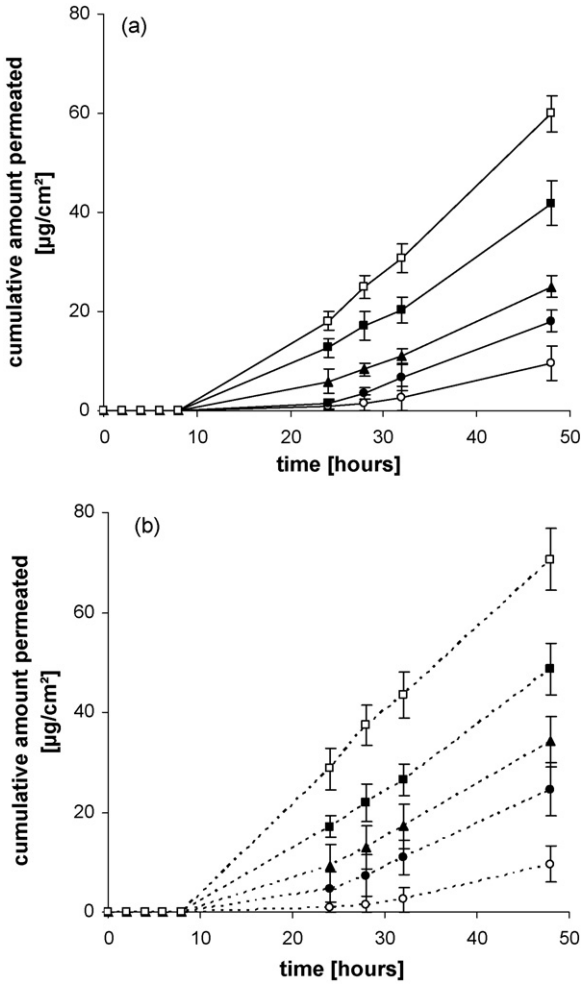

Fig. 3. Effect of cyclodextrin α, β and γ alone (a) and in combination with sucrose stearate (SS) (b) on in vitro skin permeation rates of progesterone from negatively and positively charged nanoemulsions; positively charged formulations are marked with "+". Drug permeation through porcine skin was determined by HPLC. At least five experiments were performed for each formulation ($n \geq 5$) using Franz-type diffusion cells. Indicated values are means ± SD. *Abbreviations*: (a) α prog (●); β prog (▲); γ prog (■); γ + prog (□); control prog (○). (b) α-SS prog (●); β-SS prog (▲); γ-SS prog (■); γ-SS + prog (□); control prog (○).

in thermal transition of the skin lipids, as previously observed in similar investigations (Hoeller et al., 2009). In addition, negatively charged formulations containing α-, β- or γ-CD showed a slightly stronger influence on skin lipids than the control nanoemulsion. However, only minor differences between the effects of the individual CDs were detected. For more distinguished analysis of the different formulations a more sensitive method should be employed.

In positively charged formulations, the changes in terms of peak maximum and linear onset were smaller, if significant in most cases (Table 5) when compared to the water-impregnated control. This is in accordance with previous data (Hoeller et al., 2009); it can therefore be assumed that the positive effect of PS on skin permeation is caused by other interactions than those with skin lipids. However, a slightly stronger effect on skin lipids could be achieved than with previously developed formulations containing PS (Hoeller et al., 2009).

4. Discussion

The primary objective of this study was the development of stable formulations without the use of conventional synthetic surfactants like polysorbates. Emphasis was laid on the use of natural products; hence, the applicability of different sucrose esters as well as CDs was tested extensively. The positive effects induced by incorporation of CDs clearly show that not all possible benefits of CDs as compounds in topical drug delivery systems have been thoroughly investigated yet. The effect of CDs on formulation microstructure might offer applications apart from drug solubilisation or stabilisation. However, it should be brought to mind that the effect of CDs on a complex multiphase system cannot be generally predicted, but has to be investigated separately for every novel formulation. It is of utmost importance to perform CD formulation studies in media that closely resemble the final drug-loaded formulation (Jansook and Loftsson, 2009). The present study is the first evaluation of the effect of CDs on complex nanoemulsion systems on a long-term basis.

Table 5
Thermal transition changes of porcine skin induced by pre-impregnation with positively and negatively charged nanoemulsions containing progesterone (1%, w/w) for 24 h. Porcine skin pre-impregnated with water served as a control. Experiments were performed at least in quadruplicate ($n \geq 4$). Indicated values are means ± SD. T_m indicates the temperature of the transition's maximum. Gravimetric analysis of the samples prior to DSC measurements showed average water content of about 55% (w/w).

Treatment ($n \geq 4$)	DSC T_m (°C)	Linear onset (°C)
Skin treated with water (control)	79.64 ± 0.75	65.83 ± 0.28
Formulations		
Control prog	75.99 ± 0.48	63.06 ± 0.66
α prog	73.63 ± 0.58	62.53 ± 0.44
β prog	74.39 ± 0.94	62.83 ± 0.48
γ prog	75.22 ± 0.66	62.53 ± 0.74
α-SS prog	74.53 ± 0.29	62.97 ± 0.33
β-SS prog	74.49 ± 0.71	62.64 ± 0.06
γ-SS prog	75.13 ± 0.38	62.74 ± 0.55
α + prog	79.51 ± 0.53	64.48 ± 0.73
β + prog	78.85 ± 0.45	64.40 ± 0.55
γ + prog	77.04 ± 0.72	63.74 ± 0.52
α-SS + prog	77.74 ± 0.91	63.89 ± 1.15
β-SS + prog	77.32 ± 0.64	63.65 ± 0.28
γ-SS + prog	76.07 ± 0.68	63.12 ± 0.62

First off, we succeeded in developing basic negatively charged formulations stabilised by lecithin only. Corresponding nanoemulsions with positive particle surface charge were created with the help of the cationic phytosphingosine. However, incorporation of progesterone led to destabilisation and skin permeation rates were rather poor. Therefore, CDs and sucrose esters were tested as additional stabilising agents. This strategy proved to be successful. In particular, a stabilising effect of the incorporated CDs was noticeable in positively charged formulations containing progesterone. The addition of CD α, β or γ led to increased physicochemical stability compared to the control nanoemulsion, which was unstable soon after production. This stabilising effect, however, was not as pronounced in negatively charged formulations during the observation period; it was possibly obscured by the effects of the other surfactants. All of the negatively charged formulations were stable for over 1.5 years and stability evaluations are still ongoing.

In order to visualise the differences in formulation properties caused by incorporation of CDs, the nanoemulsion microstructure was analysed by cryo TEM. Indeed, additional multilamellar structures were found in nanoemulsions containing β-CD while none of these were detected in the control formulation without CD. Furthermore, formulations with CD seemed to exhibit stronger surface tension and their freezing behaviour was completely different from the behaviour of formulations without CD. These two phenomena are most certainly related as carbohydrate containing solutes possess higher cohesion and a certain cryoprotective ability.

Therefore, vitrification of larger volumes of formulations stabilised by carbohydrates, such as the additional cyclodextrins, is possible. In contrast, crystallisation of water almost always occurred in the control nanoemulsions before the cryofixation was completed. The stronger surface tension of the CD containing formulation also might be related to the presence of the additional multilamellar structures. A possible explanation for these formations could be the tendency of lecithin to self-aggregate and form multilamellar structures or other structural units (Shchipunov, 1997; Meier and Schreiber, 2005). These multilamellar layers formed by lecithin molecules can be regarded as an effective structural-mechanical barrier (Shchipunov, 1997). After all, an important issue in terms of destabilisation of lecithin-based emulsions is the separation into an aqueous layer and a concentrated emulsion phase (Shchipunov, 1997). During the present study, this phenomenon was only observed in positively charged nanoemulsions and could be delayed by addition of CDs. In negatively charged formulations, sufficient stability was apparently achieved irrespective of the additional CDs. In this case only extended studies might reveal a more distinctive stabilising effect of the CDs in the long term.

Why the observed multilamellar structures were only formed in the presence of CDs remains to be investigated. Possibly, occupation of interfacial regions by CDs leads to an excess of free lecithin molecules which consequently form said vesicles. It is a well-known fact that amphiphilic molecules like lecithin tend to form higher structural units when dispersed in water at high concentrations; the formation of multiple bilayer membranes can lead to flat lamellar phases or vesicles (Shchipunov, 1997; Meier and Schreiber, 2005). It might be assumed that larger CDs displace more lecithin molecules and therefore lead to increased formation of additional multilamellar structures. The sugar ester present in the investigated formulations might contribute to the formation of these multilamellar or perhaps even micelle-like structures. Literature reports that similar phenomena have been observed in formulations containing elastic vesicles based on a sucrose ester (Bouwstra et al., 2003).

As a second aim of this study, the influence of CDs as well as sucrose stearate on skin permeation of progesterone was evaluated. Indeed, these compounds led to enhanced skin permeation rates of the drug. The enhancement effect of a positive particle surface charge induced by phytosphingosine was not consistent throughout the studies and will be further investigated. Overall, the achieved skin permeation rates from both positively and negatively charged formulations were satisfying. The permeation rates of progesterone from nanoemulsions with γ-CD exceeded those of previously developed formulations such as liposomes when tested under similar conditions (Biruss and Valenta, 2006).

The sucrose ester S-970, namely sucrose stearate, was found to enhance skin permeation from all formulations. In consideration of its HLB value, even stronger permeation enhancement effects might have been achieved with sucrose esters of higher HLB values, such as sucrose laureate L-1695, if they had led to stable formulations in the first place. Previous studies show a satisfying enhancement effect of sucrose stearate S-970 for the release of metoprolol from transdermal therapeutic systems. However, sucrose esters with higher HLB values of around 15–16 which are esterified with short chain fatty acids performed even better in this respect (Csoka et al., 2007). Within the scope of the present study, the use of sucrose stearate S-970 seemed to constitute the best compromise to achieve satisfying results both in terms of formulation stability and skin permeation.

In this context it has to be recollected that the HLB value of lecithin is comparable to that of sucrose stearate S-970. It can therefore be concluded that the addition of sucrose stearate S-970 did not alter the HLB value of approximately 9 of the emulsifying agents within the system. Consequently, the observed enhancement of skin permeation can rather be ascribed to the additional presence of sucrose stearate than to a change in overall HLB value of the emulsifier system used.

Interestingly, the observed skin permeation enhancement was correlated to the cavity size of the incorporated CDs. The mechanism of permeation enhancement through CDs is normally ascribed to the complexation and thus better dispersion of drug molecules within the formulation (Loftsson and Bodor, 1995). In the present formulations, however, it is rather unlikely that the permeation enhancement is caused only by solubilisation of progesterone as the molar amount of CD is very low when compared to the molarity of the drug. It might therefore be assumed that the CDs do not mainly solubilise the drug, but rather contribute to the stabilisation of the interfacial regions between oil droplets and water. According to recent studies, insertion of CD-oil complexes as additional surfactants in this interfacial film can lead to increased release of drugs (Rother, 2009). The amphiphilic molecule complexes supposedly lead to areas of decreased thickness within the interfacial film through which drug molecules can permeate more rapidly. Furthermore, the additional multilamellar structures might promote interactions between skin and formulation in general and could thus exert a positive influence on skin permeation.

5. Conclusion

The results of the present study show that it is possible to develop eudermic nanoemulsions with optimised long-term stability without the use of conventional synthetic tensides. In this respect, emulsifying additives such as sucrose esters are highly recommendable both in terms of stabilisation and skin permeation. Furthermore, the natural CDs α, β and γ proved to be of great interest as additional compounds. The CDs seemed to induce fundamental changes in formulation structure as confirmed by cryo TEM, thus leading to increased skin permeation rates of progesterone. In addition, a positive effect of CD incorporation on formulation stability was noticed for certain formulations.

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