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Research paper

Development of sucrose stearate-based nanoemulsions and optimisation through γ-cyclodextrin

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ABSTRACT

Nanoemulsions aimed at dermal drug delivery are usually stabilised by natural lecithins. However, lecithin has a high tendency towards self-aggregation and is prone to chemical degradation. Therefore, the aim of this study was to develop nanoemulsions with improved structure and long-term stability by employing a natural sucrose ester mixture as sole surfactant. A thorough comparison between the novel sucrose stearate-based nanoemulsions and corresponding lecithin-based nanoemulsions revealed that the sucrose ester is superior in terms of emulsifying efficiency, droplet formation as well as physical and chemical stability. The novel formulations exhibited a remarkably homogeneous structure in cryo TEM investigations, as opposed to the variable structure observed for lecithin-based systems. The in vitro skin permeation rates of lipophilic drugs from sucrose stearate nanoemulsions were comparable to those obtained with their lecithin-based counterparts. Furthermore, it was observed that addition of γ-cyclodextrin led to enhanced skin permeation of the steroidal drug fludrocortisone acetate from 9.99 ± 0.46 to 55.10 ± 3.67 μg cm^{-2} after 24 h in the case of sucrose stearate-based systems and from 9.98 ± 0.64 to 98.62 ± 24.89 μg cm^{-2} after 24 h in the case of lecithin-based systems. This enhancement effect was significantly stronger in formulations based on lecithin ($P < 0.05$), which indicates that synergistic mechanisms between the surfactant and the cyclodextrin are involved. Cryo TEM images suggest that the cyclodextrin is incorporated into the interfacial film, which might alter drug release rates and improve the droplet microstructure.

1. Introduction

Classical O/W nanoemulsions can be produced by high-pressure homogenisation using small amounts of natural lecithin as emulsifier, which accounts for their high skin friendliness [1–3]. Although particle sizes below 100 nm are hardly reached with lecithin-type emulsifiers, the term nanoemulsion is commonly used for such systems with droplet sizes in the lower submicron range. Since phospholipid mixtures possess superior emulsifying properties compared to purified phosphatidylcholine, they are preferred for nanoemulsion production [4,5]. However, these classical nanoemulsion surfactants pose certain problems in formulation development. On the one hand, phospholipids are prone to oxidative and hydrolytical degradation [6], which results in an unfavourable visual and olfactory appearance after a certain storage time. On the other hand, lecithin mixtures have a high potential for self-aggregation phenomena, which leads to the formation of vesicular or multilamellar structures during the production of nanoemulsions [4]. Although numerous studies report the presence of liposomal vesicles in lecithin-based O/W nanoemulsions [7–11], few propositions have been made on how to avoid such by-products of high-pressure homogenisation. Despite decades of research in this field, it is still unclear whether these structures can be entirely avoided [6]. Moreover, it has not been fully clarified whether the presence of such structures has a negative effect on the formulations' long-term stability. Micelle-mediated transport of oil and the occurrence of liposomes have been reported to impair nanoemulsion stability due to increased Ostwald ripening [12–15]. Technological issues aside, the occurrence of liposomes in parenteral nanoemulsion systems is held responsible for dyslipidemias and embolisms [6,16]. The development of homogeneous, vesicle-free O/W nanoemulsions might therefore be desirable to achieve reproducible properties and storage stability.

Although many aspects of lecithin behaviour are hard to predict, few studies discuss the issue of nanoemulsion homogeneity. Common particle size measurement techniques such as dynamic

Abbreviations: CD, cyclodextrin; DLS, dynamic light scattering; PDI, polydispersity index; ZP, zeta potential.

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light scattering fail to distinguish between oil droplets and other nano-sized structures. In addition, information on how to avoid the formation of unwanted surfactant aggregates is scarce. While lecithin molecules have a pronounced tendency towards self-aggregation, other natural and eudermic emulsifiers might be more suitable to form reproducible O/W droplets. Hardly any information on the disposition of different emulsifier types to form these by-products of high-pressure homogenisation can be found. The present investigation addresses this shortcoming by a thorough comparison of a commonly used lecithin mixture and a sucrose ester mixture. It has been shown recently that sucrose esters can successfully be employed as co-surfactants in nanoemulsions [17–19]. However, the use of these natural emulsifier mixtures as main surfactants for classical nanoemulsion systems has not been thoroughly investigated yet. Especially, sucrose stearate S-970 has been shown to exert positive effects on stability and skin permeation of lecithin-based nanoemulsions [20]. Its HLB value of around 9 is comparable to that of lecithin; therefore, it should be determined whether the previously employed natural lecithin mixture lecithin E-80 could be replaced as sole emulsifier. To this end, a thorough characterisation of blank and drug-loaded nanoemulsions in terms of physicochemical formulation parameters, microscopic appearance and long-term stability was conducted.

As a second major focus of this study, the in vitro skin permeation of four model drugs from the novel sucrose stearate-based nanoemulsions was investigated and compared to the permeation from lecithin-based systems. Although O/W nanoemulsions are generally more suitable for the delivery of lipophilic drugs, hydrophilic drugs were also included for reasons of comparison. Moreover, the effect of additional γ-cyclodextrin (CD) was investigated to confirm a previously reported enhancement effect on the skin permeation of a lipophilic steroidal drug [20]. Thus, further insights into the suitability of natural CDs as additives in nanoemulsions are gained.

2. Materials and methods

2.1. Materials

Egg lecithin Lipoid E-80 was donated by Lipoid GmbH (Ludwigshafen, Germany). Sucrose stearate (Ryoto Sugar Ester® S-970) was supplied by Mitsubishi-Kagaku Food Corporation (Tokyo, Japan). Cyclodextrin γ (Cavamax® W8 Pharma) and cyclodextrin α (Cavamax® W6 Pharma) were obtained from Wacker Chemie AG (Munich, Germany). Cyclodextrin β (Kleptose®) was donated by Roquette frères (Lestrem, France). Fludrocortisone acetate (CAS: 514-36-3, Batch No. 075K1029) was purchased from Sigma Aldrich (St. Louis, USA). Fluconazole (CAS: 86386-73-4, Batch No. 050418), and flufenamic acid (CAS: 530-78-9, Batch No. 1619) was obtained from Kemprotec Limited (Middlesbrough, UK). Minoxidil (CAS: 38304-91-5, Batch No. 81835129) was purchased from Caesar & Loretz GmbH (Hilden, Germany). The preserving agent potassium sorbate was obtained from Herba Chemosan Apotheker-AG (Vienna, Austria). PCL-liquid (cetearyl ethylhexanoate, isopropyl myristate) was purchased from Dr. Temt Laboratories (Vienna, Austria). All other chemicals used were of analytical reagent grade and used without further purification.

2.2. Preliminary investigations and solubility studies

2.2.1. Optimisation of formulation composition

Preliminary studies were conducted to optimise formulation composition and processing parameters. Different surfactant concentrations between 1% and 5% (w/w) were tested. Likewise, the most suitable oil volume fraction for particle size reduction was determined. Oil concentrations of 10%, 15% and 20% (w/w) were investigated. Finally, the natural CDs α, β and γ were incorporated (1% w/w) in order to investigate their influence on formulation properties.

2.2.2. Solubility of model drugs: choice of the receptor medium

The suitability of the chosen phosphate buffer (pH 7.4, 0.012 M) as acceptor medium for in vitro skin diffusion studies has already been established for all employed model drugs in previous work by our group; details on solubility studies can be found in the literature [17,21,22]. The use of propylene glycol or ethanol as additives to enhance the solubility of the lipophilic drugs in the aqueous buffer was avoided to prevent solvent effects. For reasons of comparison both within this study and with previous results, the same phosphate buffer was employed for all investigated drugs even if the permeation of the lipophilic drugs might be comparatively lower than under actual in vivo conditions due to their low solubility.

2.3. Formulations

Nanoemulsions were prepared as previously described [17,20]. The aqueous phase, consisting of freshly distilled water and potassium sorbate, was stirred at 50 °C. Sucrose stearate S-970 was incorporated into the aqueous phase, while lecithin E-80 was dissolved in the oil-phase PCL-liquid of the respective formulations. Additional γ-CD was incorporated into the aqueous phase where appropriate. The lipophilic drugs fludrocortisone acetate and flufenamic acid were dissolved in the oil phase, while the hydrophilic drugs fluconazole and minoxidil were dissolved in the aqueous phase. 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 further homogenisation with a high-pressure homogeniser (EmulsiFlex C3, Avestin) for 20 homogenisation cycles at 750 bars. Longer homogenisation times led to an increase in particle size due to overprocessing. Table 1 shows the optimised 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 dynamic light scattering (DLS, 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. The obtained polydispersity index (PDI) values represent the particle size distribution within the formulations. PDI values below 0.2 indicate a narrow size distribution

Table 1
Composition of basic optimised nanoemulsion formulations and abbreviations. Drug-loaded formulations were created by incorporation of 1% w/w of fludrocortisone acetate (flud), flufenamic acid (fluf), fluconazole (fluc) and minoxidil (min). *Abbreviations*: LN, lecithin-based nanoemulsion; SN, sucrose stearate-based nanoemulsion; γ-LN, lecithin-based nanoemulsion with γ-CD; γ-SN, sucrose stearate-based nanoemulsion with γ-CD.

Excipients	Nanoemulsion composition (% w/w)			
	LN	SN	γ-LN	γ-SN
PCL-liquid	20	20	20	20
Lipoid E-80	2.5	–	2.5	–
Sucrose stearate S-970	–	2.5	–	2.5
Potassium sorbate	0.1	0.1	0.1	0.1
γ-Cyclodextrin	–	–	1.0	1.0
Model drug	1.0	1.0	1.0	1.0
Distilled water to	100	100	100	100

and thus good long-term stability due to reduction in degradation processes like Ostwald ripening [23]. The formulation parameters of interest were measured immediately after preparation. The obtained nanoemulsions were stored at 4 °C, and consecutive measurements were performed in regular intervals over a period of 6 months.

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, addition of electrolytes ensures reproducible measurement conditions [23,24]. The ZP roughly characterises the surface charge of the emulsion particles. High absolute values lead to repulsive forces between particles, which may improve the physical stability of multiphase systems. Absolute values higher than 30 mV generally indicate good stability, while values above 60 mV indicate excellent long-term stability [24]. Zeta potential values were determined after production and in regular intervals over 6 months.

2.4.3. Cryo transmission electron microscopy (cryo TEM)

Standard nanoemulsion samples containing either 2.5% of lecithin or 2.5% of sucrose stearate were compared in order to establish differences in particle formation and microstructure of the formulations. Corresponding nanoemulsions containing additional γ-CD (1% w/w) were investigated as well to investigate the effect of the CD on the nanoemulsion structure. The samples were dissolved (1:10 as well as 1:5 v/v, respectively) 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

2.5.1. Chemical stability of incorporated drugs

Both the drug content and the chemical stability of all drug-loaded formulations were investigated. Potential differences between the emulsifiers in terms of drug solubilisation and of the long-term stability of the incorporated drugs should be revealed in this fashion. The results should, however, rather be regarded as an overview than as a comprehensive stability study since the latter would require far more sophisticated methods of analysis which are validated for the detection of anticipated degradation products. The drug content was analysed immediately after preparation and set as 100%. The nanoemulsions were stored at 4 °C. Samples were taken in regular intervals over 6 months. Briefly, 10 mg of nanoemulsion was 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 ($n \geqslant 3$).

2.5.2. Chemical stability of the formulations

Oil components as well as surfactants may be affected by chemical degradation through hydrolysis or oxidation. Since such phenomena result in a decrease in pH [6], pH values of all nanoemulsions were determined in regular intervals over 6 months. At least three formulations ($n \geqslant 3$) were investigated at room temperature (25 °C) with a pH meter (Orion 420A, Bartelt, Austria).

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 [25,26]. The porcine abdominal skin was freed from hair and treated with a dermatome (GB 228R, Aesculap) set at 1.2 mm. The skin was stored at −20 °C until use and thawed prior to the experiments. 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 phosphate buffer (pH 7.4). The diffusion cells were kept at skin surface temperature (32 °C) and stirred with magnetic bars for 24 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 were replaced by fresh receptor medium. In the case of minoxidil, the whole acceptor fluid of 2 ml was removed and replaced in order to ensure sink conditions. At least five parallel experiments were performed for each formulation ($n \geqslant 5$). The samples were analysed for their drug content by HPLC. Permeation profiles of the drugs were constructed by plotting time (hours) against the cumulative amount of the drug (μg/cm^2) as measured in the receptor solution. In addition, the steady-state flux (J, $\mu g\, cm^{-2}\, h^{-1}$) was calculated by linear regression after the respective lag times.

2.7. HPLC analysis

All samples were analysed for their drug content by HPLC (Series ISS-200, Perkin Elmer, USA), consisting of an autosampler, an lc pump and an UV-diode array detector (235C). Previously reported methods were used using a Nucleosil 100-5 C18 column (250 mm × 4 mm, Macherey–Nagel, USA) plus a Nucleosil 100-5 C18 pre-column (CC8/4, 40 mm × 4 mm, Macherey–Nagel, USA). For all analyses, the oven temperature was set at 50 °C and the injection volume was 20 μl. The analysis of the data was performed using the TotalChrom Navigator 6.2.0 software. Standard solutions of the drugs were prepared, and calibration curves were calculated by plotting the analysed drug concentrations against the obtained peak area values. The limit of quantification, set well above the limit of detection in all cases, is represented by the respective lowest point of the standard solution measurements for each drug as given below.

The quantification of fludrocortisone acetate was performed as previously described [21,27].The mobile phase consisted of acetonitrile and water (40/60 v/v). The detection wavelength was 240 nm, and the retention time was around 11 min at a flow rate of 0.8 ml/min. The concentration range of the standard solutions was between 1.84 μg/ml and 1340.0 μg/ml with a coefficient of determination of $R^2 = 0.9999$.

The quantification of flufenamic acid and minoxidil was conducted according to previously described methods with slight adaptations [21,22]. In both cases, the mobile phase consisted of methanol/water (75/25 w/w); glacial acetic acid was added until a pH value of 3.2 was reached. The flow rate was 1.0 ml/min. For flufenamic acid, the detection wavelength was set at 245 nm with a retention time of 4.5 min. A calibration curve was calculated

based on peak area measurements of diluted standard solutions ranging from 0.82 μg/ml to 104.40 μg/ml with R^2 = 0.9990. In case of minoxidil, the detection wavelength was set at 255 nm with a retention time of 4 min. The calibration curve was calculated using standard solutions from 0.6 μg/ml to 207.10 μg/ml. The obtained coefficient of determination was R^2 = 1.00.

The quantification of fluconazole was performed as reported [21]. The mobile phase consisted of freshly prepared phosphate buffer (0.012 M, pH 7.4) and methanol (55/45 v/v) with addition of 1 mmol of octanesulfonic acid. The detection wavelength was set at 260 nm with a retention time around 4.5 min and a flow rate of 1.0 ml/min. The concentration range of the standard solutions was between 17.3 μg/ml and 548.0 μg/ml with R^2 = 0.9999.

2.8. Statistical data analysis

Results are expressed as means of at least three experiments ± SD. Statistical data analyses were performed with the software program GraphPadPrism3. Parametric data were analysed using the Student's *t*-test with $P < 0.05$ as minimum level of significance, while non-parametric data were analysed using the Mann–Whitney test with $P < 0.05$.

3. Results

3.1. Formulation optimisation

The first aim of the study was to develop a stable nanoemulsion system with one or more sucrose esters as emulsifying agents. Since satisfying results could already be obtained with S-970 as sole emulsifier, no co-surfactants were necessary. Further preliminary studies were thus conducted with either sucrose stearate S-970 or lecithin E-80 as individual emulsifiers in simple nanoemulsions. Different amounts of 1%, 2.5% or 5% (w/w) of the respective surfactant were incorporated. The results are given in Table 2. The particle size measurements revealed that sucrose stearate is a superior emulsifying agent when compared to lecithin. Smaller particle sizes could be obtained especially at low concentrations. The ZP values obtained with the different emulsifiers showed that sucrose stearate is likewise superior in terms of electrochemical stability. Overall, the ZP values of sucrose stearate-based nanoemulsions were higher than those of their lecithin-based counterparts at all concentrations and increased concomitantly with increasing surfactant concentration. In contrast, the ZP of lecithin-based nanoemulsions remained at low values for 1% and 2.5% (w/w) of surfactant and only increased when higher amounts of 5% (w/w) of lecithin were added. This tendency is in good agreement with the changes in particle size at the respective surfactant concentrations.

At increasing surfactant-to-oil ratios, the emulsifying efficiency of both surfactants increased and smaller particle sizes were obtained. At 5% (w/w) of surfactant, the performance of lecithin improved notably, if not enough to compete with sucrose stearate. Since high-pressure homogenisation of nanoemulsions with 5% (w/w) of sucrose stearate proved to be hardly feasible due to its increased viscosity, the formulations with 2.5% (w/w) of surfactant were chosen for all further studies.

Different oil volume fractions were investigated as well. Although literature reports otherwise [28], no particle size reduction was achieved by reducing the oil content from 20% to 15% or 10% (w/w). Quite the contrary, a slight increase in particle sizes with a concomitant decrease of ZP values was observed. Therefore, the oil content was maintained at 20% (w/w), which is advantageous in terms of solubilising capacity for lipophilic drugs.

Finally, the influence of the natural CDs α, β and γ on formulation properties was investigated. Neither of these CDs affected formulation parameters such as particle size or ZP in a negative way when included at 1% (w/w), as can be seen in Table 3 for γ-CD. Since previous studies had shown that γ-CD had the strongest enhancement effect on the skin permeation of a steroidal drug [20], it was chosen as additive for further investigations.

3.2. Formulations

Blank and drug-loaded nanoemulsions were created using an established method [17]. Table 3 shows the physicochemical properties of the different nanoemulsions. The effect of drug incorporation was comparable for all investigated drugs. Therefore, only systems with fludrocortisone acetate and fluconazole are shown as representative examples for a lipophilic and a hydrophilic drug. The incorporation of γ-CD did not alter formulation properties notably except for systems with minoxidil, which could not be homogenised due to viscosity alterations and were therefore excluded from further investigations.

3.3. Nanoemulsion characterisation

All formulations were highly fluid and homogeneous upon visual inspection. Their optical appearance was translucent to whitish. A bluish touch was noticeable for formulations based on sucrose stearate due to Rayleigh scattering, an optical effect caused by the nano-sized emulsion droplets [29].

Table 2
Physicochemical properties of basic blank nanoemulsions investigated in preliminary studies: comparison of different surfactant concentrations. Values are means ± SD of three formulations (n = 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 below 0.05 mS/cm. Parameters shown are mean particle size (MPS), polydispersity index (PDI), zeta potential (ZP) and conductivity (Cond).

Parameters	Mean particle size (nm)	PDI	Zeta potential (mV)	Conductivity (mS/cm)
Lecithin				
1.0% (w/w)	281.40 ± 02.92	0.180 ± 0.027	−25.20 ± 02.20	0.016 ± 0.007
2.5% (w/w)	231.39 ± 14.28	0.269 ± 0.012	−26.33 ± 02.43	0.030 ± 0.005
5.0% (w/w)	132.52 ± 26.03	0.118 ± 0.024	−56.43 ± 03.81	0.027 ± 0.001
Sucrose stearate				
1.0% (w/w)	193.79 ± 01.76	0.065 ± 0.003	−48.36 ± 01.19	0.014 ± 0.003
2.5% (w/w)	141.56 ± 09.02	0.075 ± 0.007	−57.28 ± 05.77	0.016 ± 0.008
5.0% (w/w)	120.39 ± 09.15	0.134 ± 0.017	−70.81 ± 13.25	0.020 ± 0.006

Table 3
Physicochemical properties of different nanoemulsions after 20 homogenisation cycles: comparison of blank versus drug-loaded formulations shown on systems containing fludrocortisone acetate or fluconazole. Values are means ± SD of at least three formulations ($n \geqslant 3$). 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 below 0.05 mS/cm. Parameters shown are mean particle size (MPS), polydispersity index (PDI), zeta potential (ZP) and conductivity (Cond).

Parameters	Mean particle size (nm)	PDI	Zeta potential (mV)	Conductivity (mS/cm)
LN blank	186.41 ± 11.06	0.13 ± 0.05	−21.72 ± 1.83	0.023 ± 0.002
LN flud	183.13 ± 05.50	0.11 ± 0.04	−26.64 ± 4.86	0.027 ± 0.002
LN fluc	156.87 ± 09.73	0.05 ± 0.01	−24.70 ± 3.41	0.029 ± 0.004
γ-LN flud	175.82 ± 00.47	0.09 ± 0.04	−30.19 ± 4.12	0.027 ± 0.003
γ-LN fluc	155.60 ± 07.96	0.07 ± 0.02	−22.50 ± 2.20	0.022 ± 0.002
SN blank	141.21 ± 08.73	0.08 ± 0.02	−57.28 ± 5.77	0.016 ± 0.008
SN flud	146.66 ± 08.53	0.13 ± 0.03	−63.38 ± 9.14	0.020 ± 0.001
SN fluc	136.79 ± 04.49	0.09 ± 0.03	−56.68 ± 0.05	0.022 ± 0.005
γ-SN flud	144.77 ± 10.61	0.11 ± 0.02	−63.77 ± 6.22	0.020 ± 0.008
γ-SN fluc	135.74 ± 04.57	0.08 ± 0.01	−65.19 ± 5.50	0.016 ± 0.007

3.3.1. Particle size, polydispersity index and zeta potential

The different nanoemulsions were analysed directly after production. Critical formulation parameters such as particle size, PDI and ZP were determined (Table 3). Blank lecithin-based nanoemulsions exhibited a mean particle size of around 180 nm, which remained unchanged or was even slightly decreased upon incorporation of the different drugs. In the case of sucrose ester-based nanoemulsions, the mean particle size was found to be very consistently around 140 nm for blank as well as for all drug-loaded formulations. The incorporation of γ-CD had no impact on the particle size of either of the nanoemulsion systems ($P > 0.05$ in all cases). The PDI values of all systems were far below 0.2. Overall, the mean particle size of sucrose stearate-based nanoemulsions was significantly smaller for all systems ($P < 0.05$).

The particle surface charge values were determined for all formulations. Nanoemulsions containing lecithin showed average ZP values around −20 to −30 mV irrespective of the nature of the incorporated drugs. In contrast, nanoemulsions stabilised by sucrose stearate exhibited significantly higher ZP values of over −50 mV up to −65 mV, which indicates an improved electrochemical stability ($P < 0.05$).

The physicochemical long-term stability of the formulations was monitored over 6 months. The particle size of blank formulations with either lecithin or sucrose stearate remained largely constant during the whole observation period. However, Fig. 1 clearly demonstrates that the mean ZP values remained only constant in the case of sucrose stearate-based nanoemulsions. The lecithin-based systems showed a noticeable increase in absolute ZP values from around −20 to −40 mV.

The incorporation of the model drugs hardly influenced the destabilisation process of either nanoemulsion system. Although drug-loaded lecithin-based nanoemulsions generally exhibited larger particle sizes and a more pronounced increase in mean particle size over the course of 6 months, none of the observed changes were statistically significant ($P > 0.05$). Table 4 shows the development of the mean particle size as well as PDI and ZP values of formulations with fludrocortisone acetate. The particle sizes of all drug-loaded systems remained largely constant, while the ZP increased in lecithin-based systems due to chemical degradation. Sucrose stearate-based nanoemulsions showed a slow decrease in absolute ZP values as commonly observed during the ageing process of nanoemulsions.

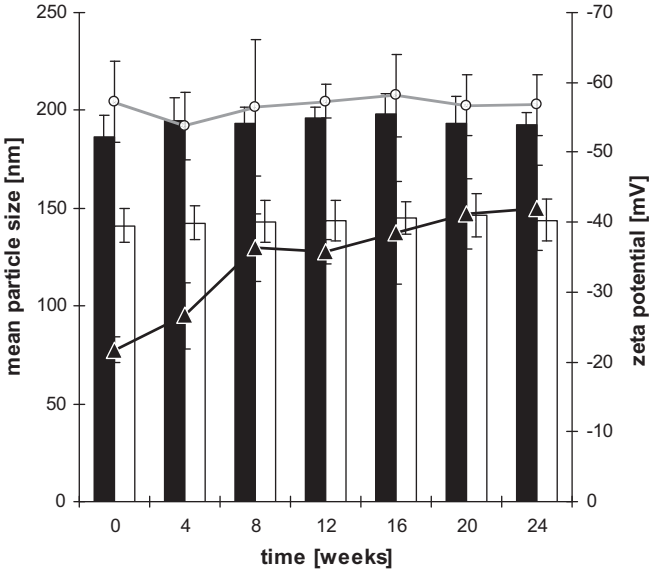

Fig. 1. Influence of surfactant on long-term stability in the case of lecithin (LN) or sucrose stearate (SN): development of mean particle size in nm and zeta potential in mV over a storage time of 6 months as observed on blank formulations with lecithin or sucrose stearate. The bars represent the particle size as indicated on the left-hand scale (black bars ■: LN, white bars □: SN). The lines show the development of the mean zeta potential (black symbol —▲—: LN, white symbol —○—: SN) as indicated on the right-hand scale of the figure. The drug-loaded formulations showed a highly similar trend. Measurements were performed at least in triplicate ($n \geqslant 3$; Zetasizer Nano) every two weeks. Numbers are given ±SD. For the sake of clarity, only monthly measurements are shown.

Table 4
Physical stability of drug-loaded nanoemulsions with either lecithin (LN) or sucrose stearate (SN) shown on systems containing fludrocortisone acetate (flud) (**3a**). The corresponding systems with additional γ-CD are shown for comparison (**3b**). Experiments were performed in triplicate ($n = 3$) in regular intervals over an observation period of 6 months. The indicated parameters are the mean particle size (MPS), polydispersity index (PDI) and zeta potential (ZP). Numbers are given as means ± SD.

(4a) Time (weeks)	MPS (nm) ± SD	PDI ± SD	ZP (mV) ± SD
LN flud			
0	183.13 ± 05.50	0,11 ± 0.04	−26.64 ± 04.86
4	184.01 ± 05.44	0.11 ± 0.06	−26.62 ± 02.18
8	188.66 ± 08.03	0.13 ± 0.08	−31.06 ± 03.03
12	185.87 ± 09.61	0.09 ± 0.04	−33.40 ± 00.58
16	186.01 ± 04.57	0.08 ± 0.02	−39.38 ± 03.91
20	197.73 ± 25.50	0.09 ± 0.04	−35.92 ± 03.70
24	189.58 ± 18.77	0.09 ± 0.04	−41.51 ± 03.70
SN flud			
0	146.66 ± 08.53	0.13 ± 0.03	−63.38 ± 09.14
4	144.10 ± 06.00	0.09 ± 0.02	−55.81 ± 05.79
8	143.26 ± 05.83	0.10 ± 0.03	−52.88 ± 01.13
12	143.24 ± 07.08	0.08 ± 0.01	−55.38 ± 06.74
16	141.89 ± 07.38	0.08 ± 0.02	−54.99 ± 02.40
20	147.08 ± 07.35	0.10 ± 0.05	−54.89 ± 08.56
24	142.88 ± 08.24	0.11 ± 0.02	−51.77 ± 12.36
(4b)			
γ-LN flud			
0	175.82 ± 00.47	0.09 ± 0.04	−30.19 ± 04.12
4	179.17 ± 08.76	0.05 ± 0.01	−30.34 ± 00.84
8	182.80 ± 13.11	0.06 ± 0.02	−35.98 ± 07.11
12	185.27 ± 16.82	0.07 ± 0.02	−34.21 ± 03.82
16	187.20 ± 21.36	0.07 ± 0.06	−37.07 ± 03.01
20	185.02 ± 17.28	0.11 ± 0.05	−36.18 ± 01.95
24	184.59 ± 14.70	0.09 ± 0.07	−34.91 ± 06.01
γ-SN flud			
0	144.77 ± 10.61	0.11 ± 0.02	−63.77 ± 06.22
4	154.44 ± 04.43	0.07 ± 0.02	−58.36 ± 02.23
8	155.86 ± 05.66	0.10 ± 0.01	−60.26 ± 03.69
12	163.24 ± 03.68	0.12 ± 0.04	−62.41 ± 13.26
16	158.72 ± 03.67	0.09 ± 0.03	−62.80 ± 04.67
20	159.20 ± 02.05	0.11 ± 0.04	−64.60 ± 01.95
24	161.93 ± 00.87	0.10 ± 0.02	−59.74 ± 06.14

3.3.2. Cryo transmission electron microscopy (cryo TEM)

Blank nanoemulsions stabilised with lecithin or sucrose stearate were investigated by cryo TEM. This technique is frequently used to visualise colloidal systems such as nanoemulsions or liposomes [7]. Fig. 2a and b illustrates the effect of the different emulsifiers on the nanoemulsion structure. It was found in several rounds of analysis that the lecithin-based formulations were far from homogeneous in their microstructure. The left-hand side of Fig. 2a shows not only irregularly shaped nanoemulsion oil droplets but also large numbers of vesicular structures such as various types of liposomes and multilamellar phospholipid layers. These images confirm that a large variety of such structures emerge during high-pressure homogenisation if lecithin E-80 is used as a surfactant. In contrast, the nanoemulsions stabilised by sucrose stearate S-970 as shown on the right-hand side of Fig. 2b had a perfectly reproducible structure. Several consecutive rounds of analysis revealed that these formulations consisted almost exclusively of perfectly spherical oil droplets.

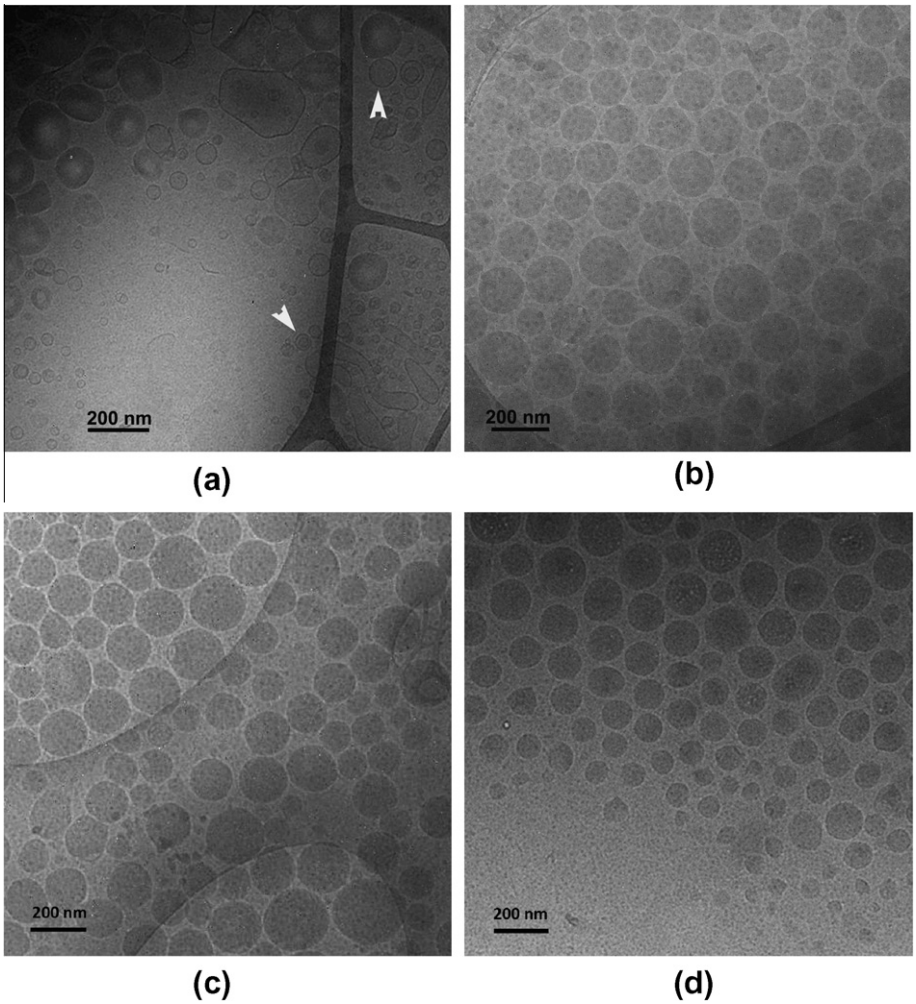

Fig. 2. Cryo TEM photographs of the microstructure of blank nanoemulsions (a) with 2.5% lecithin E-80 and (b) with 2.5% sucrose stearate S-970 as sole emulsifying agent. The corresponding formulations containing additional γ-cyclodextrin are shown below for lecithin (c) as well as for sucrose stearate (d). The magnification is illustrated by the black scale bars. Dark spheres represent nanoemulsion oil droplets. Other vesicular structures such as uni- or bilamellar liposomes (light spheres) or multilamellar structures (light lamellar layers) are indicated by white arrows. Occasional dark crystals are due to ice formation during cryo preparation of the sample.

Interestingly, the addition of γ-CD to these emulsion systems apparently had a remarkable influence on the process of emulsion formation in the case of lecithin (Fig. 2c). A much more homogeneous emulsion structure was observed than for the nanoemulsion based on lecithin alone. The sample contained remarkably less vesicular structures, and the shape of the oil droplets was more regular and spherical, if slightly rough on the surface. The images were analysed with the help of the DigitalMicrograph 3.4 software (Gatan) for their content of oil droplets and vesicular structures. In case of lecithin-based formulations without γ-CD (Fig. 2a), a total of 62.45 ± 33.12% of vesicular structures were observed per image (n = 3 analysed images, total number of structures n = 981). In case of the lecithin-based formulations with additional γ-CD (Fig. 2c), merely 13.39 ± 10.76% of vesicles were found per image (n = 5 analysed images, total number of structures n = 701). In contrast, no visual differences in the highly homogeneous structure of sucrose stearate-based nanoemulsions were noticeable after addition of γ-CD (Fig. 2d).

3.4. Chemical stability

3.4.1. Chemical stability of incorporated drugs

After the initial determination of the drug content, the chemical stability of all incorporated drugs was analysed in regular intervals over 6 months. The average content of all drugs remained between 80% and 116% of the initial value for all nanoemulsions during the observation period (data not shown). The slight fluctuations in drug content are rather due to inhomogeneous drug dispersion and methodological issues in analysis than to any actual degradation processes. Indeed, no degradation products were detected by HPLC. Neither the type of surfactant nor the presence of γ-CD exerted a noticeable influence on the recovered drug content. However, it has to be kept in mind that more sophisticated analytical methods are needed to give a full insight into the individual drugs' actual fate within the formulations over the course of time. The presented data merely serve to give an overview about the basic quality of the formulations.

3.4.2. Chemical stability of the formulations

Additional information about the physicochemical stability of the nanoemulsions was derived from the change in pH value over the course of time. A continuous decrease in pH over the course of 6 months was observed. The influence of the surfactant type on the chemical stability of the nanoemulsions was assessed on all blank and drug-loaded formulations. Although both types of nanoemulsion exhibited a significant decrease in pH value after 6 months ($P < 0.05$), lecithin-based nanoemulsions showed a more pronounced change from 6.98 ± 0.31 to 5.81 ± 0.36 (n = 18) when com-

pared to sucrose stearate-based systems with a drop from 6.75 ± 0.33 to 6.26 ± 0.24 (n = 19). Apparently, lecithin molecules have a stronger disposition towards chemical degradation than the sucrose ester surfactant. The formation of lyso-lecithin and free fatty acids through hydrolysis of lecithin molecules not only increases the negative ZP values but also causes a drop in pH and promotes further degradation [6].

3.5. Skin permeation experiments

Permeation profiles of all drugs from lecithin-based and sucrose stearate-based nanoemulsions were established after 24 h. A comparison of the cumulative drug amounts clearly shows that the hydrophilic drugs permeate through the model skin more rapidly and to a larger extent. The permeated amounts of lipophilic drugs were confined to percentages between 0.2% and 0.8% of the applied dose. In contrast, around 30–40% of the applied hydrophilic drug amounts were recovered from the receptor medium. Although nanoemulsions are generally recommended for the incorporation of lipophilic drugs, they may also be employed for the delivery of hydrophilic drugs. Table 5 shows the mean cumulative permeated drug amounts after 24 h as well as the corresponding mean drug fluxes for all investigated formulations. The concentrations of fludrocortisone acetate and flufenamic acid that were able to permeate through the porcine skin were comparatively small. The released amounts of both fluconazole and minoxidil are around 20–40 times higher than the corresponding amounts of the lipophilic drugs.

What can be further derived from these data is the fact that incorporation of γ-CD influenced mainly the skin permeation rate of the steroidal drug fludrocortisone acetate. The effect of γ-CD on skin permeation was investigated for fludrocortisone acetate, flufenamic acid and fluconazole. The incorporation of the additional compound γ-CD led to significantly enhanced skin permeation rates of the lipophilic fludrocortisone acetate ($P < 0.05$) (Fig. 3). Interestingly, this was not the case for the lipophilic flufenamic acid although a slight trend was noticeable. As expected, the skin permeation rate of the hydrophilic drug fluconazole was not increased by incorporation of the CD. In fact, the skin permeation was even slightly decreased for both lecithin-based and sucrose stearate-based nanoemulsions.

A comparison of the skin permeation efficiency of the different emulsifiers shows that the skin permeation of the hydrophilic drugs fluconazole and minoxidil was significantly higher from lecithin-based nanoemulsions ($P < 0.05$). The skin permeation of the lipophilic drugs fludrocortisone acetate and flufenamic acid was the same for both types of surfactant ($P > 0.05$). A statistical analysis was performed for the cumulative permeated drug amounts as well as for the corresponding drug fluxes. Both evaluations led to consistent results.

Table 5
Skin permeation rates of fludrocortisone acetate (flud), flufenamic acid (fluf), fluconazole (fluc) and minoxidil (min) from both lecithin-based nanoemulsions (LN) and sucrose stearate-based nanoemulsions (SN) expressed as cumulative permeated drug amounts (μg cm^{-2}) and mean drug fluxes (J, μg cm^{-2} h^{-1}). At least five experiments were performed for each formulation ($n \geqslant 5$); indicated values are means ± SD.

Formulation	Cumulative drug amount after 24 h ± SD (μg cm^{-2})	Mean drug flux ± SD (J, μg cm^{-2} h^{-1})
SN flud	9.99 ± 0.46	0.55 ± 0.02
LN flud	9.98 ± 0.64	0.55 ± 0.04
γ-SN flud	55.10 ± 3.67	2.56 ± 0.14
γ-LN flud	98.62 ± 24.89	4.53 ± 0.99
SN fluf	48.88 ± 18.27	1.55 ± 0.87
LN fluf	42.22 ± 20.47	1.21 ± 0.62
γ-SN fluf	56.62 ± 17.35	1.83 ± 0.87
γ-LN fluf	45.97 ± 18.63	1.42 ± 0.69
SN fluc	1993.45 ± 256.77	88.39 ± 9.40
LN fluc	2481.67 ± 297.86	109.55 ± 11.30
γ-SN fluc	1885.14 ± 132.48	81.74 ± 5.92
γ-LN fluc	2157.13 ± 108.32	93.63 ± 3.80
SN min	1957.93 ± 296.12	84.27 ± 11.99
LN min	2402.55 ± 236.00	102.56 ± 9.41

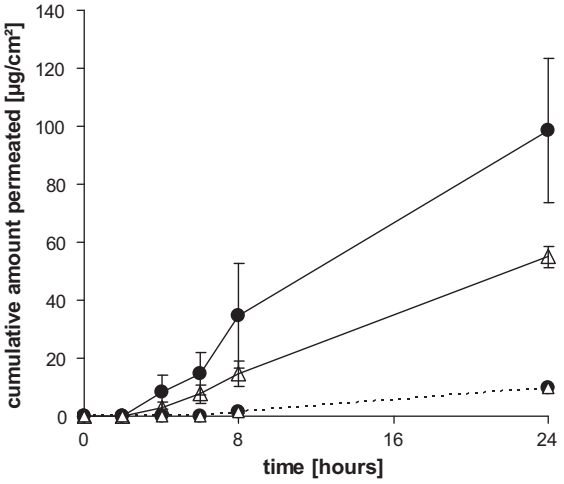

Fig. 3. Effect of γ-cyclodextrin on the in vitro skin permeation profile of fludrocortisone acetate from lecithin-based nanoemulsions (LN flud) and sucrose stearate-based nanoemulsions (SN flud). The drug permeation through porcine abdominal skin over the course of 24 h was determined by HPLC. The cumulative permeated drug amounts are plotted versus time. At least five experiments were performed for each formulation ($n \geqslant 5$) using Franz-type diffusion cells. Indicated values are means ± SD. Abbreviations: LN flud: dashed line (- -●- -); SN flud: dashed line (- -△- -); γ-LN flud: full line (—●—); γ-SN flud: full line (—△—).

4. Discussion

4.1. Comparison of emulsifying efficiency: sucrose stearate versus lecithin

Although the surfactants lecithin E-80 and sucrose stearate S-970 possess similar HLB values, the produced formulations differed in their physicochemical properties. The novel sucrose stearate-based systems exhibited smaller mean droplet sizes and a higher electrochemical stability. This tendency was observed for both blank and drug-loaded formulations. Likewise, these systems were less prone to chemical degradation, as both visual observation and monitoring of the pH indicated. The nanoemulsion production process was more reliable and controllable in the case of sucrose stearate since lecithin is more sensitive to changes in temperature. The storage of lecithin-based nanoemulsions at room temperature leads to changes in colour and odour. This destabilisation was decelerated by refrigerated storage but was nevertheless noticeable after 6 months. The chemical degradation of the emulsifier rendered the system less appealing despite the largely constant particle size. In contrast, sucrose ester nanoemulsions showed a clean white colour and no changes in odour after 6 months. The PDI of all systems remained below or around 0.2, which indicates a very homogeneous droplet size distribution and high physical stability [20].

The high negative ZP of sucrose stearate-based systems was directly correlated with the amount of sucrose ester. This might be ascribed to the presence of larger amounts of residual, non-esterified fatty acids in the sucrose ester mixture. An observed decrease in pH at increasing sucrose stearate concentrations as well as a comparison of the manufacturers' product specifications supports

this theory. While lecithin E-80 contains a maximum of 0.05% of free fatty acids, sucrose stearate S-970 may contain up to 10% of residual fatty acids, free sucrose, moisture and ash.

In terms of long-term stability, the ZP of sucrose stearate-based systems remained stable while the negative droplet surface charge of lecithin-based systems increased. This increase was most likely caused by hydrolysis of lecithin molecules. The chemical degradation of the surfactant results in increasing concentrations of lysolecithin and free fatty acids, which confer more negative charges to the droplets' surfaces [30]. This phenomenon has therefore to be considered a sign of physicochemical destabilisation.

Since the analysis of particle size distributions through DLS alone may provide incomplete information [31,32], additional cryo TEM investigations were performed. Thus, the exact nature of the measured structures and remarkable differences caused by the use of the different surfactants were visualised. Systems stabilised by sucrose stearate exhibited perfectly homogeneous nanoemulsion droplets, while systems stabilised by lecithin had a variable morphology. Sucrose stearate apparently possesses a better packing geometry at the O/W interface and is therefore more suitable for the formation of curved surfaces. In terms of droplet sizes, the images confirmed the results obtained by DLS.

Previously conducted cryo TEM analyses of lecithin-based nanoemulsions showed more regular droplet shapes, most likely due to the presence of additional emulsifiers and stabilisers [20]. The presented results confirm that the lecithin mixture alone is less efficient in nanoemulsion formation and that co-surfactants should be employed. In this context, it was shown that γ-CD might serve as a co-stabilising agent.

4.2. Effect of γ-CD on the nanoemulsion structure

Further cryo microscopic images revealed that addition of γ-CD to the lecithin-based system remarkably improved the homogeneity of the formulation. This indicates that the CD might be involved in the formation of the interfacial film which apparently became more suitable to form curved surfaces. Neither composition nor production process were altered in any other way; thus, the effect can only be ascribed to the presence of additional surface-active molecule complexes formed by CD molecules and fatty acid residues of the oil phase [33–37]. Since the rather voluminous CD molecules represent the hydrophilic region of the newly formed "surfactants", the resulting surface-active agents may have a suitable interfacial packing parameter to promote the formation of droplets. These observations are in good agreement with recent data [20]. It may be assumed that the previously detected excess of lecithin aggregates in the bulk water phase was caused by CD molecules which were inserted into the interfacial film of the O/W droplets, thus forcing surplus lecithin molecules into the aqueous phase. No such effect was observed in the present study since the overall amount of surfactant was low.

4.3. Effect of γ-CD on nanoemulsion stability

An additional aspect of the stability monitoring was the incorporation of γ-CD. The results showed that emulsion stability was not impaired by the presence of the CD. Cyclodextrins have a high potential for undesired interactions with various excipients. A high affinity of the CD towards lipophilic moieties of surfactant molecules may lead to complexation and subsequent inactivation [38]. Likewise, the decomposition of certain drugs may be accelerated by CDs [39]. It is therefore of utmost importance to investigate the specific effect of CD incorporation on every new system. Previous investigations have shown that CDs may have a stabilising effect on nanoemulsions [20]. In the present case, the addition of γ-CD seemed to stabilise ZP values of lecithin-based systems, thus conferring increased electrochemical stability to the nano-sized droplets.

4.4. Effect of the different emulsifiers and γ-CD on skin permeation

Since the skin permeation of drugs is influenced by factors such as their molecular mass and their log *P* value, the skin permeation of the investigated drugs differed considerably. The skin permeation rates of minoxidil were highest, followed by fluconazole and fludrocortisone acetate as well as flufenamic acid. Although nanoemulsions are aimed at the incorporation of lipophilic drugs, larger amounts of hydrophilic drugs could be delivered by incorporation into the aqueous phase. In the present experimental setup, this is probably related to the higher solubilities of the hydrophilic drugs in the aqueous receptor medium. In addition, the increased hydration of the skin in the experimental setting may have contributed to the enhanced permeation of the hydrophilic drugs [40]. Apart from these general aspects, the performance of sucrose stearate-based nanoemulsions in terms of skin permeation was evaluated and compared to that of lecithin-based systems. In case of both lipophilic drugs, similar skin permeation rates were achieved with lecithin- and sucrose stearate-based nanoemulsions ($P > 0.05$). Interestingly, the skin permeation of both hydrophilic drugs was higher from lecithin-based nanoemulsions than from their sucrose ester-based counterparts ($P < 0.05$), possibly due to the presence of liposomes as confirmed by cryo TEM. The hydrophilic drugs might be incorporated into the liposomal structures to a certain extent, which may enhance their skin permeation from lecithin-based systems. In addition, lecithin molecules themselves are known to interact with skin lipids and promote the skin permeation of actives [1,41,42].

In the context of the skin permeation studies, the enhancement effect of γ-CD on various drugs was investigated. A highly significant permeation enhancement was found for fludrocortisone acetate ($P < 0.05$), which confirmed previous results [20]. This indicates that γ-CD can indeed be employed to enhance the skin permeation of steroidal drugs from O/W nanoemulsions. No such effect was observed for the lipophilic flufenamic acid. Interestingly, the release of fluconazole from lecithin-based nanoemulsions was significantly decreased by the addition of γ-CD ($P < 0.05$). This might be explained by the observed strong decrease in liposomal structures through addition of γ-CD. It may thus be concluded that the superior release of hydrophilic drugs from lecithin-based systems was indeed caused by the additional liposomes.

In summary, the lack of permeation enhancement for drugs other than steroidal ones leads to certain useful conclusions. It can be assumed that the extraction of cholesterol or other skin components by the CD [43,44] can be excluded as a possible mechanism of action since such an effect would have affected the skin permeation of all drugs. Accordingly, an occlusion effect [45,46] through increased film-building capacity of the CD-containing formulations can be ruled out as well. However, it is well known that CDs can enhance the skin permeation of drugs by forming an inclusion complex and thus increasing drug solubility and dispersion within the formulation [43,47,48]. Hence, more drug is available at the skin surface to enter the stratum corneum through diffusion. However, such an effect appears unlikely if only small molar amounts of CD are incorporated, such as the presented amount of CD/drug of around 1:3.

It is particularly interesting that the permeation enhancement effect of γ-CD was linked to the nature of the employed emulsifier. The effect was significantly higher in lecithin-based nanoemulsions than in sucrose stearate-based systems ($P < 0.05$). This leads to the conclusion that some kind of underlying synergistic mechanism must be taken into account. As previously mentioned, the CD molecules can complex fatty acid residues of the oil phase, thus

forming new surface-active molecule complexes [33–37]. It is most likely that the CDs are incorporated into the interfacial film in this fashion, as the cryo TEM images suggest. The insertion of such additional emulsifiers may facilitate drug release [49]. It is commonly known that mixed interfacial films are more flexible and thus more suitable to form spherical droplets [50]. It might be deduced that this structural flexibility might also promote the release of specific drugs from the oil core, especially if they have an affinity for the CD cavities. Such a dynamic system might lead to an accelerated release of the incorporated drug, as is obviously the case for steroidal drugs in the presence of γ-CD. The complexation affinity between drug and CD may play a significant role in this context. The release of compounds from structures like solid lipid nanoparticles or nanoemulsion droplets is slower from the core than from the surface of the phospholipid layer [51]. If the CD acts as a "transporter" and enhances the affinity of the drug to leave the oil core through complexation, accelerated skin permeation may result. However, the exact mechanism behind the enhancement effect remains to be investigated. It has to be kept in mind that the involved processes are of a dynamic nature. In any case, the addition of γ-CD represents a skin-friendly way to promote the permeation of steroidal drugs without affecting the skin barrier function.

5. Conclusion

Sucrose stearate is a highly suitable emulsifier for nanoemulsion production and superior to lecithin in several aspects. Excellent skin friendliness can be expected from the novel formulations which contain only 2.5% of the mild surfactant. In addition, it was found that the formation of nano-sized droplets could be promoted by the addition of γ-CD in the case of lecithin while the number of vesicular structures was decreased. The concomitantly enhanced release of a steroidal drug from the oil core might be associated with its complexation affinity to the CD, which will be subject of further investigations.

6. Conflict of interest

The authors declare that no conflicts of interest occurred during this work and no funding sources were involved.

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