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NLCs as a potential carrier system for transdermal delivery of forskolin*

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Nanostructured lipid carriers (NLC) composed of the substances generally recognized as safe (GRAS) were obtained by using a hot high-pressure homogenization technique (HPH). The influence of the number of homogenization cycles and concentration of a decyl glucoside surfactant on the NLC properties were studied. The system's stability was assessed by macroscopic observation, light backscattering and zeta potential measurements. NLC particle size was measured using dynamic light scattering (DLS). The kinetically stable formulations were loaded with forskolin and selected for *in vitro* drug permeation study using the Franz cell method. Concentration of forskolin in the receptor solution (i.e. ethanol/PBS mixture) was analyzed with high performance liquid chromatography (HPLC) with UV detection. The obtained results have shown that NLC formulations could be used as effective carriers for forskolin permeation through the skin.

Key words: Forskolin, NLC, nanostructured lipid carrier, skin permeation

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Abbreviations: NLC, nanostructured lipid carrier; SLN, solid lipid nanoparticle; HPH, high pressure homogenization

INTRODUCTION

Nanostructured lipid carriers (NLCs) one of the main types of lipid nanoparticles, are alternative carrier systems next to emulsions, liposomes and polymeric nanocapsules. And as a second generation of lipid nanoparticles they have many advantages over solid lipid nanoparticles (SLN). NLCs are produced using blends of solid and liquid lipids (oils) in contrast to SLNs, which contain only solid lipids. A certain amount of oil in NLCs leads to a less perfect crystal structure, which contributes to an increased active loading capacity and minimizes, or even prevents, the expulsion of drug during storage (Müller *et al*., 2002a; Zheng *et al*., 2013).

Therefore, lipid nanoparticle formulations with solid matrix have shown great potential as carriers for topical administration of poorly soluble active ingredients. Over the past few years they have been studied intensively for dermal applications, for both pharmaceutical and cosmetic applications (Müller *et al*., 1995; Müller *et al*., 2002a; Pardeike *et al*., 2009; Müller *et al*., 2007). They have many advantages important for this kind of products, e.g. they increase skin hydration and occlusive properties (Wissing & Müller, 2003) enable the modified release profile (Jenning *et al*., 2000; Souto *et al*., 2004) increase skin penetration related to a targeting effect and avoidance of systemic absorption (Liu *et al*., 2007; Chen *et al*., 2006). Moreover, lipid nanoparticles enhance the stability of chemically labile drugs and active ingredients (Souto & Müller, 2005; Üner *et al*., 2005; Junyapraserta *et al*., 2009).

Recent research has indicated that nanoparticular systems, such as lipid nanoparticles (SLNs, NLCs) show improved uptake and skin targeting (Gelfuso *et al*., 2016). Nanoparticles like liposomes (De Leeuw *et al*., 2009) oil-based dispersions (Konan *et al*., 2002) polymeric particles (Gomes *et al*., 2007) or gold nanoparticles (Cheng *et al*., 2008) have been successfully applied to create a new drug delivery system for treatment of skin cancer. The main focus has been put on diagnosing and treating metastatic melanoma, which is the deadliest skin cancer (Vyas *et al*., 2012). Many chemotherapeutics administrated systematically are cytotoxic to healthy cells, therefore nanomedicine aims to design nanoparticles which could selectively deliver drug specifically to the melanoma cells (Chen *et al*., 2010; Dhar *et al*., 2011).

Forskolin is a diterpene produced by the Indian Coleus plant (*Coleus forskohlii*) and an interesting active compound showing potential to protect skin from the UVB damage. It activates adenylyl cyclase and therefore increases the intracellular levels of cAMP (cyclic adenosine monophosphate) (Burlando *et al*., 2010). It has been demonstrated that inducing pigmentation with forskolin provides effective protection against UVB-induced DNA damage and skin cancer in mice deficient for a DNA repair enzyme. Passeron and others (Passeron *et al*., 2009) demonstrated in their study that forskolin protects keratinocytes from UVB induced apoptosis independently of the amount of melanin in the skin. They proved that it enhances the removal of cyclobutane pyrimidine dimers and 6,4-photoproducts, which are the two major types of UVB-induced DNA damage, and facilitates DNA repair. These results imply new preventive approaches with topical formulations containing forskolin, which could be applied to the skin before sun exposure. Moreover, forskolin has appeared in the literature as a natural substance to obtain a healthy tan (Spry *et al*., 2009).

The objective of this study was to develop and optimize stable NLC formulations based on mixture of

beeswax and caprylic/capric triglycerides as the carriers for topical administration of forskolin, which could be potentially used as an alternative drug carrier.

MATERIALS AND METHODS

Materials. In this study we used the following solid lipids: Apifil® (PEG-8 beeswax; Gattefossé GmbH, Weil am Rhein, Germany) Cutina®CP (cetyl palmitate, BASF Chem Trade GmbH, Burgbenheim, Germany) Compritol®888ATO (glyceryl behenate, Gattefossé GmbH, Weil am Rhein, Germany) Carnauba wax (Kahlwax, Kahl GmbH & Co. KG, Trittau, Germany). Labrafac®CC (caprylic/capric triglycerides, Gattefossé GmbH, Weil am Rhein, Germany) and Cetiol®V (decyl oleate, BASF Chem Trade GmbH, Burgbenheim, Germany) were liquid lipid used. PlantaCare®2000UP (decyl glucoside, BASF Chem Trade GmbH, Burgbenheim, Germany) was applied as a surfactant. An active substance, Forslean (*Coleus Forskohlii* Root Extract containing 95% of forskolin) was purchased from Sabinsa Europe GmbH, Langen, Germany). The ultra-purified water was freshly prepared by a MiliQ® System (Millipore, Schwalbach, Germany).

Forskolin solubility. Prior to the NLCs production a lipid screening was performed to determine the most suitable lipids with respect to the solubility of forskolin to be incorporated. This was done by heating the solid lipid, 5°C above its melting point, and dissolving an increasing amount of forskolin therein. After dissolution, the mixture of lipids and the active ingredient was cooled down to room temperature for solidification and then visually examined for the presence of crystalline forskolin. Additionally, to exclude presence of forskolin crystals in the lipid matrix, we used Leica Reichert Polyvar 2 microscope with a hot plate and a polarizer.

Preparation of NLC. Free and forskolin-loaded NLCs were prepared using hot high-pressure homogenization method (HPH). A certain amount of solid lipid (Apifil®) and liquid oil (Labrafac®CC) were melted in various solid to liquid lipid ratios (Table 1) at 80°C, to get a uniform oil phase. When needed, forskolin was added to the oil phase. Next, the melted lipid phase was dispersed in the hot surfactant water solution by using high speed magnetic stirrer (Agimatic-N, P Selecta) at 750 rpm, for 5 min. The obtained pre-emulsion was subsequently homogenized at 75°C, using the high-pressure homogenizer (Microfluidics Corporation, Newton Massachusetts) at 275 bar. The number of homogenization cycles ranged from 3 to 6. For a comparative study, a nanoemulsion was prepared, using Labrafac®CC as an oil phase, and production parameters that were the same as for NLCs with 3 homogenization cycles.

Zeta potential analysis. Zeta potential (ZP) of NLC dispersions was determined by the measurement of the electrophoretic mobility, using Malvern 4700C Sub Micron Particle Analyzer. The conversion into the ZP was performed using Helmholtz-Smoluchowski equation. ZP was measured at room temperature after dilution of samples with deionized water. The measurements for each sample were repeated three times.

Particle size measurements. Mean particle size of the lipid dispersions (z-ave) and the polydispersity index (PI) which is a measure of the width of the size distribution, were determined using Dynamic Light Scattering (DLS) method, using Malvern 4700C Sub Micron Particle Analyzer. Analyses were performed using a 90° scattering angle at 25°C. Prior to the measurements, all samples were diluted with deionized water to have a suitable scattering intensity. During the experiment, refractive index of the samples was set at 1.450. For each sample the analysis was performed three times to determine mean values.

NLC stability studies. The stability of NLC formulations was firstly evaluated by macroscopic observation and estimating the "cream" increasement in time by measuring the height of delamination. The samples of equal volume were observed daily over three weeks, and any destabilization processes (creaming or coalescence) were measured.

After macroscopic observation, the stability of the most stable NLC formulations was additionally assessed by light backscattering, by means of a Turbiscan Lab® Expert (Formulation SA, France) at constant temperature (32°C). Transmission and backscattering data were acquired for 24 h, in intervals of 2 hour, according to the method proposed by Caldero and others (Caldero *et al*., 2011).

***In vitro* skin permeation studies**. For the skin permeation study, human skin samples obtained by abdominoplasty surgeries were kindly provided by Clínica Sagrada Familia, Barcelona, Spain. Before each experiment, skin integrity was evaluated by measuring the transepidermal water loss (TEWL) of skin pieces. *In vitro* permeation through human epidermis (0.4 mm) from the same donor was assessed using the MicroettePlusR system (Hanson Research, USA). The experiments were performed at 32°C±0.5, 400 rpm, using mixture of PBS

Table 1. The NLC systems' composition and obtaining parameters.

Formulation name	Ingredients (%wt.)			Number of homogenization cycles
	oil	solid lipid	surfactant	
NLC-3	3	7	2	3
NLC-4	3	7	4	
NLC-5	4.5	10.5	2	
NLC-6	6	14	2	
NLC-7	4.5	10.5	4	
NLC-8	6	14	4	3
NLC-9				4
NLC-10				5
NLC-11				6

*water up to 100% wt., 270 bar, 75°C

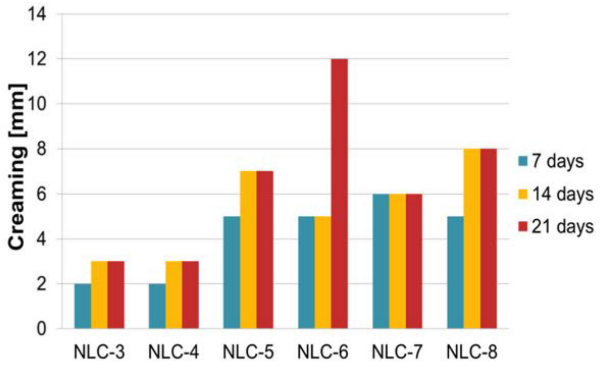

Figure 1. Effect of NLC's composition on creaming phenomena (3 cycles, p=275 bar, T=75°C).

(pH 7.4)/ethanol (60:40, V/V) as receptor solution (sink conditions). An adequate amount of formulation (0.350 ml) was placed in the donor part at Franz diffusion cells. Samples of 700 µl were withdrawn automatically from the receptor compartment. The acceptor fluid was collected subsequently during 24 h. The number of replicates per formulation was n=6.

Estimated permeation parameters were: flux at steady state (J_{ss}) by means of a linear regression (cumulative permeated amount *vs.* time, slope) lag time (TL) (X-intercept) permeability coefficient ($K_p=J_{ss}/C_{formulation}$) partition parameter P_1 and the apparent length of diffusion parameter P_2 (according to: Okamoto (Okamoto *et al.*, 1986) and Selzer (Selzer *et al.*, 2013). The steady state flux through the membrane is given by: $J_{ss}=C_0 \cdot K_p=C_0 \cdot (K \cdot D)/L$, where C_0 is the constant forskolin concentration in the donor compartment, K is the partition coefficient vehicle/skin, D is the diffusion coefficient of the permeant in the skin and L is the effective diffusion path length. Since L is unknown, the expression $(K \cdot D)/L$ can be replaced by the product of P_1 and P_2 parameters, being $P_1=K \cdot L$ and $P_2= D/L^2$; the calculation of P_2 is obtained from $1/6 \cdot T_L$.

The parameters calculated were compared by a non-parametric statistical assay (Kruskall-Wallis Z) ($p<0.05$) according to Williams and others (Williams *et al.*, 1992) and followed by Kruskal-Wallis Multiple-Comparison Z-Value Test.

HPLC analysis. Concentration of the active ingredient in acceptor medium of the permeation assays was analyzed using HPLC Waters instrument, operated at room temperature, consisting of an automatic auto sampler system, equipped with UV detector and Spherisorb ODS column (5 mm×15 cm×0.46 cm). The mobile phase was isocratic (60 volumes of acetonitrile and 40 volumes of water) which remained constant throughout the entire analysis. The flow rate was set to 0.5 ml/min. The assay was monitored at the wavelength of 210 nm, sample injection volume was 20 µl and run time 10 min. The active ingredient's content was identified by comparing its retention time and UV spectra. The calibration curve was constructed from linear plots of peak area versus concentration.

RESULTS AND DISCUSSION

Forskolin solubility in lipids

A precondition for a successful encapsulation of forskolin into NLC system is its applicable solubility in the lipid. Therefore, the four chosen solid lipids (Apifil®, Cutina®CP, Compritol®888ATO, Carnauba wax) and their mixture with liquid lipids (Labrafac®CC, Cetiol®V) in the ratio 70:30 were screened. The obtained results showed that the best solvent for forskolin was Apifil®/ Labrafac®CC mixture (Table 2). It was further chosen to produce forskolin-loaded NLCs.

Table 2. Solubility of forskolin in solid and liquid lipids.

Lipids	max solubility, mg/mL
Apifil®	3.2
Cutina CP®	n.d.
Compritol® 888 ATO	1.3
Canauba wax	n.d.
Labrafac® CC	3.5
Cetiol V	1.5
Apifil® / Cetiol V	1.3
Apifil® / Labrafac® CC	6.2
Compritol® 888 ATO / Labrafac® CC	1.2
Compritol® 888 ATO / Cetiol V	1.2
Cutina CP®/ Labrafac® CC	n.d.

*Ratio of solid to liquid lipid ratio was 70/30; n.d., not analyzed amount

NLC stability

The high-pressure homogenization technique, using a varying number of process cycles was applied for NLCs preparation. Moreover, influence of lipids' concentration, solid to liquid lipids ratio, and concentration of surfactant on NLCs' stability were studied.

Figure 1 shows the results of creaming phenomena for formulations with different composition, obtained by HPH method (3 cycles, p=275 bar, T=75°C). It has been found that increase in the solid lipid content in formulation: 7, 10.5, and 14% for NLC-3, NLC-5, and NLC-6, respectively, resulted in a creaming increasement.

NLC formulations were prepared applying a varying number of homogenization cycles (3, 4, 5, or 6). Figure 2 shows the influence of the number of homogenization cycles on NLCs' stability (composition of the formulations, pressure and temperature of homogenization were maintained constant). The obtained results show that creaming with 3 homogenization cycles and

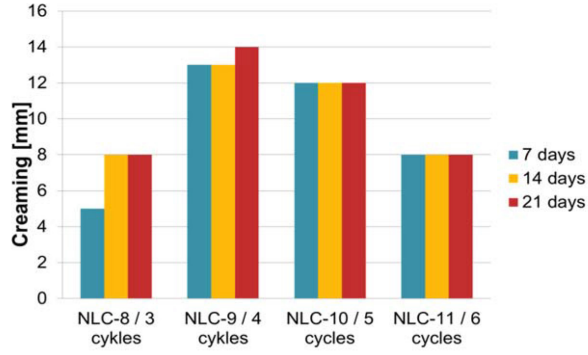

Figure 2. Influence of the number of homogenization cycles on creaming phenomena.

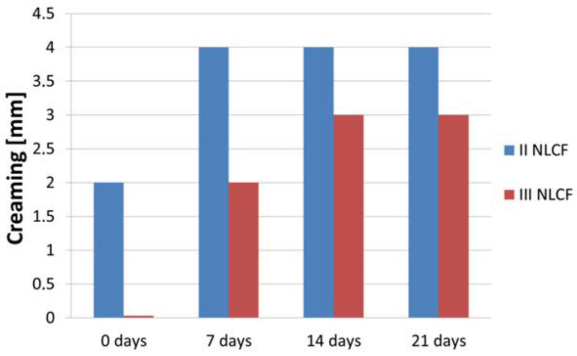

Figure 3. Influence of surfactant concentration on creaming phenomena (II NLCF, 2% of surfactant; III NLCF, 4% of surfactant).

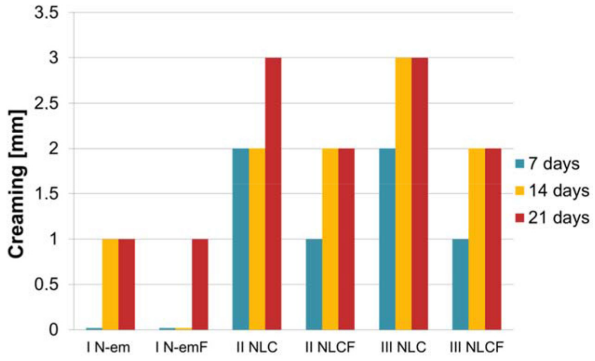

Figure 4. Influence of forskolin on creaming phenomena
I N-em, nanoemulsion; I N-emF, forskolin-loaded nanoemulsion; II NLC formulation with 2% wt. of surfactant; II NLCF, forskolin-loaded formulation with 2% wt. of surfactant; III NLC formulation with 4% wt. of surfactant; III NLCF, forskolin-loaded formulation with 4% wt. of surfactant.

6 homogenization cycles were similar. Taking into consideration the economy and efficiency of the homogenization process, 3 cycles of the microfluidizer setting were finally chosen to obtain forskolin loaded NLC.

The choice of surfactant and its concentration has an impact on the quality of NLC dispersion (Mulla & Khazi, 2009). In our work decyl glucoside (PlantaCare®2000UP) was chosen as an emulsifier because of its dermatologically compatible properties and unpreserved form, which is very important in skin care products. A sufficient amount of a surfactant must be used to cover the newly formed surfaces, created during high-pressure homogenization process. The influence of the surfactant content on creaming phenomena in the formulations of the same lipid concentration is presented in Fig. 3. Increasing the surfactant content from 2% to 4% wt. resulted in an increase of the systems stability. The concentration above 4% wt. was not considered, as it was observed that higher concentration of PlantaCare®2000UP caused foam formation during the high-pressure homogenization process.

Zeta potential (ZP) is often a key factor used to evaluate the stability of colloidal dispersion. Particle aggregation is less likely to occur for charged particles with high zeta potential (more than |30| mV) because of electric repulsion. Generally, lipid nanoparticles (SLNs, NLCs) are negatively charged on the surface (Schwarz & Menhert, 1999). In our formulations all the zeta potential values were less then –30 mV; nevertheless, destabilization processes in form of creaming were observed in many cases. Non-ionic surfactant, like alkyl polyglucoside, cannot ionize into charging group like the ionic surfactants, but indicates its ZP. It might be because of molecular polarization and adsorption of surfactant molecule on the charge in water, it was absorbed to the emulsifier layer of particle/water interface, and an electric double layer similar to ionic was formed (Han *et al.*, 2008).

Characterization of forskolin-loaded NLC

Considering the results obtained in the first part of our study, we chose the optimal formulation for the active ingredient incorporation. NLCs that contained 0.075% wt. of forskolin, 3% wt. of Labrafac®CC, 7% wt. of Apifil® and 2% (II NLCF) or 4% wt. (III NLCF) of PlantaCare®2000UP were prepared and characterized. Moreover, a nanoemulsion (I N-emF) with this same percentage of oil/surfactant was also prepared for comparison (Table 3).

During stability study of forskolin-loaded NLCs it was confirmed (Fig. 3) that 4% wt. content of surfactant in the formulations (III NLCF) is sufficient to obtain a stable system. It was also found that addition of forskolin positively affected the stability of NLC systems. Figure 4 shows the effect of forskolin presence in the formulations (II NLCF, III NLCF) compared to formulations without the active ingredient (II NLC, III NLC) on the process of creaming. It can be clearly seen, that NLCs containing forskolin (II NLCF, III NLCF) do not show the further creaming process after 7, 14 and 21 days, contrary to formulations without the active ingredient.

Additionally, the stability analysis of the pre-selected for *in vitro* skin permeation study formulation (III NLCF) and the nanoemulsion prepared for a comparison (I N-emF) were also assessed using Turbiscan Lab® Expert. This method is non-destructive, as no dilution of the sample is necessary, and gives the information on the kind of destabilization process. Turbiscan measurements are based on the variation of the droplet volume fraction (creaming/sedimentation) or mean size (coalescence) which result in the variation of backscattering and transmission signals (Paolino *et al.*, 2011). These signals occur as a function of time (and particle migration) and

Table 3. The optimal formulations for drug encapsulation.

Formulation name	Ingredients (% wt.)				ZP [mV]	Z-ave [nm] ±S.D.	PDI
	Oil	Solid lipid	Surfactant	Forskolin			
I N-emF	10	–	2	0.075	–44.2	168.5±*6.6*	0.211
II NLCF	3	7	2	0.075	–32.3	174.8±*5.3*	0.315
III NLCF	3	7	4	0.075	–36.5	184.4±*3.8*	0.309

*water up to 100% wt.

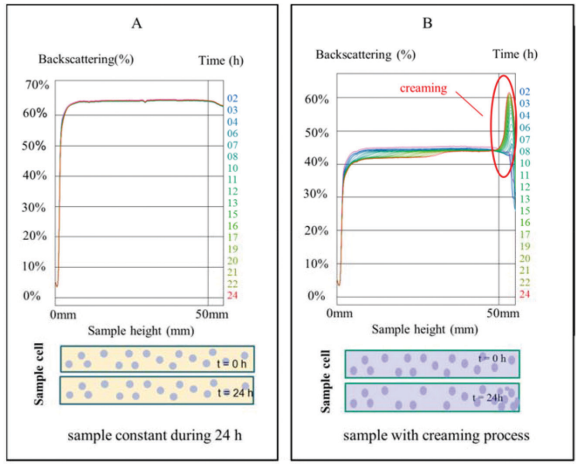

Figure 5. Comparison of backscattering data of (A) nanoemulsion (I N-emF) and (B) III NLCF

are graphically reported in the form of positive (backscattering increase) or negative peaks (backscattering decrease). No variations of particle size take place when the backscattering profile is within the interval ±2%. Variations greater than 10% represent destabilization which will occur over time (Fig. 5).

In vitro skin permeation

The skin permeation of forskolin contained in the NLC system (III NLCF) nanoemulsion (I N-emF) and Labrafac®CC was studied. The results of the *in vitro* percutaneous permeation experiments are presented in Fig. 6 and Table 4. As it can be observed, the highest permeation through the skin profile of forskolin was achieved in Labrafac CC® ($p<0.05$) and could be mainly attributed to a higher skin/vehicle partition coefficient (reflected in the value of the parameter P_1) that could favor the active ingredient's penetration through the stratum corneum. The percentage of forskolin permeated at 24 h was very high (near 80%).

Both assayed nanoformulations showed similar permeation profiles. According to the permeation parameters (Table 4) the calculated Kp enhancement ratio of IN-emF with respect to IIINLCF is close to 1 (1.12). This shows that the permeability coefficients were very similar, which would be in accordance with the high lipophilicity of forskolin and the lipophilic nature of the stratum corneum. However, the permeation was slightly higher in case of the emulsion. This higher permeation could be explained by a higher thermodynamic activity of forskolin in the oil of the nanoemulsion (Labrafac

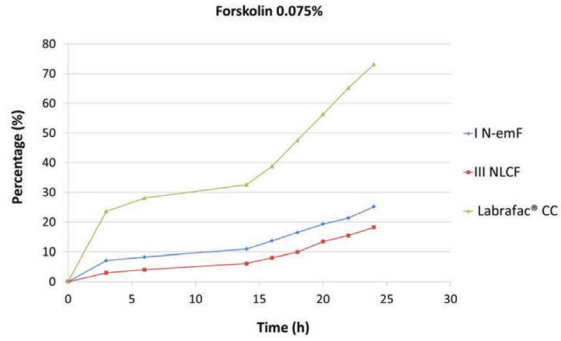

Figure 6. Mean skin permeation profile of forskolin in nanoemulsion (I N-emF) NLC (III NLCF) and oil (Labrafac CC®).

CC®) with respect to the mixture Apifil/Labrafac CC® of the NLC, according to its solubility in the oil phases (Table 2). Thermodynamic activity is the main driving force for skin permeation (Kemken, 1991) and it would lead to fast diffusion of forskolin through the skin. This is reflected in the P_2 diffusion parameter (median value of $2.74 \cdot 10^{-2}$ h^{-1} *vs.* $1.74 \cdot 10^{-2}$ h^{-1} for nanoemulsion and NLC, respectively). Moreover, in this study, the occlusive effect of the NLC component (solid lipid) that usually gives an enhancing effect on skin permeation, did not exceed the effect of thermodynamic activity in the nanoemulsion.

The nanocarrier systems composed of caprylic/capric triglycerides and a biologically compatible surfactant can be considered as good vehicles for forskolin delivery into the skin. According to the obtained results and considering that the developed formulations are for topical application and local purposes, the NLC formulation would provide less forskolin in blood than the nanoemulsion and in this sense it would be more appropriate.

CONCLUSION

The obtained results showed that not only composition (content of solid lipid, surfactant concentration) but also parameters of homogenization influence the stability of nanostructured lipid carrier formulations (NLCs). The kinetically stable NLCs for forskolin encapsulation containing 4% of emulsifier (decyl glucoside) were obtained by HPH process, at T=75°C, p=275 bar and 3 pass number of the high-pressure homogenization setting. Moreover, forskolin positively influenced the stability of NLC formulations. The skin permeation results have shown that the obtained NLC formulations could be used as effective carriers for a controlled release of forskolin to the skin, and hence also as an alternative drug carrier in the anticancer drug delivery.

Table 4. Median (and range) of permeation parameters (steady state flux (J_{ss}) lag time (T_L) permeability coefficient (K_p) P_1 and P_2 parameters, and percentage of permeated forskolin at 24 h (n=6).

Formula	J_{ss} (μg·cm^{-2} · h^{-1})	T_L (h)	$K_p \cdot 10^3$ (cm · h^{-1})	P_1 (cm)	$P_2 \cdot 10^2$ (h^{-1})	Percentage 24 h
IN-emF	1.96*±0.47	6.66±3.28	2.82*±0.42	0.119*±0.071	3.29±2.27	25.15*±3.05
IIINLCF	1.77*±0.35	8.68±2.54	2.37*±0.46	0.124*±0.045	2.18±1.10	18.21*±3.62
Labrafac®CC	6.11±0.44	6.26±1.00	8.15±0.58	0.306±0.054	2.72±0.47	73.12±5.01

*$p<0.05$ with Labrafac CC (Kruskal-Wallis Z-test)

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