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Nano-emulsions as vehicles for topical delivery of forskolin

Małgorzata Miastkowska[1], Elżbieta Sikora[1]✉, Elwira Lasoń[1], Maria Jose Garcia-Celma[2], Elvira Escribano-Ferrer[2], Conxita Solans[3] and Meritxell Llinas[3]

[1]Institute of Organic Chemistry and Technology, Cracow University of Technology, Kraków, Poland; [2] Pharmacy and Pharmaceutical Technology Department, Faculty of Pharmacy, University of Barcelona, Barcelona, Spain, IN_2UB members; [3] Institute of Advanced Chemistry of Catalonia, Consejo Superior de Investigaciones Científicas (IQAC-CSIC) and CIBER en Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Barcelona, Spain

Two O/W forskolin-loaded nano-emulsions (0.075% wt.) based on medium chain triglycerides (MCT) and stabilized by a nonionic surfactant (Polysorbate 80 or Polysorbate 40) were studied as forskolin delivery systems. The nano-emulsions were prepared by the PIC method. The mean droplet size of the nano-emulsions with Polysorbate 80 and Polysorbate 40 with oil/surfactant (O/S) ratios of 20/80 and 80% water concentration, measured by Dynamic Light Scattering (DLS), was of 118 nm and 111 nm, respectively. Stability of the formulations, as assessed by light backscattering for 24 h, showed that both nano-emulsions were stable at 25°C. Studies of forskolin *in vitro* skin permeation from the nano-emulsions and from a triglyceride solution were carried out at 32°C, using Franz-type diffusion cells. A mixture of PBS/ethanol (60/40 v/v) was used as a receptor solution. The highest flux and permeability coefficient was obtained for the system stabilized with Polysorbate 80 (6.91±0.75 $\mu g \cdot cm^{-2} \cdot h^{-1}$ and $9.21 \cdot 10^{-3} \pm 1.00 \cdot 10^{-3}$ $cm \cdot h^{-1}$, respectively) but no significant differences were observed with the flux and permeability coefficient value of forskolin dissolved in oil. The obtained results showed that the nano-emulsions developed in this study could be used as effective carriers for topical administration of forskolin.

Key words: Forskolin, nano-emulsions, medium chain triglycerides, *in vitro* skin permeation

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✉e-mail: esikora@pk.edu.pl

Abbreviations: DLS, Dynamic Light Scattering; F, forskolin; Jss, flux at steady state; K_p, permeability coefficient; MCT, medium chain triglycerides; NE, nano-emulsion; O, oil; S, surfactant; O/S, oil/surfactant; O/W, oil/water; PBS, phosphate-buffered saline; PIC, Phase Inversion Composition; PDI, Polydispersity Index; P_1, partition parameter; P_2, diffusion parameter TEWL, Transepidermal Water Losses; T_L, lag time; W, water

INTRODUCTION

Forskolin is one of the labdane diterpenoids. It is isolated from the roots of *Coleus Forskohlii*, an aromatic herb belonging to the family of mints and lavenders, originating from India. Forskolin shows ability to activate adenylate cyclase, resulting in increased levels of cyclic adenosine monophosphate (cAMP) (Bagchi & Preuss, 2012). It displays a broad spectrum of biological activities in the skin (Ciotonea & Cernătescu, 2010), e.g. forskolin protects keratinocytes from UVB-induced apoptosis (Passeron *et al*., 2009), and also influences eumelanin production in the epidermis (Spry *et al*., 2009; Amaro-Ortiz *et al*., 2014), inducing pigmentation, and providing effective skin protection against UVB-induced DNA damage and skin cancer.

In our previous work, we reported forskolin skin permeation from O/W emulsions with the same composition but differing in droplet size (10 mm and 380 nm) (Sikora *et al*., 2015). Both, macro- and nano-emulsion were prepared by a high-energy method. The obtained results showed that forskolin incorporated in the emulsions penetrates the human skin at a high percentage. Currently, nano-emulsions are being studied as effective formulations for pharmaceutical or cosmetic products (Chaudhri *et al*., 2015). They are liquid, kinetically stable colloidal dispersions, consisting of an aqueous phase, an oil phase and a surfactant, with or without co-surfactant. As literature reports, the droplet size of nano-emulsions internal phase is in the range from 20 to 500 nm (Fernandez *et al*., 2004; Usón *et al*., 2004; Kothekar *et al*., 2006; Porras *et al*., 2008; Caldero *et al*., 2011). From an application point of view, nano-emulsions present some advantages, among others, they are easy to spray and spread, moreover they show capacity to deposit uniformly on substrates. As pharmaceutical or cosmetic systems, they show some advantages comparing to classical emulsions, e.g. long term stability and ability to solubilize many compounds that are usually poorly soluble or insoluble in water and/or oil (Gugliemini, 2006). The major advantage of the nano-emulsions with respect to conventional emulsions, is their small droplet size. This parameter may enhance the percutaneous penetration of active substances, as well as their local bioavailability.

Surface active agents have been used to enhance the permeation rates of active substances through the skin. They cause modification of the skin barrier structure by solubilization of the stratum corneum lipids and interaction with keratin filaments (Nokhodchi *et al*., 2003; Som *et al*., 2012; Pandey *et al*., 2014). The penetration of the surfactant molecule into the lipid area is strongly dependent on their properties (Som *et al*., 2012). The most popular nonionic surfactants used to stabilize nano-emulsions are Polysorbates. They are a class of ethoxylated fatty esters of sorbitan that possess low toxicity and skin irritation potential contrary to anionic and cationic surfactants. Among all types of Polysorbates, polyoxyethylene sorbitan monooleate (Polysorbate 80) is the most effective, most widely used, as skin penetration enhancer of lipophilic drugs (Wu *et al*., 1996; Akhtar *et al*., 2011; Pandey *et al*., 2014). Furthermore the influence of Polysorbate 80 facilitating crossing of the blood brain barrier was reported (Wohlfart *et al*., 2012; Fornaguera *et al*., 2015).

For these reasons, in our work we have prepared forskolin-loaded O/W nano-emulsions by low-energy

Table 1. Physical properties of the surfactants (Graca *et al.*, 2007).

Commercial name	Polysorbate 80	Polysorbate 40
Chemical name	Polyoxyethylene (20) sorbitan monooleate	Polyoxyethylene (20) sorbitan monopalmitate
Molecular weight [g·mol^{-1}]	1310	1284
Density [g·ml^{-1}]	1.064	1.080
CMC [mM]	0.012	0.027
HLB	15.0	15.6
Viscosity at 25°C [mPas]	425	500
Appearance	Viscous liquid	Liquid-gel

methods, using medium chain triglycerides (caprylic/capric triglycerides) as an oil phase and stabilized by Polysorbates differing in the alkyl chain length (Polysorbate 80, Polysorbate 40). The influence of the kind of surfactant on *in vitro* forskolin permeation through human skin from the nano-emulsions was evaluated.

MATERIALS AND METHODS

Materials. Nonionic surfactants, Polysorbate 80 and Polysorbate 40 purchased from Sigma-Aldrich Sp. z o.o. (Poznan, Poland) were used (Table 1). The surfactant selection was made because of its good performance and lack of irritation and toxicity to the skin. The oil used, Labrafac CC® (Medium Chain Triglycerides), abbreviated as MCT was kindly supplied by Gattefosse (Montesquieu, France). Milli-Q® filtered water was used as the aqueous phase of the emulsions. Forslean CG (Coleus Forskohlii Root Extract containing 95% of forskolin) was purchased from Sabinsa Europe GmbH (Langen, Germany).

Preparation of the O/W nano-emulsions. Nano-emulsions were prepared using the phase inversion composition (PIC) method by stepwise water addition to mixtures of oil (MCT) and surfactant (either Polysorbate 80 or Polysorbate 40) with ratios between 90:10 and 50:50, at 25°C. Formation of transparent liquid phases was observed visually**.** Samples were considered as nano-emulsions when regardless of their viscosity they were transparent or transparent-bluish. It was confirmed that the obtained systems were not microemulsions because, in contrast to the microemulsions, their stability depended on the preparation method.

Nano-emulsion characterization and stability study. The mean droplet sizes of nano-emulsions were measured by Dynamic Light Scattering (DLS), using Malvern 4700C Sub Micron Particle Analyzer, at a scattering angle of 90º. The analysis was performed three times to determine the mean values and standard deviation. The emulsion stability was assessed by measuring droplet size as a function of time at constant temperature (25°C).

Additionally, the nano-emulsions stability was assessed by light backscattering, by means of Turbiscan LabExpert, at a constant temperature (25°C). Transmission and backscattering data were acquired for 24 h, at 2 hour intervals, according to the method reported by Caldero and coworkers (2011).

Incorporation of forskolin into nano-emulsions. The solubility of forskolin in the oil and oil/surfactant mixtures with ratios of 20/80 and 30/70 was first determined. In this assay, an excess of forskolin was added to the samples and kept under moderate magnetic stirring for 24 h, to reach equilibrium. The presence of crystals was observed by optical microscopy. In order to prepare forskolin (F) loaded nano-emulsions (0.075% wt.) an appropriate amount of the active substance as dissolved in the oil/surfactant mixture before the addition of water to form the nano-emulsion. The samples were homogenized with a vortex mixer and then kept in a water bath at 25°C.

Study of forskolin skin permeation. As in the previous work (Sikora *et al.*, 2015), the *in vitro* skin permeation studies using the MicroettePlus® system (Hanson Research, USA) were carried out. The experiments were performed at 32°C±0.5, 400 rpm, and using a mixture of PBS pH=7.4/ethanol (60/40, v/v) as the receptor medium. Abdominal human skin (0.4 mm) from plastic surgery (Clínica Sagrada Familia, Barcelona, Spain) and from the same donor was used. The transepidermal water losses (TEWL) of skin pieces were measured to assure the integrity of the skin. An infinite dose of formulation (0.350 mL) was placed in the donor compartment and samples of 700 µL were withdrawn automatically from the receptor compartment at 3, 6, 14, 16, 18, 20, 22 and 24 h and replaced with the same volume of receptor mixture at 32ºC. The number of replicates per formulation was n=4. Permeation parameters: flux at steady state (Jss) by means of a linear regression (cumulative permeated amount *vs.* time, slope), lag time (T_L) (X-intercept), permeability coefficient (K_p=Jss/$C_{formulation}$), partition parameter P_1 and the apparent length of diffusion parameter P_2 (Okamoto *et al.*, 1986; Selzer *et al.*, 2013) were estimated, and compared by the non-parametric analysis (Williams *et al.*, 1992) Kruskal-Wallis one-way Anova test followed by the Kruskal-Wallis multiple-comparison Z-test (α=0.05).

HPLC analysis. The concentration of forskolin in the receptor solution of the permeation assays was determined using an HPLC Waters instrument, operated at ambient 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, i.e. 60 volumes of acetonitrile and 40 volumes of water, which remains constant throughout the whole procedure. The flow rate was set to 0.5 mL · min^{-1}. The analysis was monitored at λ=210 nm, sample injection volume was 20 µL and run time 10 min. The forskolin content was identified by comparing the retention time and UV spectra. The calibration curve was constructed from linear plots of peak area versus concentration. The calibration curves were prepared in the receptor solution with a forskolin concentration range of 3.2 to 32 µg · ml^{-1}.

RESULTS AND DISCUSSION

Formulation of forskolin-loaded nano-emulsions

Prior to the preparation of forskolin-loaded nano-emulsions, the solubility of the active substance in Labrafac CC® (MCT), as well as in MCT/Polysorbate 80 and MCT/Polysorbate 40 mixtures, with ratios of 30/70 and 20/80, was determined. The results (Table 2) revealed that the solubility of forskolin in the MCT/surfactant mixtures was higher than in the oil. Moreover with decrease of MCT (i.e. O/S ratio) an increase of drug solubilization was observed. However, no differences in for-

Table 2. Solubility of forskolin.

Mixture of Oil and Surfactant	O/S ratio	Forskolin [mg·mL^{-1}]
Labrafac CC®	100/0	3.5
Labrafac CC® /Polysorbate 80	30/70	4.5
Labrafac CC® /Polysorbate 40		
Labrafac CC® /Polysorbate 80	20/80	6.0
Labrafac CC® /Polysorbate 40		

Table 3. Characteristics of the nano-emulsions.

Formulation	Mean diameter (nm) ±S.D. (n=3)	PDI
NE1	117±7	0.357
NE2	88±8	0.350
NE1F	118±10	0.370
NE2F	111±15	0.338

skolin solubility were observed with Polysorbate 80 or Polysorbate 40.

The O/W nano-emulsion formation region was determined in the Water/Polysorbate 80/Labrafac CC® and Water/Polysorbate 40/Labrafac CC® systems by stepwise addition of water to oil/surfactant mixtures (O/S) at different ratios, at 25°C. The results are shown in Fig. 1. O/W nano-emulsions were formed at O/S ratios comprised between 10/90 and 30/70. The water concentration in the system was above/exceeded 60% wt. in the system with Polysorbate 80 (Fig. 1A) and above 50% wt. in the system with Polysorbate 40 (Fig. 1B). Nano-emulsions became more transparent at high water contents.

Due to the higher forskolin solubility in the oil/surfactant mixture with ratio of 20/80, for further studies, nano-emulsions with 80% of water and with this oil/surfactant ratio were chosen. Two nano-emulsions containing 0.075% wt. of forskolin differing in the kind of surfactant used (abbreviated as NE1F with Polysorbate 80 and NE2F with Polysorbate 40), were prepared as described in Section: Incorporation of forskolin into nano-emulsions. The corresponding unloaded-nano-emulsions are designated as NE1 and NE2.

Nano-emulsion characterization

The results of mean droplet size of the nano-emulsions (±S.D.) and polydispersity index (PDI) characterized by DLS are shown in Table 3. The forskolin-loaded nano-emulsions NE1F and NE2F presented similar droplet diameters of around 118 ±10 nm and 111±15 nm, respectively. Incorporation of forskolin did not significantly affect the droplet size in the Polysorbate 80 system, while a slight increase in the droplet size from 88±8 to 111±15 nm was observed in the Polysorbate 40 system.

The stability of non-loaded nano-emulsions at 25°C was assessed by light transmission spectra as a function of time along the sample height. Figure 2 shows that the transmission profiles were roughly flat at the whole height of the samples, indicating that they were homogeneous. The increase in transmission at the meniscus of the sample, as well as at the convex bottom of the cell containing the sample, due to multiple light diffractions, has no physical meaning. The transmission remained practically unchanged in a period of 24 h, indicating that no significant destabilization phenomena, due to creaming or sedimentation, or due to droplet size changes occurred in this period of time.

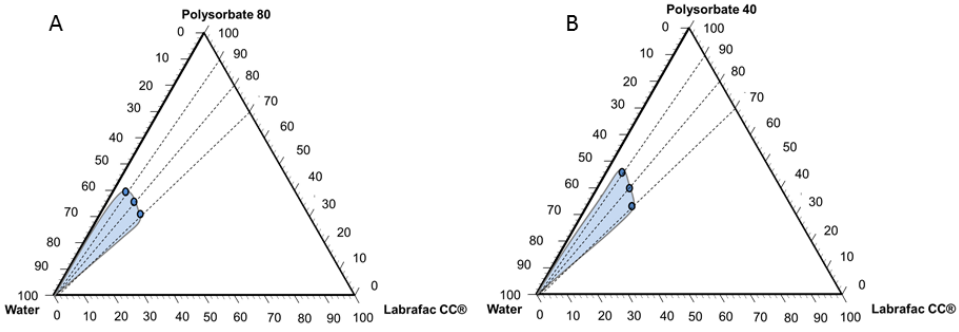

Figure 1. O/W nano-emulsion region in the Water/ Surfactant/Labrafac CC® (MCT) systems at 25°C: (A) Polysorbate 80 and (B) Polysorbate 40.

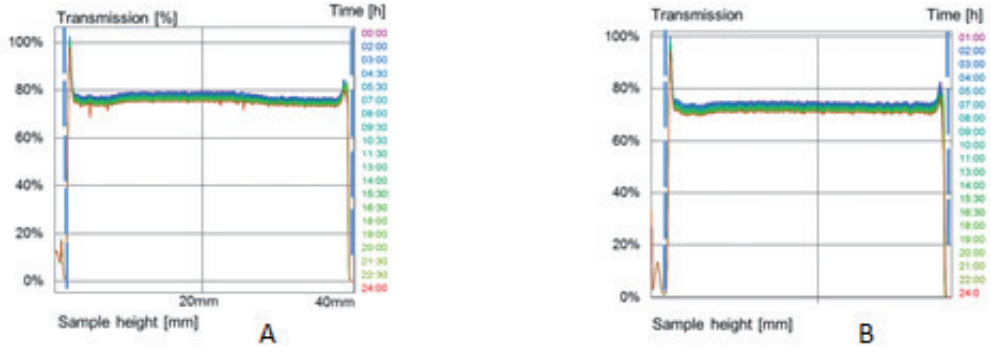

Figure 2. Transmission data of nano-emulsions of the water/surfactant/Labrafac CC® (MCT) system with 80% water and O/S ratio of 20/80 along the sample height at different times at 25°C: (A) Polysorbate 80 system (NE1) and (B) Polysorbate 40 system (NE2).

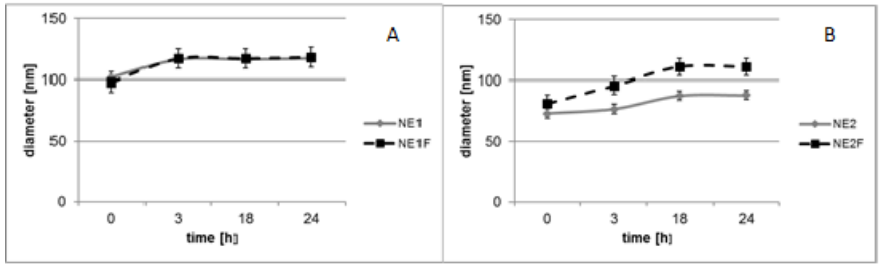

Figure 3. Droplet size of nano-emulsions of the water/ surfactant/Labrafac CC® (MCT) system, with 80% water and O/S ratio of 20/80 without forskolin (NE1 and NE2) and with forskolin (NE1F and NE2F) as a function of time: (A) Polysorbate 80 system and (B) Polysorbate 40 system.

Table 4. Mean and standard deviation of permeation parameters (n=4).

Formula	J_{ss} ($\mu g \cdot cm^{-2} \cdot h^{-1}$)	T_L (h)	$K_p \cdot 10^3$ ($cm \cdot h^{-1}$)	P_1 (cm)	$P_2 \cdot 10^2$ (h^{-1})
NE1F	6.91±0.75	8.82±0.90	9.21±1.00	0.49±0.10	1.90±0.002
NE2F	4.92*±0.44	2.75*±0.36	6.55*±0.59	0.108*±0.019	6.13*±0.80
Labrafac CC®	6.07±0.53	4.25±1.21	8.10±0.70	0.210±0.080	4.13 ±1.00

*$p<0.05$ with NE1F

Stability of the forskolin-loaded and unloaded nano-emulsions was also determined by measurements of droplets size (by DLS) with time. Figure 3A shows that in the system with Polysorbate 80, a slight increase in the droplet size was produced during the first three hours and afterwards the size was constant during the experimental time of 24 hours. The size and the evolution of size with time of the unloaded and loaded nano-emulsions of this system were identical. Also, for the nano-emulsions with Polysorbate 40 (Fig. 3B), the data confirm the stability of the systems for 24 hours, at 25°C.

Permeation results

Permeation profiles of forskolin from the two nano-emulsions and Labrafac CC®(MCT) were similar (Fig. 4). The flux was higher for NE1F than for NE2F. The percentage of permeation after 24 h was high (69.92±8.89%, 71.49±7.11% and 83.48±7.41%, for NE1F, NE2F and MCT, respectively). These results are similar to those obtained in our previous work with emulsion composed of MCT but with different surfactant and droplet size (Sikora *et al.*, 2015). We hypothesize a rapid penetration of forskolin through the *stratum corneum* (SC) facilitated by the high lipophilicity of the molecule (logP=3.89) and adequate molecular weight (410.50 g ·mol^{-1}). But as the skin is composed of the lipophilic SC and hydrophilic viable layers of epidermis, the partitioning of forskolin into the layers becomes difficult. Therefore, forskolin would make a reservoir in SC and after approximately 10 h, it would start a second step of permeation from a saturated SC where forskolin would be accumulated. This second step is possibly due to alterations in the skin mediated by the components of the formulations, and basically by the Labrafac CC® oil. Kandimala and coworkers (2010) also described a two-step profile in the permeation of melatonin in the presence of saturated and unsaturated fatty alcohols. However, it showed a higher flux in the first step than in the second. This decrease in the second step was attributed to the penetration of polar formulation components in the SC decreasing the formulation/skin partition of melatonin, which is not the case in our study.

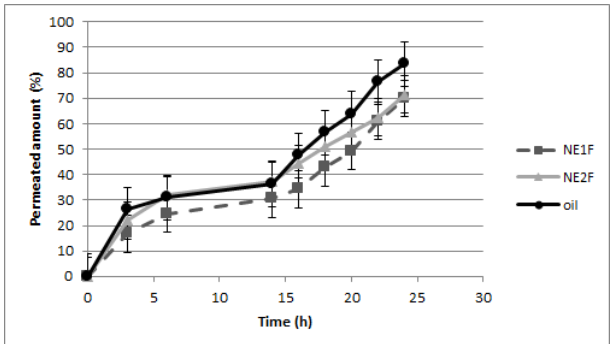

Figure 4. Skin permeation profile of forskolin from nano-emulsions and an oil solution.
NE1F: Polysorbate 80 system. NE2F: Polysorbate 40 system.

According to the permeation parameters (Table 4) obtained from the second step of the profile, NE1F presented higher values ($p<0.05$) than NE2F and Labrafac CC®, except for the P_2 parameter. Then, the substitution of Polysorbate 40 for Polysorbate 80 in the nano-emulsions increased the percutaneous penetration of forskolin through human skin. If we calculate the K_p enhancement ratio (Williams and Barry, 1991) of NE1F in respect to Labrafac CC®, the value is approximate to 1 (1.14). In this way, the permeability coefficients are very similar and would be in accordance with the high lipophilicity of forskolin. However, the *in vivo* application of forskolin in MCT would represent a non-acceptable cosmetic formulation, due to the oily feeling.

On the other hand, the lipids of the NE2F (Labrafac CC®) and surfactant Polysorbate 40 interact in the same order as the oil alone did with the intercellular lipid matrix, increasing the disruption of the packed lipid regions and rising the diffusion of forskolin through the skin and reflected in the P_2 parameter. However, when comparing the permeability coefficient of forskolin for

NE1F in respect to NE2F ($9.21 \cdot 10^{-3} \pm 1.00 \cdot 10^{-3}$ cm $\cdot h^{-1}$ *vs.* $6.55 \cdot 10^{-3} \pm 0.59 \cdot 10^{-3}$ cm $\cdot h^{-1}$) the difference can be attributed to the relatively higher $P_{1(NE1F)}/P_{1(NE2F)}$ ratio (4.53) with respect to the $P_{2(NE1F)}/P_{2(NE2F)}$ ratio (0.30). According to this, and taking into account that the composition of the formulations are the same (except for the type of surfactant), and also the droplet size and the solubility in the Polysorbate/Labrafac CC® mixture are similar, the differences observed in the permeation should be attributed to higher affinity of forskolin for the vehicle in NE2F with respect to the skin and giving a lower value of the P_1 parameter. The nano-emulsion of NE2F presented a similar behavior as MCT did in skin permeation of forskolin: in those formulations the diffusion through the skin layers is higher and also presented lower partition coefficient of forskolin skin/vehicle retaining the actives in the vehicle. The observed results can be explained by differences in hydrophilicity and the structure of the alkyl chain of the surfactants used, and related to the higher HLB number of Polysorbate 40 with respect to Polysorbate 80 (15.6 *vs.* 15, respectively) by which forskolin would have more affinity. Wu and coworkers (1996) studied the effect of the kind of surfactant on captopril percutaneous absorption. Among the nonionic surfactants (Polysorbates) used, the polyoxyethylene sorbitan monolaurate (Polysorbate 20), the most hydrophilic one (HLB=16.7), had the greatest impact on the flux of the active substance. On the other hand, in the case of Polysorbate with longer alkyl chain, the structure of the chain seems to be more significant than the surfactant hydrophilicity. Molecules with saturated fatty acids, e.g. Polysorbate 40, despite the higher hydrophilicity, have less pronounced effect than unsaturated ones (Polysorbate 80), probably due to a lesser ability to disrupt the lipid packing of *stratum corneum*. In summary, active skin permeation is related to the structure of the alkyl chain of the surfactants, their hydrophilicity and the affinity of the actives to the vehicle.

In the case of forskolin, both Polysorbates used act as skin permeation enhancers. That means that emulsions composed of caprylic/capryc triglycerides and different surfactants can be considered as good carriers for forskolin delivery systems into the skin. However, it should be noted that maximum forskolin permeation was obtained using the nano-emulsion containing Polysorbate 80.

CONCLUSIONS

Kinetically stable, forskolin-loaded O/W nano-emulsions (0.075% wt.), based on caprylic/capryc triglycerides and stabilized with Polysorbate 80 or Polysorbate 40 were obtained, with droplet diameters around 118 nm and 111 nm, respectively. Nano-emulsion droplet size was not changed by incorporation of forskolin in the system with Polysorbate 80, but a slight increase in size was observed for the Polysorbate 40 system. The *in vitro* skin permeation studies through human skin have shown that similar amounts of forskolin penetrate from both, the nano-emulsion formulations and from the oil solution. However, the substitution of Polysorbate 40 for Polysorbate 80 in the nano-emulsions increases the percutaneous penetration of forskolin through the skin. Nano-emulsions, based on caprylic/capryc triglycerides can be considered as good carriers for controlled release of forskolin for topical application.

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