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Research Paper

Evaluation of percutaneous absorption of the repellent diethyltoluamide and the sunscreen ethylhexyl *p*-methoxycinnamate-loaded solid lipid nanoparticles: an in-vitro study

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Abstract

Objectives Diethyltoluamide and ethylhexyl *p*-methoxycinnamate (OMC) are two active ingredients in insect repellent and sunscreen products, respectively. The concurrent application of these two substances often increases their systemic absorption, compromising the safety and efficiency of the cosmetic product. In this study, diethyltoluamide and OMC were incorporated into solid lipid nanoparticles, a colloidal drug delivery system, to reduce percutaneous absorption and avoid toxic effects and also maintain the efficacy of the two active compounds on the skin surface for a long duration.

Methods Solid lipid nanoparticles were prepared based on an ultrasonication technique and characterized by differential scanning calorimetry (DSC) analyses. In-vitro studies determined the percutaneous absorption of diethyltoluamide and OMC.

Key findings DSC data carried out on unloaded and diethyltoluamide- and/or OMC-loaded solid lipid nanoparticles highlighted that diethyltoluamide and OMC modified the temperature and the enthalpy change associated to the calorimetric peak of solid lipid nanoparticles. The concurrent presence of the two compounds in the solid lipid nanoparticles caused a synergic effect, indicating that the lipid matrix of nanoparticles guaranteed a high encapsulation of both diethyltoluamide and OMC. Results from the in-vitro study demonstrated that the particles were able to reduce the skin permeation of the two cosmetic ingredients in comparison with an oil-in-water emulsion.

Conclusions This study has provided supplementary evidence as to the potential of lipid nanoparticles as carriers for topical administration of cosmetic active compounds.

Keywords diethyltoluamide; differential scanning calorimetry; ethylhexyl *p*-methoxycinnamate; percutaneous absorption; solid lipid nanoparticles

Introduction

Insect repellents and sunscreens are two types of consumer care products that are extensively used by the general public. Concurrent application of these substances for outdoor activity has become a commonly practised routine to prevent vector borne diseases and sunburn. For decades diethyltoluamide (*N*,*N*–diethyl–*m*-toluamide) has been used as one of the most effective insect repellents, while ethylhexyl *p*-methoxycinnamate (octyl methoxycinnamate; OMC) is a well-known sunblocking agent present in many commercially available sunscreen preparations.[1–3] These substances, designed as topical preparations, should exert minimal transdermal and systemic absorption under ideal application conditions. Diethyltoluamide, particularly, when administered alone had a remarkably low incidence of adverse effects, but when the absorption into the circulation was high, neurotoxic symptoms including tremor, seizures and toxic encephalopathy have been reported.[4,5] The mechanism controlling the systemic neurotoxicity of diethyltoluamide is unknown. It has been suggested that diethyltoluamide may induce neuronal apoptosis or disrupt the permeability of the blood–brain barrier, but neither of these hypotheses have been proven.[6]

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There is extensive literature regarding the transdermal permeation of diethyltoluamide and sunscreens from topical application, but only recently has the influence of co-applying these substances on their systemic absorption been studied fully.[7–13]

In-vitro and in-vivo studies on transdermal permeation of diethyltoluamide and sunscreens demonstrated that these compounds acted as percutaneous absorption enhancers to each other, compromising the safety and efficiency of the cosmetic product.[12,14–16]

Ross *et al*.,[12] in an in-vitro study, demonstrated that sunscreens enhanced significantly diethyltoluamide absorption through full-thickness hairless mouse skin. Kasichayanula *et al*.,[13] in an in-vitro and in-vivo study, assessed that the combined use of diethyltoluamide and oxybenzone (a well-known sun-filter) significantly enhanced the percutaneous penetration percentages. Particularly diethyltoluamide produced a faster transdermal permeation rate and higher systemic absorption extent, but oxybenzone formed a concentrated depot within the skin and delivered the content slowly over time.

Due to these problems, several studies have been aimed at the development of an efficient means for diethyltoluamide and OMC topical administration to reduce systemic absorption so as to limit their harmful side-effects. Recently, a new approach has been described using solid lipid nanoparticles (SLN) as carriers for cosmetic active ingredients.[17,18] SLN, based on nonirritating and nontoxic lipids, provide a carrier system for various active agents with controlled release characteristics. This feature appeared to be very important since irritant substances turned out to be less irritating when applied in a controlled release system, which also reduced systemic uptake of the active agent.[17,18]

In this study, we have evaluated solid lipid nanoparticles as a carrier system to optimize diethyltoluamide and OMC concurrent application. With this aim we investigated the influence of SLN on in-vitro percutaneous absorption of OMC and diethyltoluamide compared with a conventional oil-in-water (o/w) emulsion.

Differential scanning calorimetry (DSC), a suitable technique to characterize drug-loaded SLN, was applied to determine the best experimental conditions guaranteeing the highest incorporation of OMC and diethyltoluamide in the solid lipid bulk.[19]

Materials and Methods

Materials

Stearic acid and diethyltoluamide (purity > 98%) were purchased from Sigma (Milan, Italy). Tween 80 was provided by BASF AG (Ludwigshafen, Germany). Ethylhexyl *p*-methoxycinnamate (OMC) was purchased from Cognis S.p.A. (Milan, Italy). Xanthan gum was purchased from Sigma Chemical (Milan, Italy). HPLC grade methanol and water were purchased from CarloErba reagents (CarloErba, Milan, Italy). All other chemicals were of reagent grade and used without further purification.

Preparation of solid lipid nanoparticles

SLN were prepared by ultrasonication.[20] Briefly, stearic acid (4 g) was melted at 80°C and OMC (5%) and diethyltoluamide (10%) (w/w) were added under mixing. The hot lipid phase was dispersed in a surfactant solution (8000 rev/min, 85°C for 1 min) by using a high-speed stirrer (Ultra Turrax T25). The obtained pre-emulsion was ultrasonified at 80°C by using a UP 400 S (Ultraschallprozessor, Dr. Hielscher GmbH, Teltow, Germany). The obtained nanoemulsion (o/w) was cooled down in an ice bath to form SLN and finally diluted up to 200 ml with deionized water. The SLN dispersion was stored at 4°C.

Solid lipid nanoparticle size distribution

Mean particle size and population distribution of the bulk particle dispersion were measured by photon correlation spectroscopy using a Zetamaster (Malvern Instruments Ltd, Malvern, Worcestershire, UK) equipped with a solid state laser having a nominal power of 4.5 mW with a maximum output of 5 mW 670 nm. Dimensional analyses were performed using a 90° scattering angle at 20 (± 0.2)°C. Samples were prepared by diluting 10 μl SLN suspension with 2 ml deionized water previously filtered through a 0.2 μm Acrodisc LC 13 PVDF filter (Pall-Gelman Laboratory, Ann Harbor, MI, USA). During the experiment, the refractive index of the samples was always matched to liquid (toluene) to avoid stray light.

Determination of OMC and diethyltoluamide loading

The percentage of OMC and diethyltoluamide entrapped in the lipid matrix was determined as follows: a fixed amount of SLN dispersion was filtered using a Pellicon XL tangential ultrafiltration system (Millipore, Milan, Italy) equipped with a polyethersulfone Biomax10 membrane. An amount of retained material was freeze-dried, dissolved in chloroform and analysed by UV spectrophotometry at 310 and 254 nm for OMC and diethyltoluamide, respectively (Lambda 52, PerkinElmer, MA, USA). Calibration curves for the validated UV assays of OMC and diethyltoluamide were performed on five solutions in the concentration range 2–20 μg/ml, respectively. The correlation coefficient was > 0.990. Each point represented the average of three measurements and the error was calculated as standard deviation (± SD). OMC and diethyltoluamide incorporation efficiency was expressed as drug recovery and calculated using the following equation:

$$\text{Drug recovery}(\%) = \Big((\text{Mass of drug in nanoparticles})/(\text{Mass of drug fed to the system})\Big) \times 100 \quad (1)$$

Possible lipid interferences during UV determination of OMC and diethyltoluamide were also investigated by comparing the two standard curves of each substance alone and in the presence of lipids. The differences observed between the standard curves were within the experimental error, thus inferring that no lipid interference occurred (data not shown).

Differential scanning calorimetry

DSC studies were performed using a Mettler TA STAR^e System equipped with a DSC 822^e cell and Mettler STAR^e V8.10 software (Mettler Toledo, Schwerzenbach, Switzerland). The reference pan was filled with 120 μl distilled water. The calorimetric system was calibrated, in transition temperature and enthalpy changes, by using indium and palmitic acid (purity ≥ 99.95% and ≥ 99.5%, respectively; Fluka, Switzerland) following the procedure of the Mettler STAR^e software. The DSC measurements were carried out on the following samples: unloaded SLN; OMC-loaded SLN, diethyltoluamide-loaded SLN, OMC and diethyltoluamide-loaded SLN. Stearic acid, polysorbate, OMC and diethyltoluamide, submitted to the same procedure employed to prepare SLN, were analysed.

Each sample (120 μl) was transferred into a 160 μl calorimetric pan, hermetically sealed and submitted to DSC analysis as follows: a heating scan in the temperature range 5–85°C at the rate of 2°C/min; a cooling scan in the temperature range 85–5°C at the rate of 4°C/min; for at least three cycles.

A fixed amount (corresponding to that present in the SLN) of stearic acid was weighed in the calorimetric pan, 120 μl distilled H_2O was added, the pan was hermetically sealed and submitted to the DSC analysis reported above. Each experiment was carried out in triplicate.

In-vitro study

Preparation of formulations

SLN were formulated into hydrogel using glycerol and xanthan gum as excipients. Briefly, the hydrogel formulation was produced by adding to OMC/diethyltoluamide-loaded SLN suspension (89%) 10% (w/w) glycerol and 1% (w/w) xanthan gum. The formulation was stirred for 5 min and then stored at 4°C before use.

A further OMC/diethyltoluamide-loaded topical formulation was prepared as the control form for the in-vitro percutaneous absorption study. The oil phase of the control formulation was composed of: PPG-15 stearyl ether (7 g); isohexadecane/PPG-15 stearyl ether (3 g); OMC (5 g); diethyltoluamide (10 g). Steareth 2 (3.5 g), steareth 21 (2.5 g), stearic acid (2.5 g), cetylstearylic acid (2.1 g) and xanthan gum (0.3 g) were used as surfactants and structurizing agents. The o/w emulsion was prepared by slowly adding the aqueous phase (64.1 ml) to the oil phase and to the blend of surfactants under continuous agitation; the phases were kept to 70°C. This mixture was stirred until it was cool, thus forming the emulsion formulation.

Skin membrane preparation

Samples of adult human skin (mean age 36 ± 8 years) were obtained from breast reduction operations. Subcutaneous fat was carefully trimmed and the skin was immersed in distilled water at 60 ± 1°C for 2 min, after which the stratum corneum and epidermis were removed from the dermis using a dull scalpel blade.[21] Epidermal membranes were dried in a desiccator at approximately 25% relative humidity. The dried samples were wrapped in aluminum foil and stored at 4 ± 1°C until use. Previous research had demonstrated the maintenance of the stratum corneum barrier characteristics after storage in the reported conditions.[22] Preliminary experiments were carried out to assess stratum corneum and epidermis samples for barrier integrity by measuring the in-vitro permeability of [^{3}H]water through the membranes using the Franz cells described below. The value of calculated permeability coefficient (P_m) for [^{3}H]water agreed well with those reported previously.[23]

In-vitro skin permeation experiments

Samples of dried stratum corneum and epidermis were rehydrated by immersion in distilled water at room temperature for 1 h before being mounted in Franz-type diffusion cells supplied by LGA (Berkeley, CA, USA). The exposed skin surface area was 0.75 cm^2 and the receiver compartment volume was 4.5 ml.

The receptor compartment contained a water–ethanol solution (50 : 50) to allow the establishment of the 'sink condition and to sustain permeant solubilization'.[24] Furthermore, the solution was stirred with the help of a magnetic bar at 500 rev/min and maintained at 35 ± 1°C for the duration of the experiments.

Approximately 300 mg SLN gel and control formulations were placed on the skin surface in the donor compartment and the latter was covered with Parafilm. Each experiment was run in duplicate for 24 h using three different donors (n = 3). At intervals samples (200 μl) of receiving solution were withdrawn and replaced with fresh solution. The samples were analysed for drug content by HPLC as described below. OMC and diethyltoluamide fluxes through the skin were calculated by plotting the cumulative amount of substance penetrating the skin against time and determining the slope of the linear portion of the curve and the χ-intercept values (lag time) by linear regression analysis. The fluxes of the substances (μg/cm^2 per h), at steady state, were calculated by dividing the slope of the linear portion of the curve by the area of the skin surface through which diffusion took place.

High-performance liquid chromatography

The HPLC apparatus consisted of a Shimadzu LC10 AT Vp (Milan, Italy) equipped with a 20 μl loop injector and a SPD-M 10 A Vp Shimadzu photodiode array UV detector.

Chromatography was performed using a Symmetry Shield Waters C_{18} RP column (particle size, 5 μm; 25 cm × 4.6 mm i.d.; Waters S.P.A, Italy). The mobile phase was composed of methanol and water (pH 2.83, adjusted with glacial acetic acid). Three-stage gradient steps were used, 0–3 min 50% methanol and 50% water; 3–9.5 min 70% methanol and 30% water; 9.5–25 min 90% methanol and 10% water. The flow rate was set at 1 ml/min and the detection was effected at 310 and 254 nm for OMC and diethyltoluamide, respectively. The retention time was 9.1 and 20.2 min for diethyltoluamide and OMC, respectively. The calibration linearity range was 50–2000 ng for diethyltoluamide and OMC ($r^2 \geq 0.99$).

Statistical analysis

Statistical analysis of DSC data was performed using the Kruskal–Wallis and the Dunn's tests. Statistical analysis of in-vitro data was performed using the Mann–Whitney U-test. A probability, P, of less than 0.05 was considered significant.

Results

Preparation and characterization of solid lipid nanoparticles

Mean particle size data of diethyltoluamide and OMC-loaded SLN were obtained by photon correlation spectroscopy analyses. The results confirmed the efficiency of the ultrasonication method, in fact a particle population with mean diameter of 302.6 nm was obtained (polydispersity index: 0.211 ± 0.06).

As regards the drug loading, the SLN produced by the ultrasonication method had a high incorporation efficiency showing a percentage of drug recovery of 87.3% and 79.6% for OMC and diethyltoluamide, respectively.

Unloaded and loaded SLN were characterized by DSC analysis. Unloaded SLN were analysed to obtain more information on the SLN system. To be certain that the calorimetric peak of the SLN could be ascribed to just a well-defined system composed of stearic acid and polysorbate, we submitted the single components used to prepare the SLN to DSC analysis. We then submitted the SLN prepared as outlined above to DSC analysis. The calorimetric curves are shown in Figure 1. No calorimetric peak was recorded when polysorbate was submitted to calorimetric analysis in the temperature range employed in the study; consequently no phase transition was detected and the SLN peak should be ascribed only to the lipid component. SLN, stearic acid and treated stearic acid calorimetric peaks were quite different in temperature and enthalpy variation. SLN calorimetric peak was sharp and centred at 54.5°C, a temperature lower than that of stearic acid and treated stearic acid. In addition, the enthalpy variation was higher with respect to treated stearic acid but lower with respect to stearic acid. Stearic acid showed, as expected and in agreement with the literature, a well-defined peak at 69.73°C relating to the melting of the solid compound.[25] The treated stearic acid was characterized by a broad peak at 55.61°C, a temperature much lower than that of stearic acid.

The calorimetric curves of unloaded and loaded SLN are reported in Figure 2. Unloaded SLN were characterized by just one well-defined calorimetric peak at 54.5°C due to the stearic acid mixed with polysorbate phase change. OMC-loaded SLN show a peak shifted towards a lower temperature with respect to unloaded SLN. The calorimetric peak of diethyltoluamide-loaded SLN was broader and at a lower temperature than that of unloaded SLN. Even broader and at a lower temperature was the calorimetric peak of SLN loaded with OMC and diethyltoluamide. To check that none of the peaks were to be ascribed to OMC and diethyltoluamide, the two compounds were singularly submitted to DSC analysis, which did not give any calorimetric signal in the temperature range employed. The calorimetric peak temperature of unloaded and loaded SLN is reported in Figure 3a. It was

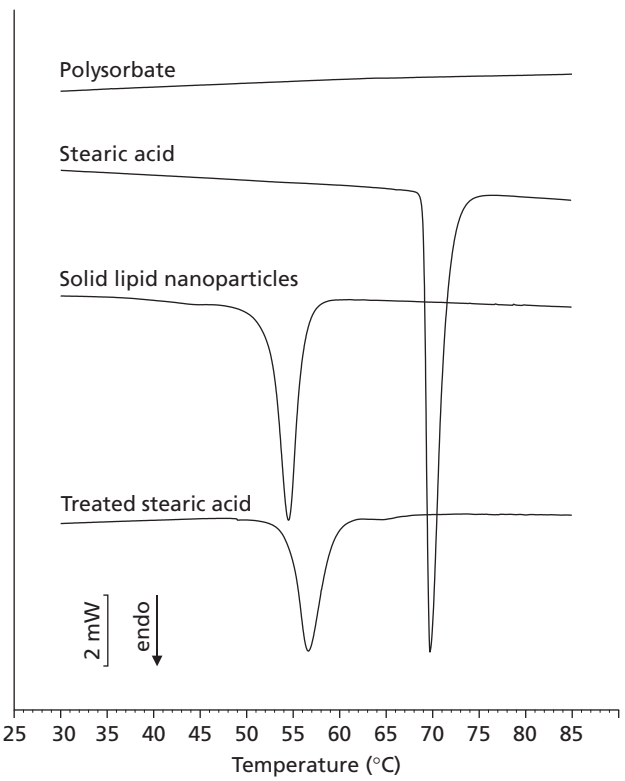

Figure 1 Calorimetric curves, in heating mode, for the prepared solid lipid nanoparticles and each of their singular components.

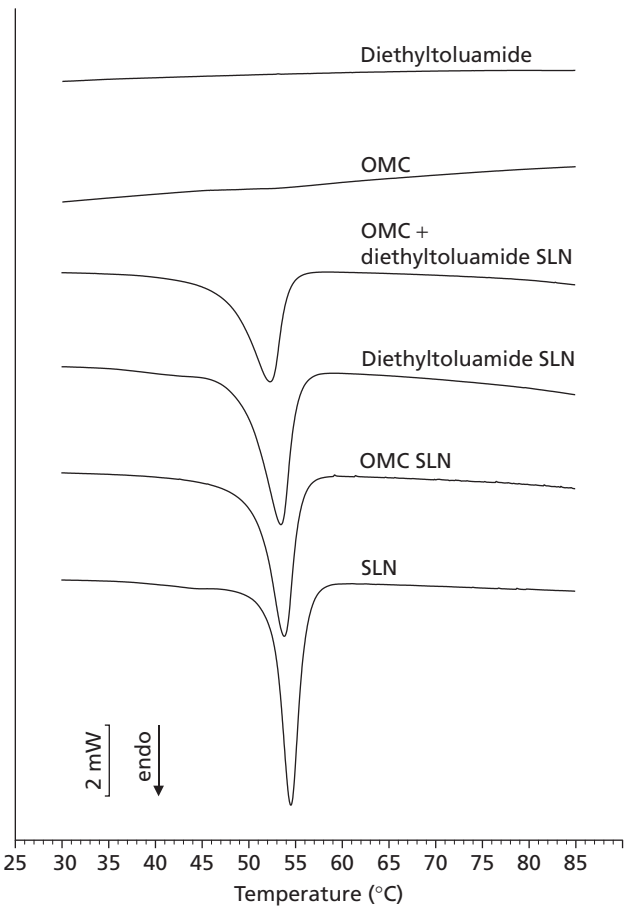

Figure 2 Calorimetric curves, in heating mode, of loaded and unloaded solid lipid nanoparticles. The calorimetric curves were for treated diethyltoluamide, treated ethylhexyl *p*-methoxycinnamate (OMC), unloaded solid lipid nanoparticles (SLN) and loaded SLN.

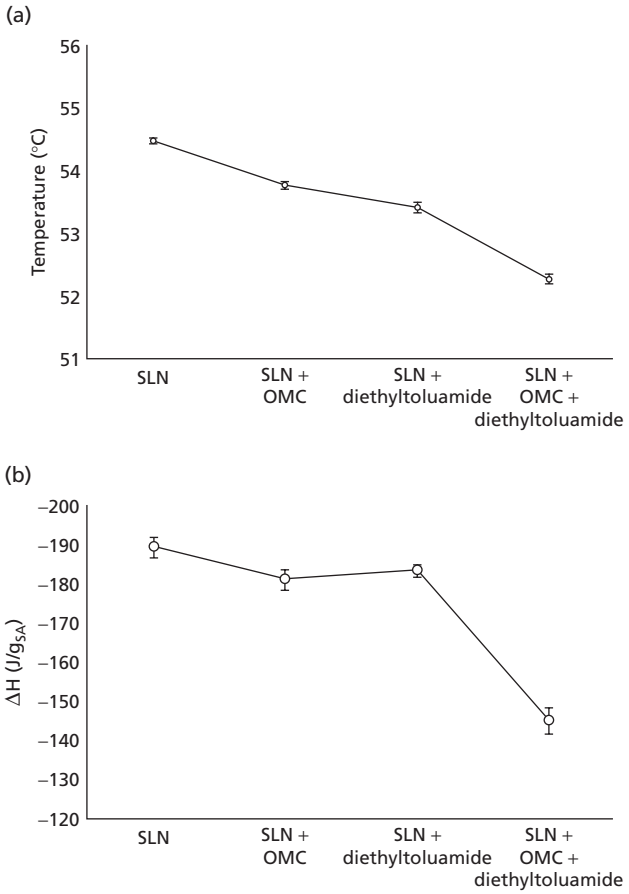

Figure 3 Calorimetric peak temperature and enthalpy variation of solid lipid nanoparticles. (a) Peak temperature and (b) enthalpy variation (ΔH) of unloaded and loaded solid lipid nanoparticles (SLN). OMC, ethylhexyl *p*-methoxycinnamate.

evident that OMC and diethyltoluamide caused a decrease of the temperature, which was more evident when diethyltoluamide was used. When OMC and diethyltoluamide were present at the same time in the SLN the temperature decrease was marked. The results were analysed using Kruskal–Wallis and Dunn's tests, which confirmed their significance with $P = 0.02$ and $P < 0.05$, respectively. The enthalpy variation values, shown in Figure 3b, indicated an enthalpy variation decrease of loaded SLN with respect to unloaded SLN. When the compounds were singularly present in the SLN the enthalpy change decrease was insignificant, as confirmed by Dunn's test ($P > 0.05$), whereas the decrease was more evident and statistically significant ($P < 0.05$, Dunn's test) when the two compounds were present together in the SLN.

In-vitro study

Plotting the cumulative amounts of compounds permeated through human stratum corneum and epidermal membranes as a function of time, flux values at the steady state from diethyltoluamide/OMC-loaded SLN and from the o/w emulsion were obtained (Figure 4). Diethyltoluamide and

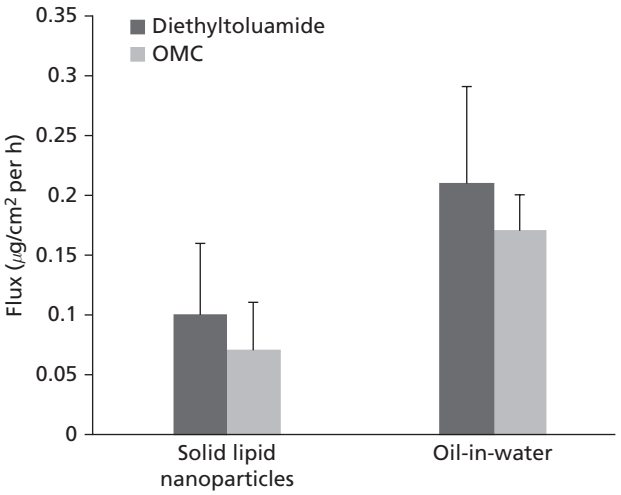

Figure 4 Diethyltoluamide and ethylhexyl *p*-methoxycinnamate fluxes at steady state. The diethyltoluamide and ethylhexyl *p*-methoxycinnamate (OMC) were from solid lipid nanoparticles and from oil-in-water formulations.

OMC incorporated in viscosized SLN dispersions exhibited a lower flux with respect to the o/w formulation containing the same amount of substances.

Discussion

Diethyltoluamide and OMC-loaded SLN were prepared with stearic acid as the solid lipid and Tween 80 as the surfactant. We decided to use these ingredients according to the work of Iscan *et al.*[26] in which the researchers, after a lipid screening for the identification of matrices for diethyltoluamide incorporation, chose stearic acid to prepare diethyltoluamide-loaded SLN. In fact those authors assessed that lipids such as cetylpalmitate and Dynasan 116 were not good candidates to prepare SLN as diethyltoluamide was quickly expelled from the matrices.[26] Those results were similar to the ones obtained in this study, even though the use of ultrasound increased the amount of diethyltoluamide incorporated into SLN (data not shown).

Notwithstanding that the hot high pressure homogenization method is recognized as the most suitable procedure to produce lipid nanoparticles because of its easy scalability, the ultrasonication method, in our experience, is a 'cheap and fast' method suitable for the production of lipid nanoparticles.[20,27–29] The results obtained in this study by photon correlation spectroscopy analyses and drug loading determination confirmed the efficiency of the ultrasonication method.

The SLN and each singular component were submitted to DSC analysis and the calorimetric curves were compared. With regard to treated stearic acid, both the peak broadening (with an enthalpy variation decrease) and the shift towards lower temperature, with respect to stearic acid, could be related to a less structured system whose formation was due to the treatment to which stearic acid was submitted. In fact, the enthalpy variation and the temperature decrease depended on the microdispersion of the solid phase in the solvent during the treatment. Unloaded SLN exhibited a

sharp calorimetric peak that could indicate the formation of a well-ordered structure in which a high cooperativity among the molecules exists; this evidence was supported by the enthalpy variation increase compared with treated stearic acid. The comparison between unloaded SLN and stearic acid highlighted the decrease of the peak temperature and of the enthalpy variation of the former which indicated that polysorbate was part of the SLN ordinate structure.

From the comparison between unloaded and loaded SLN several considerations can be made. The decrease of the temperature of the OMC-loaded SLN and diethyltoluamide-loaded SLN with respect to unloaded SLN indicated that OMC and diethyltoluamide were incorporated in the SLN and destabilized their ordered structure, acting as impurities among the stearic acid and polysorbate molecules; the marked decrease observed in the OMC and diethyltoluamide-loaded SLN indicated that the two compounds could be hosted together by the SLN. Further evidence of the concurrent incorporation of OMC and diethyltoluamide in the SLN system was given by the analysis of the enthalpy changes of loaded and unloaded SLN: when the compounds were singularly present in the SLN the enthalpy change decrease was insignificant with respect to unloaded SLN, whereas the decrease was more evident when the two compounds were present concurrently in the SLN, causing an increase of the disorder in the stearic acid/polysorbate system.

As regards in-vitro findings, they were in accordance with the evidence reported in the literature and confirmed the key role of the vehicle in determining the active pharmaceutical ingredient dermal or transdermal fate for topically applied formulations.[18,29–31] In fact lipid nanoparticles have shown the peculiarity to reduce and/or suppress the permeation (transdermal delivery) through the skin while producing an active pharmaceutical ingredient accumulation in the horny layer.

This feature of lipid nanoparticles has been used successfully for the topical application of different active cosmetic ingredients. Wissing and Müller[32] evaluated the influence of solid lipid nanoparticles on the rate of release of the molecular sunscreen oxybenzone, demonstrating, in an in-vitro model, that the release rate could be decreased by up to 50% with SLN compared with an o/w emulsion. The authors investigated the role of SLN as 'active carriers' for sunscreens and demonstrated that, due to their particulate character, SLN represented a physical sunscreen on their own and therefore the incorporation of a molecular sunscreen into SLN had a synergistic effect on the protective characteristics. This feature of SLN is due to the particulate character of lipid nanoparticles. The solid particles, in fact, are stronger scatterers than the liquid emulsion droplet of an o/w emulsion.[33] In a further comparison between SLN and a conventional emulsion, the amount of molecular sunscreen can be reduced by 50% in the SLN formulation maintaining the protective level of the conventional emulsion.[18]

SLN were studied as vehicles for topical formulations containing repellents. Iscan *et al*.[26] characterized SLN-loaded diethyltoluamide, demonstrating that the choice of lipid matrix was very important to increase the repellent loading. The results obtained by Iscan *et al*.[26] were confirmed by our characterization data even though the SLN were prepared using a different method.

In a more recent study, Iscan *et al*.[34] demonstrated in an in-vitro model the suitability of diethyltoluamide-loaded SLN in reducing the release rate and skin permeation of diethyltoluamide compared with a conventional o/w formulation.

Unfortunately, all previous studies on transdermal permeation of diethyltoluamide and OMC were carried out individually, because at that time concurrent application of a repellent and sunscreen preparation was relatively infrequent among the public.

The results of our in-vitro study not only confirmed the evidence reported in the literature regarding SLN efficacy in reducing the skin permeation of the two cosmetic ingredients, but also demonstrated that diethyltoluamide and OMC concurrent application could be optimized by using lipid nanoparticles.

Conclusions

The SLN employed in our study were endowed with a high encapsulation efficacy and with a well-determined size distribution. DSC studies performed on lipid nanoparticles showed that OMC and diethyltoluamide were incorporated into a solid lipid matrix. In-vitro percutaneous absorption studies showed that OMC and diethyltoluamide loaded in solid lipid nanoparticles decreased the rate of release compared with an o/w emulsion.

Therefore it was possible to hypothesize that when incorporated in SLN the active compounds remained on the surface of the skin for longer, where they were intended to act. This study has provided supplementary evidence as regards the potential of lipid nanoparticles as carriers for topical administration of cosmetic active compounds.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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