JPP Journal of Pharmacy and Pharmacology

JPP 2010, 62: 35–45

Received March 30, 2009
Accepted October 13, 2009
DOI 10.1211/jpp/62.01.0003
ISSN 0022-3573

Research Paper

Development of an original method to study drug release from polymeric nanocapsules in the skin

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Abstract

Objectives This study aimed to investigate the distribution and release profile in the skin of a lipophilic model molecule, octylmethoxycinnamate (OMC), loaded in poly(ε-caprolactone) nanocapsules (NC) by the Franz cell method.

Methods Nanocapsules were formulated in a hydroxyethylcellulose gel and compared to the same gel containing 5% of free OMC as control. A new extraction method was used to discriminate the OMC still entrapped in the NC from free OMC released in the skin strata. The OMC extraction from the skin was performed using acetonitrile, which broke the NC, or isopropyl myristate, which kept the NC intact.

Key findings When isopropylmyristate was used to determine the OMC released from NC, the results showed that more than 80% of the OMC was released from the NC at the skin surface after 6 h, whereas only 30% was released in the stratum corneum and epidermis.

Conclusions It is suggested that the mechanism of release is different at the surface and in viable skin, probably due to the different local environments surrounding the NC. The small amount of OMC that reached the dermis was no longer encapsulated, suggesting that the NC did not reach the dermis. The viable epidermis seemed to be the limiting barrier against NC diffusion into the skin.

Keywords controlled release; nanoparticles; octylmethoxycinnamate; percutaneous absorption; UVB filters

Introduction

Particulate drug delivery systems such as liposomes,[1,2] microparticles,[3] solid lipid nanocapsules (NCs)[4,5] and polymeric NCs[6,7] have received increasing interest as carriers for topical delivery of drugs to the skin. Among them, NC delivery systems have demonstrated several advantages, such as improving the stability of the encapsulated molecule, increasing the covering effect and limiting direct contact of the drug with the skin.[8–10] These nanocarriers can modulate drug penetration of the skin by either improving or limiting its permeation; depending on the drug application, nanoencapsulation aims to either improve the transdermal delivery of the drug (for vaccines,[11,12] hormones,[13] antihypertensives such as nitrendipine[14] and so on) or to limit its penetration by improving the skin uptake and reducing systemic absorption (for example psoralen,[15] vitamin A,[16] and antiacneic agents such as tretinoin and isotretinoin[17,18]) and by favouring its accumulation in the horny skin layer (e.g. sunscreens[5,19]). Furthermore, NCs may allow a sustained release of the entrapped ingredient into the skin. Sustained-release profiles appear important for active substances that are irritating at high concentrations to supply the skin with the drug over a prolonged period of time and to reduce systemic absorption. For example, Mandawgade *et al.* have demonstrated that solid lipid nanoparticles (SLN) can slow down skin penetration of a lipophilic drug, tretinoin, which is used for acne therapy and is responsible for major side effects when administrated by the oral route.[18] Tretinoin has to be localised in the outer skin layers without systemic

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absorption. SLN tretinoin presents an interesting tool to limit tretinoin skin penetration, allow its controlled release into the skin and reduce side effects from systemic absorption.

The controlled release behaviour of drugs in the skin using nanocarriers is widely described by authors since NC allow only limited skin penetration of active compounds compared to classical formulations. However, the different studies in the literature usually describe skin permeation of the drug without knowing if it remains encapsulated or if it is released from NC. For the present study we developed a new method of extraction of the drug from the skin in order to follow drug release from the NC. The objective of this study was firstly to investigate the classical skin penetration of a lipophilic model compound, octylmethoxycinnamate (OMC) (log P = 5.96), encapsulated in polymeric NC (poly(ε-caprolactone, PCL) and secondly to determine its release behaviour from these NC in the skin using this new method of drug extraction.

OMC is widely used in sunscreen formulations because of its high extinction coefficient in the UVB region (290–320 nm).[20] It appears to be an interesting candidate as a lipophilic compound for a nanoparticulate skin delivery system for several reasons. OMC, like many other active compounds for topical delivery, needs to accumulate on the skin surface to increase its effectiveness and to limit both its toxicity towards deeper skin layers and its systemic absorption. Thus, its entrapment in NC could restrict its penetration of the skin. Moreover, OMC is a very light-sensitive molecule,[21,22] and reactive products issuing from its photodegradation may promote phototoxic or photoallergic contact dermatitis. OMC-nanocapsules (OMC-NC) were developed to circumvent these drawbacks. It has previously been demonstrated that the encapsulation of OMC in polymeric NC such as poly(lactic-co-glycolic acid),[9] polylactic acid[10] and PCL[8,23] NC improves photostability of OMC. Furthermore, the efficiency of the encapsulated OMC is not modified by encapsulation since OMC-NC maintains the same sun protection factor as non-encapsulated OMC formulations.[8,10]

Several skin permeation studies using the Franz cell method have been performed on OMC-NC formulations, showing that these NC decreased OMC accumulation in pig skin compared to classical emulsions.[19] However, in these studies only the total OMC was quantified in the various skin layers and there was no determination of the release behaviour of the OMC from the NC in the skin. To investigate the release profile of OMC from PCL-NC and to quantify the distribution of free OMC in the skin we developed a new method for skin permeation studies adapted from the classical Franz cell method. Usually, the evaluation of the amount of OMC in each skin compartment requires the complete destruction of NC by an appropriate solvent, leading to the evaluation of the total OMC content (i.e. both free and encapsulated OMC). In this study, the new method allowed extraction of OMC while preserving the NC shells and therefore enabled discrimination of free OMC released from the NC and OMC that remained encapsulated. Skin permeation of the OMC-NC formulation was compared to a classical formulation containing free OMC (OMC-gel).

Materials and Methods

Materials

PCL (Mw 65 000 Da) and phosphate buffer saline (PBS, 0.01 M, pH 7.4) were purchased from Sigma-Aldrich (St Quentin-Fallavier, France). OMC (Escalol 557) was provided by ISP (Tremblay en France, France). Span 60, hydroxyethylcellulose (HEC) (Natrosol 250 HHX) and isopropyl myristate (Crodamol IPM) were purchased from Uniqema (Emmerich, Germany), Aqualon (Dusseldorf, Germany) and Croda (Trappes, France), respectively. Oramix BG 14 (butylglucoside) and Montanox 80 were a gift from Seppic (Paris, France). Acetone and acetonitrile were provided by Carlo Erba (Val de Reuil, France).

Preparation and characterization of nanocapsules

Nanocapsule preparation

Nanocapsules were prepared using the nanoprecipitation method described by Fessi *et al*.[24] Briefly, OMC, PCL and Span 60 were dissolved in acetone. This organic phase was added under magnetic stirring to an aqueous phase composed of distilled water. The NC were instantaneously formed by the rapid solvent diffusion. Acetone and a large proportion of water were eliminated at 40°C under reduced pressure (Rotavapor RE-140, Büchi, Switzerland), and the final volume of suspension was adjusted to obtain 32.8 mg/ml of OMC.

Nanocapsule characterization

Particle size and polydispersity

The mean diameter and polydispersity of NC were assessed by quasi-elastic light scattering (QELS) using a Zetasizer 3000 (Malvern Instruments, UK). Measurements were performed at room temperature (20°C) in triplicate.

OMC encapsulation efficiency and drug entrapment in nanocapsules

Free OMC was separated from loaded NC by ultracentrifugation (Optima MAX-E, Beckman Coulter, UK) at 20 000 rev/min for 30 min. After ultracentrifugation, the supernatant was isolated and the sediment containing the NC was dissolved in acetonitrile (ACN). OMC content in the supernatant and in the NC was determined by high-performance liquid chromatography (HPLC).

Encapsulation efficiency of the OMC in NC was determined as the ratio between the actual OMC content in the NC and the total amount of OMC introduced in the preparation. The drug entrapment of OMC in the NC was expressed as the ratio between OMC retrieved from the NC and the corresponding weight of particles (milligrams per 100 mg).

Formulations for percutaneous penetration studies

Preparation of formulations

For skin permeation experiments, the suspension of NC was incorporated into a 2% HEC gel (OMC-NC-gel). The gel was prepared by dispersing HEC in purified water under agitation (Turbotest, Rayneri/VMI, France) at room temperature. The OMC-loaded NC were then incorporated into the gel under

gentle agitation in order to obtain an OMC content of 5% (w/v) in the final formulation.

OMC-NC-gel formulation was compared with a control HEC gel containing 5% (w/v) of free OMC (OMC-gel). The control gel was prepared as described above by dispersing 5% (w/v) of free OMC into the gel with 1% of Montanox 80.

Stability of OMC-loaded nanocapsules in formulations

The stability of OMC-loaded NC in the initial suspension of NC and after its incorporation in HEC gel was investigated by evaluating the OMC content in the NC and the external phase after preparation, after 24 h and after 7 days of storage at room temperature for both preparations. For this purpose, NC were isolated from the OMC-NC-gel by ultracentrifugation at 20 000 rev/min for 30 min. The OMC contents in the isolated NC and in the supernatant were then determined by HPLC after dilution in ACN.

In-vitro skin permeation studies

Skin preparation

Pig flank skin (Landras and Pietrain breeds) was used for the permeation studies. Full-thickness female or male pig skins were obtained from the animal house of the physiology laboratory (University of Lyon, France). The pig skin model is usually used to predict percutaneous penetration in humans.[25] After collection, the skin was cleaned and shaved, and the subcutaneous fat was removed. The epidermal side was cleaned with a 1% aqueous solution of sodium lauryl sulfate. The skin was washed with distilled water, blotted with soft paper and then stored flat at –20°C. The full-thickness skin (1.30 ± 0.06 mm mean thickness) was measured before use with a dial thickness gauge (Mitutoyo, Japan). Skin integrity was verified by measuring the transepidermal water loss (TEWL) with a skin station apparatus (La Licorne, France). Skin samples with TEWL values greater than 15 g/m^2 per h were discarded. The TEWL were measured in triplicate for 1 min and were all lower than 10 g/m^2 per h.

Distribution of nanocapsules in the skin

The skin samples were placed in static diffusion cells (Franz cells) with a surface area of 2.54 cm^2 and a receptor volume of 10 ml. The receptor fluid (RF) consisted of a PBS solution (0.01 M, pH 7.4) containing 4% (w/w) of Oramix BG14. The solubility of the tested compounds in the receptor fluid was examined before the experiment to ensure sink conditions (OMC solubility in RF = 6.8 μg/ml). Quantities of 2 mg/cm² of the formulations (OMC-gel and OMC-NC-gel) were applied to the skin surface. The cells were placed in a thermostatic water bath at 32°C, with horizontal agitation inside each receptor compartment to ensure the homogeneity of the receptor fluid. Two absorption times were investigated (3 and 6 h) and each experiment was performed six times. After 3 h and 6 h of exposure, the cells were removed from the water bath, and the receptor fluid was filtered (0.45 μm) and analysed by HPLC.

The stratum corneum (SC) of the treated area was removed from the viable epidermis by 19 successive tape-strippings with D-Squame adhesives (diameter 22 mm, Monaderm, Monaco).[19,26] According to the Scientific Committee on Consumer Products (SCCP),[27] the SC is fully removed by 10 to 20 strips. The formulation remaining at the skin surface (SS) was removed with a spatula and pooled with the first strip to estimate the quantity of OMC that had not penetrated into the SC. The 18 others strips (S2–S7) were pooled three by three in a vessel and dissolved with the appropriate solvent, according to the relevant protocol described below. After the tape-strippings, the distribution of OMC-loaded NC in the viable skin (viable epidermis + dermis + receptor fluid) was evaluated using two different protocols, A or B.

Protocol A

Protocol A is the classical method usually performed for skin permeation studies.[6,19] ACN, which is a solvent of OMC and also of PCL, was used as the OMC extraction solvent. The formulation remaining at the skin surface and the strips were dissolved in ACN to extract the total OMC. Once the stratum corneum was removed, viable epidermis (VE) was separated from dermis (D) by immersion in hot water (60°C) for 45 s. Each skin compartment was treated with ACN under magnetic stirring for 10 min. The resulting fluid was filtered and analysed by HPLC. This protocol allowed evaluation of the skin distribution of total OMC when derived from OMC-gel and OMC-NC-gel (OMC-encapsulated and released from NC) formulations. When Protocol A was applied to OMC-NC-gel, samples were referred to as 'BNC', standing for 'broken nanocapsules'.

Protocol B

In order to avoid the dissolution of the NC polymer shells and to discriminate the OMC released from the NC and the OMC remaining encapsulated in particles, Protocol B used isopropyl myristate (IPM). IPM is a solvent of OMC and a non-solvent of PCL. In this method the OMC contained in the strips was extracted with IPM for 10 min under magnetic stirring and the extraction fluid was centrifuged (15 min at 14 000 rev/min) to separate the OMC remaining encapsulated in the NC from free OMC released into the medium. The supernatant was diluted, filtered and analysed by HPLC.

After separation of VE and D as described above, they were respectively mixed with 1 and 2 ml of IPM for 10 min under magnetic agitation. Free OMC dissolved in IPM was separated from NC by centrifugation for 15 min at 14 000 rev/min. The supernatant was diluted and filtered, and the OMC was quantified by HPLC. This protocol was applied to the OMC-NC-gel formulation and samples were referred to as 'UBNC', standing for 'unbroken nanocapsules'.

In order to chose the appropriate solvent for Protocol B, comparative extraction studies were performed for IPM and receptor fluid (PBS 0.01 M, pH 7.4 + Oramix BG14 4% (w/w)). IPM was subsequently retained for this study since no extraction of the encapsulated OMC was observed whereas the presence of the surfactant Oramix BG 14 in the receptor fluid led to the extraction of the OMC from NC.

Analytical method for OMC quantification

OMC quantification in samples was performed by a validated HPLC method previously described by Jimenez *et al.*[19] The

HPLC setup from Waters (France) comprised a Waters 717 Plus autosampler, a Waters 600 controller pump, a reverse phase column X-Terra MS C18 (4.6 × 250 mm – 5 μm) maintained at 30°C, and a Waters 2996 photodiode array UV detector working at 310 nm wavelength. The elution with ACN : water (85 : 15 v/v) mobile phase at a flow rate of 1 ml/min gave a retention time of 9.5 min for OMC. The calibration curve for quantitative analysis was linear up to 50 μg/ml.

Calculations and statistics

The penetration results were expressed as a percentage of the applied dose. Each measurement was made six times. Statistical analysis was performed with the ANOVA test. $P < 0.05$ was considered statistically significant.[28]

Method validation

Non-extraction of OMC from nanocapsules by isopropyl myristate

Firstly, the ability of IPM to extract OMC from the NC suspension was considered. OMC is 'highly soluble' in IPM according to the EU Pharmacopoea (6th edition). As the NC shell is very thin, it was necessary to verify that OMC cannot be extracted by IPM as a surfactant does. For this purpose, an aqueous suspension of NC (5.95 mg/ml) was diluted in IPM (1/80 v/v). This suspension was vortexed for 2 min and stirred for 10 min before ultracentrifugation at 20 000 rev/min for 30 min. The OMC content in the supernatant was quantified by HPLC analysis. The results were compared with those obtained using ACN as described for the OMC encapsulation efficiency determination. The comparison between the results allowed estimation of the extractive capacity of IPM.

Ability of isopropyl myristate to extract OMC from free OMC formulations

This experiment was conducted on the OMC-gel. Free OMC content in percentage terms was measured in each formulation with either ACN or IPM as the extraction solvent. IPM is a good solvent of OMC but its higher viscosity (10 mPa.s) compared to ACN (0.38 mPa.s) could prevent good extraction from the formulations. For this purpose, 5 mg of OMC-gel was dissolved in ACN or IPM (2 mg/ml) and then agitated for 10 min under magnetic stirring at 500 rev/min. Samples were diluted and filtered, and the OMC content analysed by HPLC. The same experiment was conducted using ACN as the dilution solvent. The experiment was repeated six times.

Ability of isopropyl myristate to extract OMC from skin strata

It was necessary to evaluate the comparative abilities of IPM and ACN to extract the free OMC located in each skin compartment during the skin absorption studies. For this purpose, skin permeation studies (t = 3 h) were conducted on free OMC-gel using either ACN or IPM as the extractive solvent of OMC. Comparison was made of the OMC amount retrieved from each skin layer after 3 h with both solvents. The new method was considered as validated if the results obtained with IPM were not significantly different from those obtained with ACN (ANOVA, $P < 0.05$). The experiment was performed six times.

Results and discussion

Nanocapsule characterization

OMC-loaded NC have a mean diameter of 263 ± 2 nm and the total OMC amount retrieved in the NC suspension, using ACN as solvent to break the NC shell, was 92.5 ± 1.3%, which corresponds to 5.95 mg/ml of OMC. The amounts of OMC in the supernatant and the pellet (NC) after ultracentrifugation were 0.70 ± 0.02% and 99.3 ± 2.2%, respectively, of the total OMC. The entrapment efficiency of OMC in NC was 99.3 ± 2.2% and the drug entrapment was 61.6 ± 1.4 mg per 100 mg of NC (Table 1). These results are consistent with previous studies in which similarly high loading efficiencies were obtained for encapsulation of lipophilic substances such as OMC.[6,29]

Stability of OMC-loaded nanocapsules in formulations

The choice of the formulation containing OMC-NC was also considered. Interactions between NC and ingredients could lead to a release of OMC in the continuous phase of the vehicle. The stability of NC was therefore determined at 24 h and 7 days, both in the initial suspension and after their incorporation in the HEC gel, by evaluating the fraction of free OMC in the preparations. The results showed that the percentage of OMC rose from 0.7 to 2.5% after 7 days. After incorporation in the HEC gel, the percentage of free OMC increased to almost 15%, probably because of the effect of the mechanical stirring necessary to produce a homogeneous gel. This may be explained by the polymeric shell thickness of the NC, which can be broken under shear. This result was dramatically increased when the OMC-NC were introduced in a conventional o/w emulsion containing PEG-5 glyceryl stearate as surfactant and a mixture of mineral oil, C12–C15 alkyl benzoate and caprylic/capric triglyceride as the lipophilic phase. Indeed, the fraction of free OMC in the NC-loaded emulsion reached 52% after 24 h. This fast release can be attributed to the presence of the surfactant, which can extract OMC from the NC. Moreover, OMC has a great affinity for the lipophilic phase of the emulsion, therefore an aqueous gel was chosen as a vehicle for the topical application of OMC-NC.

Validation of the new method (Protocol B)

Non-extraction of OMC from nanocapsules by isopropyl myristate

In order to verify the ability of IPM to extract OMC from NC or otherwise, the determination of the amount of OMC retrieved from the supernatant was performed after incubation of NC in IPM. After treatment with IPM and separation of the NC by centrifugation, 0.69 ± 0.02% of the total OMC amount

Table 1 Results of nanocapsule characterisation

NC mean diameter (nm ± SD)	263 ± 2
Encapsulation efficiency (% ± SD)	96.57 ± 2.24
OMC entrapment (mg/100 mg of NC ± SD)	61.62 ± 1.43

NC, nanocapsules; OMC, octylmethoxycinnamate; $n = 3$.

was detected in the supernatant. However, when the amount of non-encapsulated OMC in the native suspension was determined without any contact with IPM, we also found $0.70 \pm 0.02\%$ of OMC in the supernatant. Since the same amount of OMC was retrieved from supernatant in both cases, this experiment demonstrates that the IPM does not extract the OMC from the NC, where it remains encapsulated.

Ability of isopropyl myristate to extract OMC from the free OMC formulations

The theoretical OMC percentage in the OMC-gel and OMC-NC-gel was 5%. The OMC percentage in the OMC-gel was estimated using IPM and ACN as solvent. These percentages were, respectively, $5.14 \pm 0.05\%$ and $5.13 \pm 0.06\%$ ($n = 6$). It can therefore be concluded that IPM is a good extraction solvent for quantification of free OMC in these formulations.

Ability of isopropyl myristate to extract OMC from skin strata

Before skin permeation experiments can be conducted, the ability of IPM to extract OMC from the skin strata has to be determined. For this purpose, a skin absorption study was performed on the OMC-gel using either Protocol A or Protocol B. Results are presented in Figure 1. Whatever the protocol used, the same amounts of OMC were retrieved from each skin compartment. Indeed, as shown in Figure 1, there was no statistical difference between the percentage of OMC at the skin surface, in the strips or in the viable skin layers (VSL = VE + D + RF) for both experiments. It can be concluded therefore that both solvents, classical (ACN) and new (IPM), can be used to quantify free OMC in the different skin layers. IPM appears to be a good solvent for the quantification of the OMC released from the NC in the skin layers.

The results of the three experiments described above allowed us to conclude the validity of our new method of

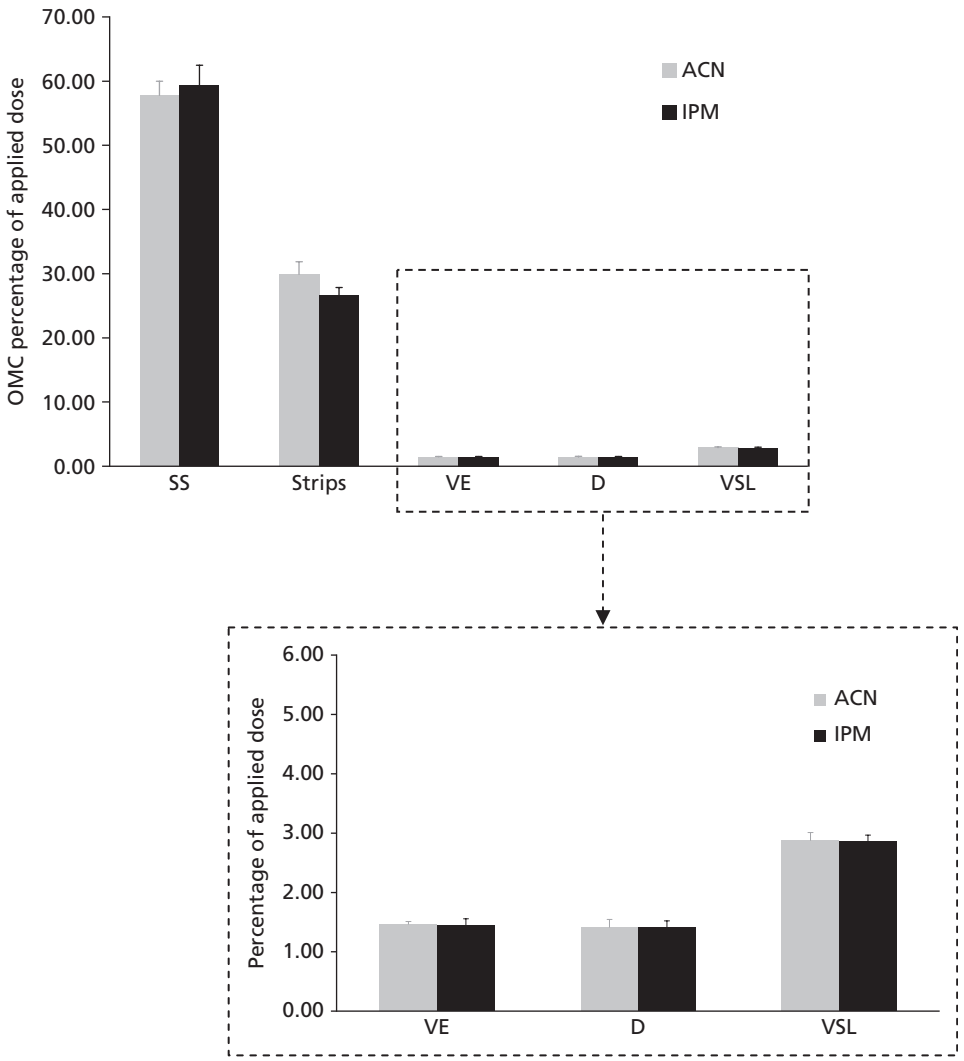

Figure 1 Protocol B validation. Percutaneous penetration of octylmethoxycinnamate for the control gel (OMC-gel) at 3 h using either acetonitrile (ACN) or isopropyl myristate (IPM) as extractive solvent. Percentages of the octylmethoxycinnamate (OMC) applied dose (% ± SD) in various skin layers: skin surface (SS), stratum corneum (SC), viable epidermis (VE), dermis (D) and viable skin layers (VSL = VE + D + RF receptor fluid), $n = 6$.

using IPM as extraction solvent for OMC in skin permeation experiments.

Skin permeation studies

The main purposes of the percutaneous penetration experiments were, firstly, to investigate the comparative distributions of free and encapsulated OMC in the skin using a gel as vehicle (OMC-gel and OMC-NC-gel) and, secondly, to investigate the release behaviour of OMC from NC and its deposition in the skin layers as a function of time. The new extraction method using IPM was designed to estimate the OMC released from the NC inside the skin. The results of skin permeation studies for both protocols are shown in Table 2.

In-vitro distribution of total OMC in skin layers

The distribution of OMC in the skin after application of an OMC-NC-gel (BNC) or OMC-gel was investigated with the Franz cell method using ACN. In this way, the total OMC amount that penetrated (Protocol A) the skin was quantified. The experiments were stopped after 3 and 6 h, which roughly corresponds to spending one half day (3 h) or the entire day (6 h) under UV exposure. As shown in Figure 2, the penetration of OMC 3 h after application of the OMC-gel was low. Only 1.40% of the applied dose penetrated the VSL, with the remainder remaining at the skin surface (~70%) and the stratum corneum (almost 20%) (Table 2). No OMC was found in the receptor fluid after 3 or 6 h, mainly because of the lipophilic property of OMC (log P = 5.96), which diffuses very slowly in the hydrophilic environment of the deeper skin layers.[30] This result is in accordance with results obtained by Jimenez, Alvarez and Potard who did not find OMC in receptor fluid after 3, 6 or 16 h or even after 24 h under some conditions.[6,19,31]

A significant increase of the absorbed amount was noticed after 6 h in the skin compartments (SC, VE and D) since 4.86% of the applied dose was recovered in the VSL (against 1.40% after 3 h). The amount found in the SS decreased from around 70 to 59% between 3 h and 6 h, whereas it increased from 18 to 25% in the stratum corneum. The difference was larger in the first three tapes (S2) (Table 3). The mean quantity accumulated in the SC after 3 or 6 h was around 15–20 μg/cm². This result is in accordance with those reported by Potard *et al.* and Benech-Kieffer *et al.*, who found the same OMC amount of 15–20 μg/cm² whatever the exposure time.[30,31]

The results of penetration studies of OMC from the OMC-NC-gel (BNC) are shown in Figure 3. The encapsulation of OMC in the NC did not modify the OMC distribution in the skin after 3 h, since no significant differences were found between OMC distributions using either OMC-gel or OMC-NC-gel. However, after 6 h, a significant difference was observed in the dermis (Table 2). The mean amount recovered in this compartment was higher in the case of the control gel (1.67% versus 0.43%). Consequently, the total amount which permeated the VSL after 6 h was significantly higher in the case of the OMC-gel (4.86% versus 2.99%).

The OMC distribution in the skin layers for BNC was quite similar between 3 and 6 h, except for the mean amount accumulated in the VE, which increased from 1.26 to 2.56% (Figure 3). Interestingly, this increase had no impact on the amount recovered from the dermis or receptor fluid. It seems that the VE limits the diffusion of OMC. This result is in accordance with the preliminary study carried out by Jimenez *et al.*, who showed that the major part of the OMC was found in the epidermis when NC-based formulations were used.[19] They studied the distribution of OMC after 3 and 24 h with OMC-loaded NC incorporated in a conventional oil/water (o/w) emulsion and with the same emulsion containing 5% of free OMC as control. The comparison of their penetration experiments with the present study showed the influence of the 'final vehicle' containing the NC on the OMC distribution in the skin. The distribution of OMC after 3 h using these different NC-loaded vehicles (a conventional emulsion in the case of Jimenez *et al.* and a gel in the present study) was quite similar. Similarly, 1.76% of the applied dose was recovered from the VSL in this study against 1.69% for the same compartment for Jimenez *et al.* However, in the upper skin levels (SC + SS), although the total amount recovered was larger than 80%, the distribution of OMC between SC and SS was different: Jimenez *et al.* found 8.5% of the total amount (SS + SC) in the SC whereas we found around 22% in the SC for NC-loaded formulations.

Interestingly, a significant difference was noticed at 3 h between the skin distribution in the control emulsion studied by Jimenez *et al.* and the control gel used in this study. The global amount recovered from the SS + SC was greater than 80% (as previously noticed for OMC-NC-formulations). However, the ratio between SS and SC was very different:

Table 2 In-vitro skin distribution of octylmethoxycinnamate

Skin portion	OMC-gel		OMC-BNC		OMC-UBNC	
	3 h	6 h	3 h	6 h	3 h	6 h
Surface skin	69.78 ± 1.06	58.80 ± 2.30	65.08 ± 10.49	60.30 ± 1.43	51.89 ± 9.77	54.58 ± 4.39
Stratum corneum	18.16 ± 0.06	25.00 ± 1.19	18.60 ± 4.21	20.65 ± 0.90	8.81 ± 0.79	6.17 ± 0.92
Viable epidermis	1.04 ± 0.06	3.19 ± 0.22	1.26 ± 0.15	2.56 ± 0.39	0.33 ± 0.02	0.68 ± 0.09
Dermis	0.36 ± 0.03	1.67 ± 0.21	0.50 ± 0.20	0.43 ± 0.06	0.29 ± 0.07	0.26 ± 0.10
Viable skin layers	1.40 ± 0.06	4.86 ± 0.10	1.76 ± 0.31	2.99 ± 0.40	0.62 ± 0.12	0.94 ± 0.17
Receptor fluid	0.00	0.00	0.00	0.00	0.00	0.00
Cumulative amount	89.34 ± 2.31	88.66 ± 1.52	85.44 ± 7.72	83.94 ± 2.26	—	—

Values are mean percentage of applied dose ± SD, $n = 6$. Viable skin layers = viable epidermis + dermis + receptor fluid. OMC, octylmethoxycinnamate; BNC, broken nanocapsules; UBNC, unbroken nanocapsules.

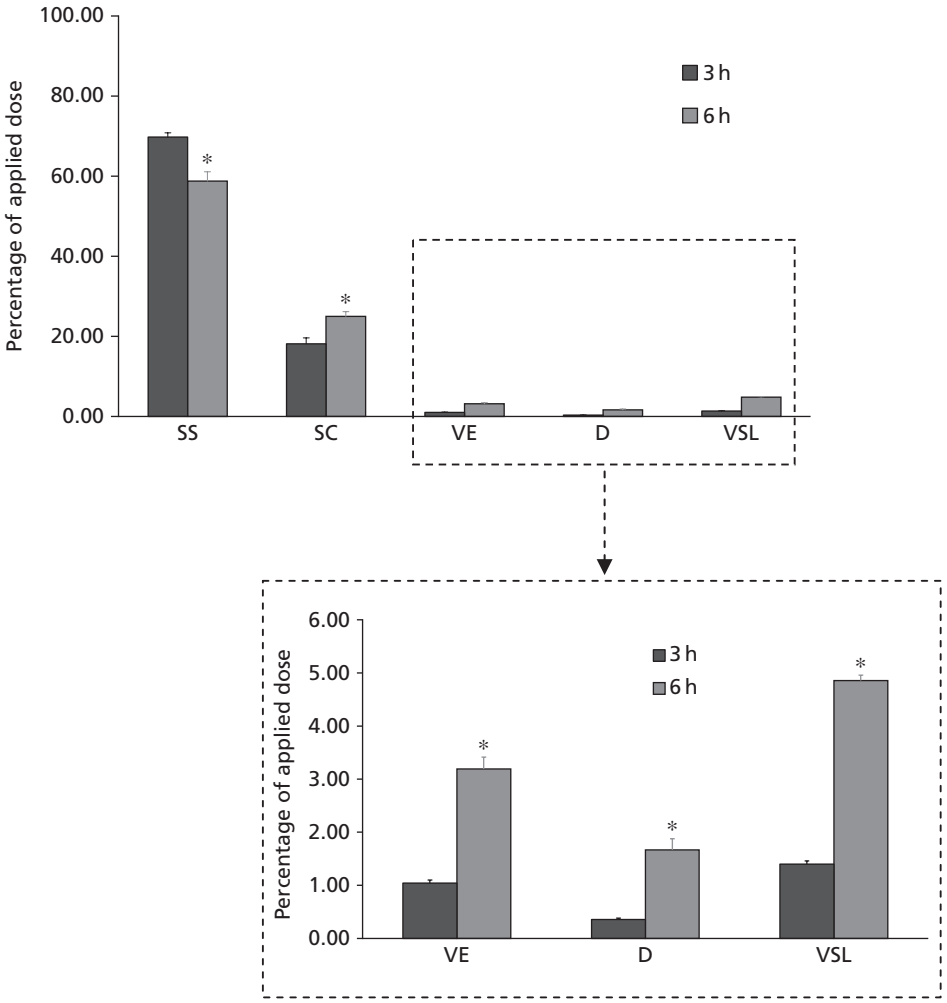

Figure 2 Percutaneous penetration of octylmethoxycinnamate for the control gel (OMC-gel) at 3 and 6 h. Percentages of the octylmethoxycinnamate (OMC) applied dose (% ± SD) in various skin layers: skin surface (SS), stratum corneum (SC), viable epidermis (VE), dermis (D) and viable skin layers (VSL = VE + D + RF receptor fluid) (n = 6). *P < 0.05, 6 h compared with 3 h.

around 21% was distributed in the SC for the gel whereas the ratio was almost 50 : 50 with the emulsion used by Jimenez *et al.* It can be concluded that the control gel is a better vehicle than their emulsion because it improves the OMC accumulation at the skin surface. The degree of penetration of sunscreens into the skin depends greatly on the vehicle in which they are incorporated.[32–35] This observation has been previously reported by Montenegro *et al.*, who demonstrated that the distribution of OMC in the skin varied significantly depending on the nature of the silicone emulsifier used in the

Table 3 In-vitro skin distribution of octylmethoxycinnamate in the stratum corneum

Stratum corneum portion	OMC-gel		OMC-BNC		OMC-UBNC	
	3 h	6 h	3 h	6 h	3 h	6 h
S2	13.56 ± 0.95	17.82 ± 1.29	12.76 ± 3.07	13.79 ± 0.98	6.17 ± 0.85	4.21 ± 0.75
S3	1.97 ± 0.51	3.58 ± 0.62	2.81 ± 0.70	2.93 ± 0.22	1.19 ± 0.29	0.77 ± 0.11
S4	1.40 ± 0.23	1.74 ± 0.52	1.29 ± 0.33	1.82 ± 0.25	0.48 ± 0.05	0.33 ± 0.14
S5	0.65 ± 0.15	0.83 ± 0.10	0.87 ± 0.18	0.91 ± 0.06	0.44 ± 0.08	0.48 ± 0.10
S6	0.59 ± 0.16	0.66 ± 0.07	0.52 ± 0.10	0.62 ± 0.10	0.36 ± 0.07	0.30 ± 0.06
S7	0.00 ± 0.00	0.38 ± 0.05	0.35 ± 0.07	0.59 ± 0.13	0.17 ± 0.06	0.09 ± 0.04
SC (S2–S7)	18.16 ± 1.48	25.00 ± 1.19	18.60 ± 4.21	20.65 ± 0.90	8.81 ± 0.79	6.17 ± 0.92

Values are mean percentage of applied dose ± SD, n = 6. S2 to S7 represent the 21 tapes strips, from skin surface to viable epidermis, pooled 3 by 3. OMC, octylmethoxycinnamate; BNC, broken nanocapsules; UBNC, unbroken nanocapsules.

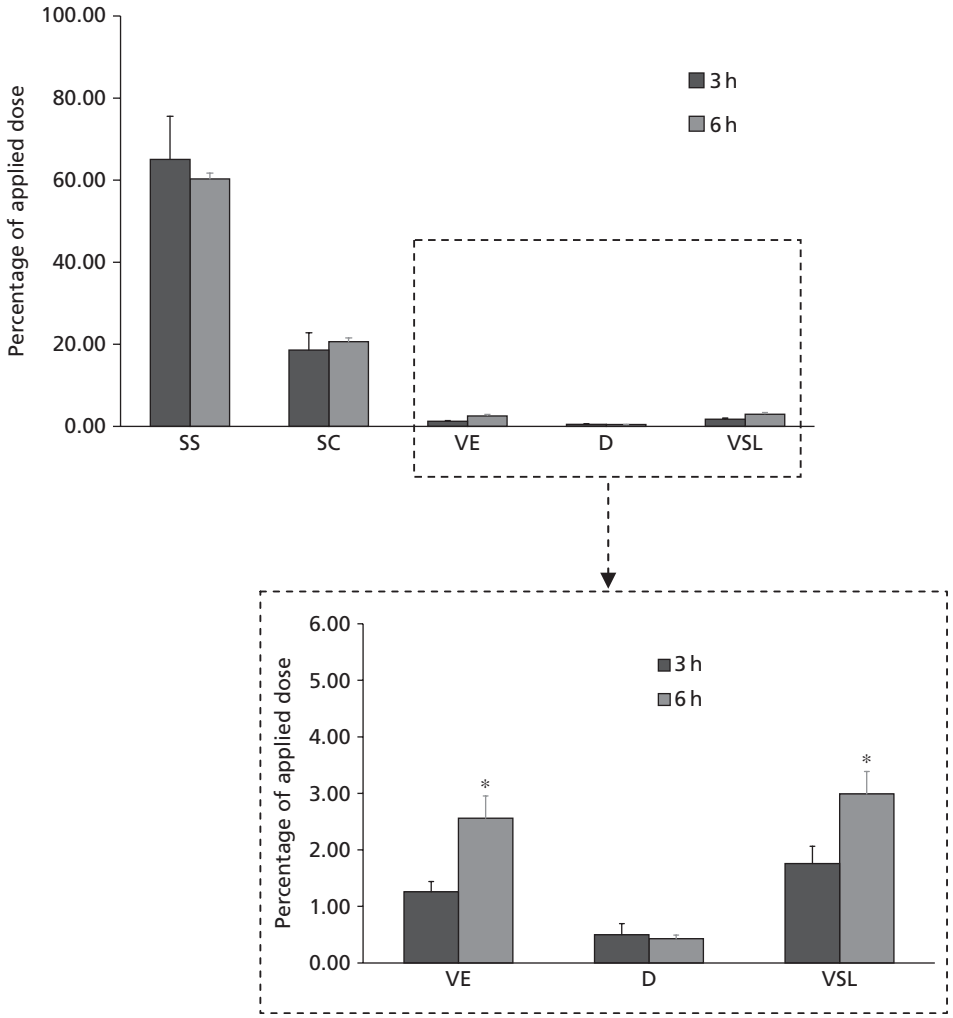

Figure 3 Percutaneous penetration of octylmethoxycinnamate for broken nanocapsules (Protocol A) at 3 and 6 h. Percentages of the octylmethoxycinnamate (OMC) applied dose (% ± SD) in various skin layers: skin surface (SS), stratum corneum (SC), viable epidermis (VE), dermis (D) and viable skin layers (VSL = VE + D + RF receptor fluid), $n = 6$. *$P < 0.05$, 6 h compared with 3 h.

preparation.[33] In the same way, Yener *et al*. have demonstrated *in vitro* with Franz diffusion cells that OMC-loaded microspheres formulated in an oleaginous cream or a Carbopol gel released OMC in greater quantity than an o/w emulsion.[35] However, the OMC amounts released from the OMC-loaded microspheres in the gel or the oleaginous cream were always lower than the amounts released from the same vehicles containing free OMC.

To summarise the present study, after 3 h the skin distribution did not differ between the OMC-gel and the OMC-NC-gel, whereas the distribution described by Jimenez *et al.* for the OMC-emulsion and the OMC-NC-emulsion was very different.[19] It seems that the gel is the limiting factor for UV-filter permeation because it slows down OMC skin penetration. Conversely, it is obvious that the penetration rate limiting factor in the study of Jimenez *et al.* is the carrier.[19,36] The same kind of difference appeared only after 6 h in the present case, probably because the rate of penetration depends more on the vehicle. Regarding the formulations, it can be stated that the emulsion is a 'faster vehicle' for delivering OMC in the skin due to its strong amphiphilic character.

In our study, the NC accumulated mostly in the epidermis after 6 h. OMC was mainly located in this compartment but it did not cross the VE level. This behaviour is in accordance with previous studies performed by Alvarez-Román *et al*., who reported that the use of particulate drug carriers such as NC appears to improve drug residence in the skin without increasing transdermal transport.[6,37] The same observations were made with other lipophilic molecules, such as retinol (log P = 5.86, close to OMC), using solid lipid NC.[38]

More recently, several authors investigating the skin penetration behaviour of particulate delivery systems have concluded that the diffusion pathway of NC is mainly via the skin appendages (follicular pathway).[39–42] For example, visualization of fluorescent NC of 20 nm, 200 nm or 400 nm by confocal microscopy reveals that NC accumulate preferentially in the follicular openings and in the upper skin layers (10 μm).[7,8] The authors of that paper suggest that the enhanced delivery into the skin of lipophilic

compounds such as OMC from NC formulations is probably not caused by the penetration of the particles but rather to an efficient release of the lipophilic substances into the SC. Furthermore, Lademann *et al.* observed the follicular storage behaviour of NC in human skin for up to 10 days, while non-particle forms were detected only for 4 days. Penetration and accumulation of NC in the follicular ducts occurs in a time- and size-dependent manner.[7] In the same way, Toll *et al.* and Knorr *et al.* recently described the follicular pathway as a mean for targeted drug delivery using NC.[41,43] This follicular pathway can explain NC accumulation in the VE level and then the apparent barrier effect of the epidermis.

Discrimination of free and encapsulated OMC in skin layers

The extraction of OMC with isopropylmyristate (Protocol B) allowed the titration of the free OMC only, therefore the comparison of results recorded according to Protocols A and B allowed evaluation of the relative contributions of the encapsulated- and free-OMC transport and the penetration of the NC as an indirect consequence.

At the skin surface, the OMC released from the NC represented around 52% of the applied dose after 3 h and 54.6% after 6 h (Table 2). Comparatively, for BNC, the total amounts of OMC remaining at the surface after 3 and 6 h were 65.1 and 60.3%, respectively. This result indicates that almost 80% of OMC remaining at the skin surface after 3 h was in the free form (i.e. OMC released from NC) and that this quantity rose to 90% after 6 h (Table 4).

In the SC, 18.6% of the applied dose of OMC was accumulated after 3 h and rose to 20.6% after 6 h when Protocol A was applied. However, only 8.8% of the applied dose of OMC was in the released form in the SC after 3 h and 6.2% after 6 h (OMC-UBNC). This OMC released from NC represented around 47% of the total amount of OMC retrieved in the SC with NC formulations at 3 h and 30% at 6 h.

The distribution of free OMC evaluated with Protocol B was quite similar between 3 and 6 h (Figure 4), except in the VE, as noted previously for BNC (0.33% after 3 h versus 0.68% after 6 h). However, if the amount of OMC in the VE increased between 3 and 6 h in both cases (UBNC and BNC), the release profile of OMC seemed to be constant after 3 or 6 h, with 26% of the total OMC released in the VE in both cases (see Table 4 for the ratio of OMC-UBNC to OMC-BNC). The significant difference observed between 3 and 6 h in the VE for BNC and UBNC experiments (Table 2) indicates that OMC was still entrapped in intact NC, therefore a sustained release was observed, which delayed the OMC permeation in the D or the RF. In the D, after 3 and 6 h, no significant differences were noticed between the OMC quantified for BNC and UBNC. Moreover, no statistical differences were observed between 3 and 6 h in the D for either protocol. These results are interesting because they indirectly show the absence of NC in the D, since it appears that the OMC quantified in this compartment is free OMC only. In the RF, whatever the protocol applied, no OMC was detected, therefore, in this experiment, the VE seems to be the limiting barrier for the diffusion of NC in the dermis. Furthermore, the encapsulation in NC is an effective way to slow down the release of OMC in deeper skin levels.

The results of these experiments suggest that the release profiles of OMC from NC are not similar in the different skin layers investigated. The release profile is higher near the skin surface (from 80 to 90% of OMC released) and lower in the viable skin (~30–35%) (Table 4). A high release near the surface improves protection against UV-radiation, whereas the low release verified in the deeper layers strongly limits the contact of OMC with the viable skin and therefore its toxic effect towards these tissues. The differences observed in the permeation profiles can be attributed to the local environment. At the surface, the NC are formulated in a gel and deposited on the skin at 32°C. Due to the evaporation of water at the SS, after 3 h the NC were in a dry environment and could aggregate, leading to their breakdown and consequently the release of OMC. NC that penetrated the skin were isolated and consequently could remain intact. Moreover, at the SS and even in the SC, the local environment is rather lipophilic and the water content is low, between 10 and 13% by weight. In the VSL, the water content increases, to reach almost 70% in the dermis, a level that could limit OMC diffusion from NC to skin compartment. It can be suggested, moreover, that the breakdown of the OMC-NC in such an environment takes place according to a different mechanism, possibly by means of biodegradation of the PCL shell, which is very slow and leads to a sustained release in the VSL of a lipophilic molecule such as OMC.

Conclusions

Skin penetration experiments of OMC-loaded NC incorporated in different vehicles have shown that these particulate delivery systems are promising for the dermal administration

Table 4 Estimation of the octylmethoxycinnamate released from nanoparticles using the new protocol

Skin portion	OMC-BNC		OMC-UBNC		Release estimate[a]	
	3 h	6 h	3 h	6 h	3 h	6 h
Surface skin	65.08 ± 10.49	60.30 ± 1.43	51.89 ± 9.77	54.58 ± 4.39	79.7%	90.5%
Stratum corneum	18.60 ± 4.21	20.65 ± 0.90	8.81 ± 0.79	6.17 ± 0.92	47.4%	29.9%
Viable epidermis	1.26 ± 0.15	2.56 ± 0.39	0.33 ± 0.02	0.68 ± 0.09	26.2%	26.6%

The results in columns 1–4 represent the mean percentage of the OMC applied dose ± SD as in Table 2. [a]Estimate of the percentage of octylmethoxycinnamate released from NC: ratio of OMC-UBNC to OMC-BNC. OMC, octylmethoxycinnamate; BNC, broken nanocapsules; UBNC, unbroken nanocapsules.

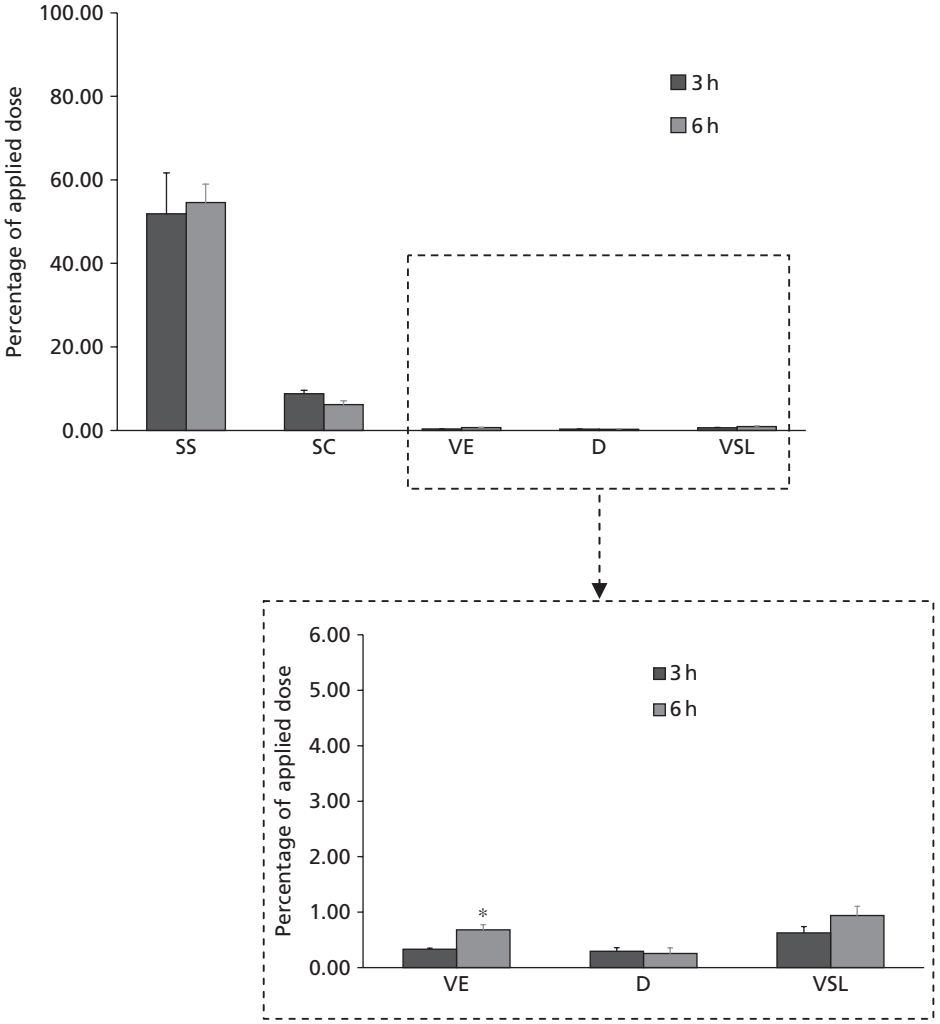

Figure 4 Percutaneous penetration of octylmethoxycinnamate for unbroken nanocapsules (Protocol B) at 3 and 6 h. Percentages of the octylmethoxycinnamate (OMC) applied dose (% ± SD) in various skin layers: skin surface (SS), stratum corneum (SC), viable epidermis (VE), dermis (D) and viable skin layers (VSL = VE + D + RF receptor fluid), $n = 6$. *$P < 0.05$, 6 h compared with 3 h.

of lipophilic molecules. The influence of the type of vehicle was an important factor considered in this study, which demonstrates that a gel form allows a faster release of OMC on the skin than other formulations.

The new methodology developed in this study has highlighted the sustained release behaviour of OMC from the NC in each skin layer. OMC release was more important in the upper skin layers, where UV radiation has to be absorbed by sunscreens. Conversely, the NC limited release of OMC in the deeper skin layers, minimising the toxicity of sunscreens. This effect can be explained by the small size of the NC and by the hydrophobic character of the PCL. Both factors contributed to the accumulation of NC at the skin surface.

Declarations

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

Funding

This work was supported by Rede Nanocosméticos-CNPq/MCT-Brazil, PROPESQ-UFRGS and Rede Cooperativa Brasil França-França em Processos de Produção de Nanopartículas para Aplicação em Saúde (CNPq).

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