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4-METHYLBENZYLIDENE CAMPHOR MICROSPHERES: RECONSTITUTED EPIDERMIS (SKINETHIC®) PERMEATION AND DISTRIBUTION

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Abstract

Objective The UV filter 3(4-methylbenzylidene) camphor (4-MBC) is a common ingredient in sunscreen cosmetic products. However, different "in vitro" and "in vivo" studies suggest that 4-MBC can cause endocrine disrupting effects. Therefore, there is a need for new systems able to minimize the skin penetration of this UV filter. The aim of this study was to evaluate cutaneous permeation and distribution, through and into EPISKIN reconstituted epidermis (RE) from an O/W emulsion containing 4-MBC free or encapsulated in polymeric substantive microspheres.

Methods Microspheres containing 4-MBC were prepared using the emulsification-solvent evaporation method and characterized for shape and surface morphology and encapsulation efficiency. O/A emulsions containing sunscreen free or encapsulated in microspheres were undergone to permeation tests through RE using vertical diffusion cells. At the end of the in vitro permeation experiments, the skin was subjected to tape stripping procedure to separate stratum corneum from viable epidermis. Each part was properly treated to extract the sunscreen retained and subject it to quantitative analysis.

Results The encapsulation of the sunscreen in the microspheres remarkably reduced the permeation of 4-MBC and increased its retention on the skin surface where its action is more desirable.

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Conclusions The results of this study confirm the validity of substantive microspheres as an ideal formulation candidate to use in sunscreen preparation since they appear minimising its systemic uptake and the potential associate toxicological risks. Therefore more of the active sunscreen remains on the surface of the skin where it is intended to act and a higher activity it will esplicate.

Key Words

Microspheres; 4-Methylbenzylidene camphor; Reconstituted epidermis; Permeation; Skin distribution

Introduction

Sunscreen products can be effective in preventing sunburn, the damage linked to photoageing, some type of skin carcinoma and can protect against induced photo-immunosuppression [1-4] To avoid harmful effect of sun exposure health authorities emphasised the importance of the link between the correct application of sunscreen products and the efficacy of the sun protection factor claimed. In particular, frequent application and re-application after contact to water are recommended [5]. This leads to use of large amount of sunscreens and a possible systemic absorption. Since these preparations are often applied on large skin areas even low penetration rates can cause significant amount of chemical UV absorber to enter the body [6]. Since sunscreens are intended to act on the surface of the skin with the site of action restricted to the skin surface or to the uppermost part of the stratum corneum, they should penetrate as little as possible into the viable epidermis, the dermis and into the systemic circulation.

The degree of penetration depends strongly on the physicochemical properties of the active compound and the nature of the vehicle in which the sunscreen is applied, e.g. polarity of the solvent, particle size, type of vehicle [7, 8]. In fact it has been demonstrated that penetration into skin, permeation through skin and retention of UV filters in the skin from topical products can differ significantly among formulations used [9]. Jiang et al. [10] found that diffusion of UV filter across the epidermis varied significantly with formulation type.

Therefore, a safer application of sunscreens is needed that allows for the achievement of maximal skin protection from UV radiation concurrent with minimal penetration of these actives into the skin. Thus the development of suitable products which prevent penetration of the sunscreens into the skin is a challenge for manufactures of cosmetic products. For this purpose Microencapsulation of sunscreens has been considered a promising approach in photoprotection because it is safer (because of the lack of percutaneous absorption and the reduced photodegradation) and more effective (because of the lasting

effect on the skin and stability of the sunscreen). Microspheres [11, 12], micro- and nanocapsules [12-15], lipid particles [16-19], hydrotalcite-like anionic clays [20] and inclusion complexes [21-24] have all attracted interest in recent years as vehicles for sunscreens. Microparticulate carriers were prepared to embed UV chemical blockers using both hydrophilic (chitosan and gelatine) and hydrophobic (polymethylmetacrylate), polymers [25] Encapsulated sunscreens offer various advantages: better photostability and substantivity, less contact with skin, homogeneous skin distribution.

The UV filter 3-(4-methylbenzylidene) camphor (4-MBC) is a common ingredient in sunscreen cosmetic products. However, various opinions, reported by European Scientific Committee for Consumer Products, are focused on the suitability of the use of this filter [26-28].

Recent investigations have shown that in rat as well as in man, 4-MBC is systematically absorbed after topical application [29-31]. Some studies showed that 4-MBC can penetrate through human skin and be excreted in urine [32]. Different “in vitro” and “in vivo” studies suggest that 4-MBC can cause endocrine disrupting effects [33]. Some “in vitro” experiments showed that 4-MBC increased cell proliferation in MCF-7 breast cancer cells and an estrogen antagonist blocked its proliferative effects [34]. Regarding “in vivo” studies they suggested that exposure of rats to 4-MBC affected the regulation of estrogen target genes [35], interfered with sexual dimorphic gene expression in brain in a sex- and region-specific manner [36], had an influence on the development of male reproductive organ and reproduction [37], and promoted prostate growth [38].

In addition there are many studies informing a strong anti-osteoporotic effects after cronic application [39]. Hamann [40] observed that 4-MBC was a potent inhibitor of the pituitary-thyroid-axis, due to the fact that TSH serum levels were significantly elevated, and the weight of the thyroid glands was remarkably increased. Therefore, there is a need for new systems able to minimize the skin penetration of 4-MBC. Some studies suggest that the encapsulation of this filtering agent in different delivery systems can reduce the percutaneous absorption [14, 21, 41].

The aim of this study was to evaluate the 4-methylbenzylidene camphor cutaneous permeation and distribution, through and into reconstituted epidermis (SKINETHIC®, RE), from an O/W emulsion containing 4-MBC free or encapsulated in polymeric substantive microspheres.

Materials and methods

Materials

Materials were obtained from commercial suppliers and used as received. The following were used to prepare microspheres: co-polymers of poly(ethylacrylate, methylmethacrylate) and trimethyl aminoethyl methacrylate chloride (Eudragits RS 100, Mol. Wt. ~150,000, Röhm Pharma Polymers), polyvinyl alcohol (PVA: Mol. Wt. 13-23,000 and 22,000, 87-89%, Fluka), 4-MBC (Parsol 5000, DSM). The excipients for the emulsion were obtained as follows: tri- $C_{12\text{-}13}$ alkyl citrate (Cosmacol ECI) from Sasol Italy S.p.A. (Milan, Italy); cetearyl glucoside, cetearyl alcohol (Montanov 68) from Seppic S.A. (Paris, France); potassium cetyl phosphate (Amphysol K) from ResPharma (Trezzo sull'Adda, Milan, Italy). Sodium dodecylsulfate (SDS) and polyoxyethylene-20-oleyl-ether (Brij®98) were purchased from Sigma-Aldrich (Sigma-Aldrich S.r.l., Milan, Italy). All other chemicals and solvents were of analytical grade.

Preparation of microspheres

Microspheres containing 4-MBC were prepared using the emulsification-solvent evaporation method [42], employing a synthetic co-polymer of poly(ethylacrylate, methyl methacrylate) and trimethyl aminoethyl methacrylate chloride and 4-MBC (80% filter:polymer w/w).

The polymer (1.0 g) and the filter were dissolved in 10 mL of dichloromethane (DCM) (10% v/v). Separately, an aqueous solution was prepared dissolving PVA (1% w/v) in 100 mL of water. The organic phase was slowly injected, using a syringe, into an aqueous phase containing PVA as dispersing agent, and emulsified. The O/W emulsion was maintained under continuous magnetic stirring for about 24h, to ensure complete evaporation of the organic solvent and formation of the microspheres. At the beginning, the critical step of emulsification was carried out at room temperature but subsequently, to avoid premature solvent evaporation, an ice-bath at 4-5°C was used. Finally, the hardened microspheres were recovered by centrifugation (3600 rpm, 30 min) and washed three times with deionized water. The microspheres, suspended in a small amount of deionized water, were frozen and lyophilized, or collected by filtration under vacuum and dried at room temperature in a desiccator under reduced pressure for 48h.

Unloaded microspheres, used as reference, were prepared following the procedure described above. All batches of microparticles were produced at least in triplicate.

Characterization of microspheres

Microspheres were characterized for shape and surface morphology by scanning electron microscopy (SEM) (XL 20 SEM, Philips, The Netherlands and XL 30 SEM, Philips, CDU Leap Detector). Particle size was analysed with an Accusizer 770 (Particle Sizing System, Santa Barbara, CA, USA). Microsphere loading was investigated by UV analysis: 2.5 mg of 4-MBC-loaded microspheres, accurately weighed, were dissolved in a mixture of THF/H_2O (9:1 v/v), under sonication, to a filter concentration in the range of 5-30 $\mu g \cdot ml^{-1}$ (a calibration curve of 4-MBC in THF/H_2O 9:1 v/v was used as reference). The sunscreen concentration was determined by measuring the absorbance at 297 nm (Varian Cary 1E ver.3.03) in quartz cuvettes (path length 1 cm). Unloaded microspheres gave insignificant absorbance at the same wavelength. The amount of the filter in the microparticles was expressed as a percentage of the total weight of the sample. Means of three assays were reported. Encapsulation efficiency was calculated as the percentage of the experimental and the theoretical loading.

Evaluation of 4-MBC release from microspheres

To compare free and encapsulated filter release from the vehicle, two different receiver fluids were used: hydrophilic, using phosphate buffer, pH 5.9, employing a dialysis bag [43], and lipophilic, using caprylic/capric triglyceride and Strainer cells [44].

The hydrophilic receiver fluid was a buffered solution (pH 5.9, NaH_2PO_4 + Na_2HPO_4); it was chosen to mimic the skin pH. The filter or microspheres were exactly weighed and placed in a dialysis bag, previously hydrated for 24 hours and washed with distilled water. The bag was immersed in 100 ml of the buffered solution, under magnetic stirring. This acceptor phase was periodically sampled and assayed spectrophotometrically (1 ml of ethanol was added to 1 ml of receiving buffer). The same volume of fresh receiving buffer was added to replace the samples. The analyses were conducted on 100 mg of free filter and, in the same way, on the sample of 4-MBC-loaded microsphere, prepared with 80% of sunscreen. After analysis of the release, the microspheres were recovered and spectrophotometrically assayed in the same operating conditions as for loading determination.

The lipophilic receiver fluid was caprylic/capric triglyceride (Myritol 318) because 4-MBC is soluble in it, while the microspheres remain intact. In this case two O/W emulsions (EM-1 and EM-2, which composition will be shown below), selected as model formulations, were employed as vehicles for the free or encapsulated filter. Release was evaluated using a modification of the method proposed by Casolaro et al. [44]. Briefly, 2.0 g of the sample was layered on the bottom of a Strainer cell, and placed in close contact with the surface of 20 ml of Myritol 318 at room temperature, under magnetic stirring (150 rpm)

for max 24h. Strainer cells are sterile sieves made of strong 100-micron nylon mesh. One hundred µl of the acceptor fluid were taken at each time point (0, 5, 15, 30, 60, 120, 240, 420 and 1440 min), diluted with 900 µl ethanol (v/v) and analysed by UV spectrophotometry (λ_{max}=298 nm). A solution of Myritol 318:ethanol (1:9 v/v) served as reference. After each withdrawal, 100 µL of fresh receiver fluid was added to keep the volume constant. Analyses were run in triplicate; means and standard deviations were calculated.

Formulations

In this study two O/W emulsions containing 4-MBC were prepared: one containing 3% w/w of the free sunscreen (EM-1), the other the corresponding amount of sunscreen incorporated in microspheres (EM-2). The quantitative composition (% w/w) is reported in table 1.

To prepare the emulsions, Montanov® 68 and Cosmacol® ECI (phase A) were melt at 60-70°C and Amphisol® K was dissolved in water at 70°C (phase B). Then, 4-MBC or 4-MBC-loaded microspheres were added to phase A and undergone to sonication for some seconds. Finally the last dispersion/solution was added to phase B under energetic stirring to obtain final emulsions.

Skin model

As tissue model, EPISKIN reconstructed human epidermis (RE) (EPISKIN/L/13, reconstructed human epidermis large, age day 13, tissue surface: 1.07 cm^2) supplied by SkinEthic Laboratories (Nice, France) was used. EPISKIN is an in vitro reconstructed human epidermis from normal human keratinocytes cultured on a collagen matrix at the air-liquid interface. This model is histologically similar to the in vivo human epidermis. As indicated by the manufacturer the human tissue models were removed from the agarose-nutrient solution in the shipping multiwell plate immediately after arrival and placed in a plate previously filled with SkinEthic maintenance medium at room temperature under a sterile airflow. Then culture dishes were put in the incubator at 37°C, 5% CO_2, and saturated humidity. Permeation experiments started after overnight incubation.

In vitro permeation studies

Permeation tests through RE were carried out using a system (Harvard Apparatus Inc., Holliston, MA, USA) consisting of six thermostated cells with the lower donor and the upper receptor chambers separated

by RE, the stratum corneum facing the donor chamber. 1.5 ml (corresponding to 1.4 $g \cdot cm^{-2}$) of each formulation was placed on the epidermal surface. The receiving phase consisted of 2.0 ml of isotonic, 66.7 mM, pH 7.4 phosphate buffer solution (PBS) maintained at 37 ºC. 1.0% Brij 98 was added to PBS to increase the solubility of the sunscreen. The solubility of 4-MBC in the receiving phase was 0.13 $mg \cdot ml^{-1}$.

At predetermined intervals of time (0.5, 1, 1.5, 2, 3, 4, 5 hours), 1.0 ml samples of the receiving phase were withdrawn for HPLC analysis and replaced with the same volume of fresh fluid. All experiments lasted 5 hours and were repeated four times.

Skin distribution studies

At the end of the in vitro permeation experiments, the skin was removed from the diffusion cells**,** rinsed with distilled water to eliminate excess formulation from the skin surface and gently wiped with cotton-wool tampons.

The skin specimens were then positioned on a home-made specific apparatus delimiting the drug-exposed surface. Afterwards the skin was subjected to tape stripping procedure to separate stratum corneum (SC) from viable epidermis [45]. The skin was stripped using an adhesive tape (Tesa film N. 5529, Beiersdorf, Hamburg Germany) and the tape strips were pressed on the skin by applying uniform pressure in order to obtain intimate contact between the film and the skin. The procedure was repeated two-times to remove completely the SC from the reconstituted epidermis. The remainder represents viable epidermis. Each tape strip sample consisted of SC was cut to a same small size and placed in a glass vial containing 1 ml ethanol, sonicated for 10 minutes and submitted to centrifugation (15 min, 12,000 rpm). 100 µl of supernatant was collected for HPLC analysis to determine the amount of 4-MBC.

To extract the sunscreen from the viable epidermis, the samples were treated with 1.0 ml of 2% SDS for 20 hours. After treatment with methanol (2.0 ml) for 3 hour, the mixture was centrifuged at 12,000 rpm for 15 min and appropriate aliquots of supernatant were subjected to HPLC analysis. For validation of the extraction procedure, samples of blank skin (stratum corneum or viable epidermis) was submitted to the assay, and the retention time of endogenous compounds was compared with that of 4-MBC in order to verify that there were no interferences in analyzing the molecule. Moreover, a known aliquot of 4-MBC was added to blank skin (stratum corneum or viable epidermis) and the extraction recovery was determined by computing the ratio of the amount of sunscreen extracted from the skin to the amount added. The percentage of recovery was 98.69 ± 4.12 and 89.78± 6.89 (mean ± SE) for stratum corneum and viable epidermis, respectively.

HPLC analysis

The concentration of 4-MBC in receiving fluid and in skin samples was selectively determined by HPLC (liquid chromatograph with LC-20AT pump and 20 µl Rheodyne injector, SPD-6AV detector and computer integrating system C-R4AX, Shimadzu Corp., Kyoto, Japan). The column (30 cm x 3.9 mm) was packed with LiChrocart Phrospher C18 (Phenomenex, Torrance, CA, USA). The mobile phase consisted of a mixture of acetonitrile: methanol:water (70: 25:5), the flow rate 1.0 ml/min. The retention time and the detection wavelength were respectively 14.0 min and 298 nm. The amount of sunscreen in the samples was determined by comparison with appropriate standard curves. In case of biological materials, a standard curve was obtained by adding increasing amounts of 4-MBC to blank biological samples.

The limit of quantization of 4-MBC (LOQ) was 0.098 $\mu g \cdot ml^{-1}$, 0.129 $\mu g \cdot ml^{-1}$ and 0.168 $\mu g \cdot ml^{-1}$ in receiving fluid, the stratum corneum and viable epidermis samples, respectively

Data analysis

Linear regression analysis of pseudo steady state diffusion plots allowed calculation of the following parameters: steady-state flux (*J*), given by $Q/A \cdot t$, where *Q* is the amount of permeant diffusing across the area *A* in time *t*; *lag time* (t_L), indicating the time taken by the drug to saturate the membrane and to reach the receiving phase, calculated from the X-axis intercept values of the regression lines; sunscreen amount permeated at end of the experiment (Q_{300min}). Moreover, the digestion procedure allowed calculation of the 4-MBC content as mg of sunscreen per g of skin retained in stratum corneum (Q_{sc}) and viable epidermis (Q_{ve}).

Data are the average of four determinations ± standard error (SE) for all the formulations tested ± standard error (SE). Statistical differences between permeation parameters were assessed by GraphPad Prism software (GraphPad Software Inc., San Diego, CA). The evaluation included calculation of means and standard errors, and group comparisons using the Student's two-tailed unpaired t-test. Differences were considered statistically significant at $p<0.05$.

4-MBC apparent diffusion coefficient (D_{app}) through EPISKIN was calculated according to the following relation: $D_{app} = h^2/6t_L$ where h represents the thickness of the membrane and t_L the lag time. The thickness of the EPISKIN membrane was about 130 µm, as indicated from SkinEthic Laboratories.

The permeability coefficient K_p was calculated using this equation: $K_p = J/C_o$ where J is the flux at steady-state ($\mu g/cm^2 \cdot min$) and C_o is the initial UV-filter concentration ($\mu g/cm^3$). UV filter membrane/vehicle partition coefficient, K_m, was obtained from the relationship: $K_p = K_m D_m/h$.

Systemic exposure dosage (SED) as $mg \cdot kg^{-1} \cdot day^{-1}$ was estimated as reported in the SCCS's Notes of Guidance

for the Testing of Cosmetic Substances and their Safety Evaluation 8th Revision (SCCS/1501/12) using the following equation [46]:

$$SED = \frac{DA_a\ (mg \cdot cm^{-2}) \times SSA\ (cm^2) \times F\ (day^{-1})}{60\ Kg}$$

where DA_a ($mg \cdot cm^{-2}$) represents dermal absorption as amount/cm^2 resulting from an assay under in-use mimicking conditions, SSA (cm^2) is the skin surface area expected to be treated with the finished cosmetic product (17500 cm^2 as indicated in section 4-2 of SCCS/1501/12 for SSA values per product type), F (day^{-1}) is frequency of application of the finished product (F ≥ 1), 60 kg is default human body weight.

To estimate the margin of safety (MoS), the SED was compared to the NOAEL [47]: MoS=NOAEL/SED where NOAEL is the no observed adverse effect level. NOAEL for 4-MBC was obtained from the document of SCCP Colipa n. S60 and its value was 25 mg $\cdot kg^{-1} \cdot day^{-1}$ [28].

Results and Discussion

Characterization of microspheres

The morphology of microparticles was determined. Particles were spherical with a porous surface and a compact internal matrix as seen in figure 1. The particle size obtained was between 20 and 50 µm with the majority of the population in the 30 µm range, which is proper for topical application when penetration absorption should be prevented [48, 49]. Loading data were satisfactory (39.39 % ± 0.68). Encapsulation efficiency, calculated as the percentage of the experimental and the theoretical loading, was ~50%.

Evaluation of 4-MBC release from microspheres

The interaction of the sunscreen with the skin depends on its mechanism and rate of release from the vehicles. As a consequence, assessment of in vitro 4-MBC release is a crucial step. At first 4-MBC release from microspheres to a hydrophilic receiver fluid was determined. No release was observed after 24 hours in hydrophilic environment surely due to the lack of solubility in water of the filter. As expected, the

microsphere loading values, determined by spectrophotometric analysis on the microspheres recovered, were the same before and after the release process (38.98 % ± 0.70).

Then in vitro release of 4-MBC from the microspheres in lipophilic environment was tested to simulate to actual conditions: stratum corneum, outermost layer of the skin, is known to have essentially lipophilic characteristics. In this case the release of 4MBC from the EM-1 and EM-2 formulations was tested.

Table 2 shows the amount of 4-MBC released from EM-1 and EM-2 containing free and encapsulated filter, respectively using Strainer cells. The sunscreen diffused to the lipophilic receiving fluid (caprylic/capric triglyceride). For the preparation containing microspheres, release was slow, reaching 5% in 24h, whereas the release of free sunscreen was faster and reached a peak of 10% in 24h. The data obtained highlight that 4-MBC release and diffusion was decreased from EM-2 formulation indicating that the retention capacity of the microparticles was maintained after incorporation into the emulsion.

In vitro skin permeation and distribution studies

Results of 4-MBC permeation experiments are illustrated graphically in figure 2, while the relevant permeation parameters (J, t_L, Q_{300min}, K_p, D_m, K_m) are summarized in table 3. The permeation profiles demonstrated that the incorporation of the sunscreen in the microspheres remarkably reduced the permeation of 4-MBC through reconstituted epidermis. After application of EM-1 formulation containing the free filter, the steady state flux of 4-MBC through reconstituted tissue was found to be $20.5 \cdot 10^{-2} \pm 7.7 \cdot 10^{-2}$ $\mu g \cdot cm^{-2} \cdot min^{-1}$. The permeation of 4-MBC from EM-2 containing encapsulated filter was decreased of 46 times with $0.44 \cdot 10^{-2} \pm 0.07 \cdot 10^{-2}$ $\mu g \cdot cm^{-2} \cdot min^{-1}$ flux. In addition the amount of filter permeated from EM-1 was 52.85±18.49 $\mu g \cdot cm^{-2}$ compared to 1.41±0.16 $\mu g \cdot cm^{-2}$ obtained from EM-2. In all cases the differences were statistically significant: p=0.0113 in the case of flux; p=0.0079 for 4-MBC permeated. Since 4-MBC flux through the skin was similar to its release rate, it is confirmed that the rate limiting step was the sunscreen release from the formulations and the microencapsulation influences the filter availability for the permeation.

As well as different interactions between 4-MBC/vehicle, vehicle/skin and 4-MBC/skin may affect the sunscreen permeation, to assess the mechanism of 4-MBC permeation through the skin, the influence of K_p, D_m and K_m was investigated. As shown in table 3, D_m and K_m values showed opposite trends. The data obtained pointed out that the D_m alone could not be regarded as a predictive parameter to evaluate 4-MBC permeation through the skin because it takes into account only the lag time but not what happens once the steady state is reached. A more complete frame could be obtained calculating the membrane/vehicle

partition coefficient, K_m, which was 500-fold lower for the microencapsulated sunscreen than for the free one for the same formulation demonstrating that microspheres keep the filter inside the vehicle.

The in vitro penetration data are illustrated in figure 3 as micrograms of compound per gram of skin retained in stratum corneum and viable epidermis for both formulations under study, EM-1 and EM-2 containing free UV filter or sunscreen loaded microspheres, respectively. The obtained results showed that EM-1 and EM-2 formulations produced a similar recovery of 4-MBC in the stratum corneum: 471.59±102.17 $\mu g \cdot g^{-1}$ for EM-1 vs 552.75±150.76 $\mu g \cdot g^{-1}$ for EM2. On the contrary the application of 4-MBC in microsphere form demonstrated a hampering effect on the retention of the filter in the viable epidermis with a decrease in amount retained from 959.6 ± 192.78 $\mu g \cdot g^{-1}$ for EM-1 to 439.85±114.9 $\mu g \cdot g^{-1}$ for EM-2 with statistically significant differences (p=0.0428).

Viable epidermis can be considered as a sink, therefore the amount of filter found in this tissue could be considered as absorbed. It is important to note that the incorporation of sunscreen into microspheres allowed modulate the absorption of filter in the skin, by reducing significantly the content of 4-MBC in the viable epidermis and by maintaining the filter on the skin surface and in the horny layer. The lower retention in the viable epidermis achieved by its incorporation in the microspheres should reduce the potential toxicological risk associated with skin penetration. The hampering effect on sunscreen penetration provided by the application of 4-MBC in microparticle form could be due: i) microparticles with a diameter > 10 µm do not penetrate the horny layer; ii) substantive properties of polymer that fix the sunscreen molecule on the cutaneous surface. These characteristics could also enhance the efficacy of the sunscreen confirming the role of the vehicle on the skin penetration of 4-MBC.

According to the 8th revision of the SCCP's notes of guidance for the testing of cosmetic ingredients and their safety evaluation [46], a MoS of at least 100, obtained by extrapolation from a group of test animals to an average human being, is generally accepted to declare a product safe for use. In case of 4-MBC, the SCCP evaluated acceptable a reduction of the toxicokinetic factor of the MoS from 4 to 1, therefore a MoS of 25 needs to be achieved [28]. Since there aren't any literature data comparing the permeation of 4-MBC through human and reconstituted skin, a reference MoS related to these two substrates has not been calculated. Anyway, it is noteworthy that the barrier properties of Episkin are weak when compared to human skin with a consequently higher permeability [50]. As well as our experiments could represent a worst case scenario we decided to use a MoS of 25 as threshold to reach.

MoS values calculated in our study for EM-1 and EM-2 were 0.30 and 13.09, respectively. Notwithstanding both values were less than the requested thresholds of 25, suggesting that these products could not be safe, it should be kept in mind that we have performed our permeation experiments applying

a high amount of formulation (as donor phase) and adding a solubilizer (Brij®98) in the receiving phase, thus forcing the filter transport through RE to appreciate the different behaviour of the formulations under study. Anyway when 4-MBC was encapsulated in microspheres, the MoS value was 43-folds higher than that obtained with the free sunscreen.

Moreover it could be hypothesized that in physiological conditions MoS value for EM-2 formulation might be beyond the limit accepted for safe products demonstrating the safety of the microspheres as carrier for this UV filter.

Conclusions

In daily life, UV exposure is unavoidable and sunscreen should be used regularly. Then considering the frequent and prolonged use over time, particular attention has to be paid to their efficacy and safety. An ideal sunscreen product should be exhibit high skin accumulation of UV filter with minimal permeation to the systemic circulation.

The results obtained in this study have shown that the incorporation of 4-MBC in microspheres decreases the percutaneous penetration of the sunscreen thereby minimising its systemic uptake and the potential associate toxicological risks. An additional advantage of this effect is that more of the active sunscreen remains on the surface of the skin where it is intended to act and an improved activity it will esplicate.

These cationic microspheres were able to bind to keratin or keratinaceous substances for longer period of time as showed in our previous study on microspheres containing hair dyes [51]. As a consequence, this suggested that the microspheres obtained in this work were able to prolong release of 4-MBC. By delivering the active gradually to the skin 4-MBC-loaded microsphere formulations can ensure the effectiveness of the product with minimal irritation. A comparison of the in vitro release rate of 4-MBC from EM-1 and EM-2 confirms the capacity of microspheres to maintain the chemical stability of the sunscreen as the polymeric matrix protects the filter from photodegradation, as previously demonstrated [52]. Moreover we verified that the inclusion of 4-MBC in the microspheres did not affect its efficacy. Indeed we compared the ability of the O/W emulsion containing 4-MBC-loaded microspheres and the free filter (corresponding to EM1 and EM2) to protect against UV rays. The protective efficacy, examined by measuring the *in vitro* SPF, was very similar for the sunscreen loaded in microspheres or free. Differences were non-significant between the *in vitro* SPF of the emulsion containing the sunscreen free (4.82 ± 0.45) or microencapsulated (4.25 ± 0.26) ($p < 0.05$, ANOVA) [52].

In conclusion, substantive microspheres appear to be an ideal formulation candidate to use in sunscreen preparation.

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Figure 1. SEM photograph of internal matrix of 4-MBC-loaded microspheres.

Figure 2. Permeation of 4-MBC ($\mu g \cdot cm^{-2}$) through the skin as a function of time, using reconstituted epidermis. Data are presented as mean±SE (n=4).

Figure 3. 4-MBC retained (μg/g) into skin layers (stratum corneum and epidermis) after application of EM-1 and EM-2 formulations. Data are presented as mean±SE (n=4).

Table 1. Composition of the formulations under study

COMPONENTS	INCI NAME	AMOUNT, %
4-MBC*	4-methylbenzylidene camphor	3.00
Cosmacol® ECI	Tri C_{12-13} Alkylcitrate	15.00
Montanov® 68	Cetearyl glucoside, Cetearyl alcohol	5.00
Amphisol® K	Potassium Cetyl Phosphate	0.50
Water	Aqua	q.s. to 100

*free (EM-1) or incorporated in microspheres (EM-2)

Table 2. 4-MBC released, free or loaded in microspheres, from the emulsions

	Filter released (%)	
Time (min)	**EM-1**	**EM-2**
T_0 (0)	0.0	0.0
T_1 (5)	0.16 ± 0.17	0.0
T_2 (15)	0.95 ± 0.23	0.07 ± 0.09
T_3 (30)	1.29 ± 0.79	0.12 ± 0.05
T_4 (60)	2.01 ± 1.31	0.25 ± 0.10
T_5 (120)	3.75 ± 2.07	0.69 ± 0.22
T_6 (240)	5.30 ± 0.63	1.18 ± 0.20
T_7 (420)	5.95 ± 0.37	1.64 ± 0.40
T_8 (1440)	9.66 ± 0.06	4.59 ± 1.25

Table 3. Permeation Parameters of 4-MBC from EM-1 and EM-2 formulations

Formulation	$J \cdot 10^2$ $\mu g \cdot cm^{-2} \cdot min^{-1}$	t_L min	Q_{300min} $\mu g \cdot cm^{-2}$	$Kp \cdot 10^6$ $cm \cdot min^{-1}$	$Dm \cdot 10^6$ $cm^2 \cdot min^{-1}$	$Km \cdot 10^3$
EM-1	20.49±7.7	32.92±18.14	52.85±18.49	6.83	0.85	104
EM-2	0.44±0.07*	3.16±1.90	1.41±0.16*	0.147	8.88	0.215