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Influence of solid lipid microparticle carriers on skin penetration of the sunscreen agent, 4-methylbenzylidene camphor

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Abstract

The objective of this study was to prepare lipid microparticles (LMs) loaded with the sunscreen agent, 4-methylbenzylidene camphor (4-MBC), to achieve decreased skin penetration of this UV filter. The microparticles were produced by the melt dispersion technique using tristearin as lipidic material and hydrogenated phosphatidylcholine as the surfactant. The obtained microparticles were characterized by scanning electron microscopy and differential scanning calorimetry. Release of 4-MBC from the LMs was found to be slower than its dissolution rate. The influence of the LMs' carrier system on percutaneous penetration was evaluated after their introduction in a model topical formulation (emulsion). In-vitro measurements were performed with cellulose acetate membranes in Franz diffusion cells. The 4-MBC release and diffusion was decreased by 66.7–77.3% with the LM formulation, indicating that the retention capacity of the microparticles was maintained after incorporation into the emulsion. In-vivo human skin penetration of 4-MBC was investigated by tape stripping, a technique for selectively removing the upper cutaneous layers. The amount of sunscreen penetrating into the stratum corneum was greater for the emulsion containing non-encapsulated 4-MBC (36.55% of the applied dose) compared with the formulation with the sunscreen-loaded microparticles (24.57% of the applied dose). The differences between the two formulations were statistically significant in the first (2–4) horny layer strips. Moreover, the LMs' effect measured in-vivo was less pronounced than in-vitro. The increased 4-MBC retention on the skin surface achieved by its incorporation in the LMs should enhance its efficacy and reduce the potential toxicological risk associated with skin penetration.

Introduction

The increased awareness regarding skin protection against the harmful effects (erythema, cutaneous photoaging, immune suppression and various forms of skin cancers) of sunlight UV radiation (290–400 nm) has led to a rise in the use of topical sun-protective preparations (Gasparro et al 1998; Green et al 1999). The active constituents in these products, commonly referred to as sunscreen agents or UV filters, attenuate the transmission of the solar UV rays to the skin by absorbing, reflecting or scattering the radiation (Gasparro et al 1998). High photostability and minimum percutaneous absorption are of paramount importance for the efficacy and safety of UV filters (Treffel & Gabard 1996; Bonda 2005; EC Commission Recommendation 2006). In fact, the decomposition of the sunscreen under sunlight decreases the expected UV-protective capacity and can generate harmful photolytic products (Tarras-Wahlberg et al 1999; Scalia et al 2002). Moreover, since the site of action of sunscreens is restricted to the skin surface, their permeation will leave the skin unprotected and increase the risk of systemic effects (Treffel & Gabard 1996; Nohynek & Shaefer 2001).

4-Methylbenzylidene camphor (4-MBC) is a common sunscreen agent that is included in the list of authorized UV filters in Europe and Australia and has been submitted for approval in the USA (Steinberg 2005). 4-MBC provides excellent protection against UV-B radiation (290–320 nm) and, in addition, it has been shown to exert a photostabilizing effect on butyl methoxydibenzoylmethane, the most frequently used and efficient UV-A (320–400 nm) filter (Bonda 2005). However, recent investigations have shown that in rat as well as in man, 4-MBC is systemically absorbed after topical application (Janjua et al 2004;

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Schauer et al 2006). Although sunscreen levels measured in human plasma are low (Janjua et al 2004; Schauer et al 2006), owing to the fact that sunscreens are repeatedly applied for extended periods of time, the plasma concentrations might be higher under real in-use conditions. This aspect should be considered with attention, since in-vitro and animal studies have reported on the oestrogenic activity of 4-MBC (Schlumpf et al 2004). Therefore, there is a need for new systems able to minimize the skin penetration of 4-MBC.

Lipid particles, ranging in size from nanometres (Wissing & Müller2002) to micrometres (Yener et al 2003; Tursilli et al 2007), represent an alternative and appropriate carrier system for sunscreen agents. This investigation focuses on lipid microparticles (LMs). They consist of a lipid matrix, in the solid state at room temperature, stabilized by a layer of surfactant molecules on the surface (Jaspart et al 2005). Since LMs are based on naturally occurring lipids, they are physiologically compatible and biodegradable (Jaspart et al 2005). Moreover, they attain a high entrapment yield for hydrophobic compounds, such as most of the UV filters (Yener et al 2003; Jaspart et al 2005; Tursilli et al 2007). Additional advantages of lipoparticles include good substantivity (i.e. adhesive properties) for the superficial skin layers where sunscreens perform their specific function, and proper size for reduced skin absorption (Wiechers 2000; Wissing & Müller 2002; Yener et al 2003; Toll et al 2004). The purpose of this study was to produce and characterize LMs loaded with 4-MBC. In addition, the influence of the microencapsulation process on the sunscreen percutaneous penetration was examined after incorporation of the LMs in a model topical formulation (emulsion). For the evaluation of skin permeation, both in-vitro (Franz diffusion cell) and in-vivo (tape-stripping) techniques were used.

Materials and Methods

Materials

4-Methylbenzylidene camphor was supplied by Merck (Darmstadt, Germany). Glyceryl behenate (Compritol 888 ATO) was from Gattefossé (Cedex, France). Tristearin, cetyl palmitate and polysorbate 60 were purchased from Fluka Chemie (Buchs, Switzerland). Hydrogenated soybean phosphatidylcholine was a gift from Cargill (Hamburg, Germany). Random methyl-β-cyclodextrin was purchased from Aldrich Chimica (Milan, Italy). The excipients for the cream preparations were from Sigma Aldrich (Steinheim, Germany) and Henkel (Fino Mornasco, Italy). Cellulose acetate membranes were obtained from Albet (Barcelona, Spain). Adhesive tapes (Scotch 600 crystal clear tape) were from 3M (Cergy-Pontoise Cedex, France). Methanol, acetonitrile and water were high-performance liquid chromatography (HPLC)-grade from Merck. All other reagents and solvents were of analytical grade (Sigma).

High-performance liquid chromatography

The HPLC apparatus comprised a Model LabFlow 3000 pump (LabService Analytica, Bologna, Italy), a Model 7125 injection valve with a 20-μL sample loop (Rheodyne, Cotati, CA) and a Model 975-UV variable wavelength UV-Vis detector (Jasco, Tokyo, Japan) set at 298 nm. Data acquisition and processing were accomplished with a personal computer using Borwin software (JBMS Developpements, Le Fontanil, France). Sample injections were effected with a Model 701 syringe (10 μL; Hamilton, Bonaduz, Switzerland). Separations were performed according to the method of Scalia et al (2007), using a 5-μm Zorbax SB-CN column (150 × 3.0 mm i.d.) fitted with a guard column (5-μm particles, 4 × 2 mm i.d.) and eluted isocratically, at a flow-rate of 0.4 mL min^{-1}, with methanol–acetonitrile–water (40:25:35, v/v/v). The identity of the 4-MBC peak was assigned by co-chromatography with the authentic standard. Quantification was carried out by integration of the peak areas using the external standardization method.

Microparticle preparation

Lipid microparticles were prepared by adding hot (60–75°C) water (60 mL) containing 1% (w/v) of surfactant to the melted lipid phase (4.8 g) in which 4-MBC (1.2 g) has been dissolved. The sample was subjected to high-shear mixing (10 000 rev min^{-1} for 3 min) with an Ultra-Turrax T25 (IKA-Werk, Staufen, Germany) at 75°C. The obtained emulsion was rapidly cooled at room temperature under magnetic stirring and the formed particles were recovered by centrifugation (5000 rev min^{-1} for 15 min), washed with water and freeze-dried.

Microparticle characterization

Microparticle morphological structure was examined by scanning electron microscopy (SEM; XL-40; Philips, Eindhoven, The Netherlands). The particle size was determined by computerized image analysis of at least 100 lipoparticles on SEM micrographs. An optical microscope (N-400FL; MAD Apparecchiature Scientifiche, Bergamo, Italy) was also used.

Thermal analysis was carried out on a differential scanning calorimeter (DSC-4; Perkin Elmer, Norwalk, CT) coupled with a computerized data station (Perkin Elmer, Norwalk, CT). Samples were heated in crimped aluminium pans from 30 to 100°C at a scanning rate of 10°C min^{-1} under dry nitrogen flow (30 mL min^{-1}).

The amount of 4-MBC entrapped in the LMs was determined by dissolving the microparticles (30–40 mg) in ethanol under sonication (30 min). The obtained sample was diluted to volume (20 mL), filtered and assayed by HPLC. Data were determined from the average of at least three determinations.

In-vitro release

The sunscreen dissolution and release were studied by adding previously sieved (100 μm) 4-MBC (5 mg) or LMs containing an equivalent amount of sunscreen, to propylene glycol (100 mL) under mechanical stirring at 50 rev min^{-1} and 37°C. At appropriate time intervals, 1-mL samples of the release medium were withdrawn and replaced with an equal volume of fresh medium. The samples were filtered and assayed for 4-MBC spectrophotometrically (300 nm) on a UV/Vis spectrometer

(Lambda 3B, Perkin-Elmer, Norwalk, CT). Each series of experiments was repeated at least 3 times.

Emulsion formulations

Oil-in-water (o/w) emulsions containing 1% (w/w) 4-MBC alone or loaded in microparticles were prepared according to the common procedure used in compounding practice. The emulsion excipients were: sorbitan monostearate (2%), polyoxyethylene sorbitan monostearate (4.5%), butylated hydroxyanisole (0.02%), isopropyl isostearate (9.0%), cetearyl isononanoate (8.0%), cetearyl alcohol (7.0%), sodium benzoate (0.1%), glycerin (2.0%), dehydroacetic acid (0.1%), EDTA (0.1%) and water (66%). The UV filter was dissolved in the oil phase, whereas the sunscreen-loaded LMs were dispersed in water and added in the cooling phase of the emulsion preparation at about 40°C.

In-vitro diffusion studies

These studies were performed in static Franz-type glass diffusion cells. Cellulose acetate membranes (average pore diameter, 0.2 μm) were inserted between the donor and receptor compartment of the diffusion cells (cross sectional surface area, 1.7 cm^2). The receptor chamber was filled with a known volume (approx. 8 mL) of phosphate-buffered saline (pH 7.4) containing randomly methylated-β-cyclodextrin (60 mM) to ensure sink conditions (Scalia et al 2007). The fluid was maintained at 37°C and stirred with a magnetic bar throughout the experiment. Portions (40 mg) of the emulsion formulations containing 4-MBC (1.0%, w/w) free or entrapped in LMs were evenly spread on the membrane surface in the donor chamber. At appropriate time intervals, 0.5-mL samples of the receptor phase were withdrawn and replaced by an equal volume of fresh fluid. Samples from the receptor phase were assayed for 4-MBC by HPLC. At least five replicates were used for each series of experiments.

In-vivo penetration studies

The in-vivo permeation studies were performed by the tape-stripping technique. The trial was carried out after obtaining informed written consent of the subjects, as requested by the local ethics committee (Comitato Etico Provinciale of Modena). Six female subjects, aged 24–30 years and free of dermatological disorders, participated in the study. The sunscreen preparations were applied at a dose of 2 mg cm^{-2}, according to COLIPA standard (1994), on the inner forearm region, which was previously wiped with ethanol and dried. The cream samples containing plain 4-MBC (1.0%, w/w) or the sunscreen-loaded microparticles were randomly allocated to previously marked areas (2×5 cm^2), respectively on the upper and lower part of the forearm of each subject. The formulations were homogeneously distributed by means of a gloved finger. Thereafter, the subjects rested for 30 min without covering the test areas with textiles until the tape stripping started. The remaining product was then removed from the treated area by light cleaning with a cotton swab and the skin was stripped 15 times with Scotch 600 transparent adhesive tape (Chatelain et al 2003; Weigmann et al 2005; Scalia et al 2007). The tape strips were applied to the skin and pressed with a constant pressure (i.e., a 500 g weight was rolled over them 10 times). The first strip was added to the cotton swab for the assay of the non-penetrated 4-MBC (Shah et al 1998; Chatelain et al 2003; Scalia et al 2007). The successive 14 tape strips were pooled separately into 4 groups (group 1, strips 2–4; group 2, strips 5–7; group 3, strips 8–10; group 4, strips 11–15) for analysis of the sunscreen content. The obtained samples were extracted with 3×5 mL of methanol–acetonitrile (90:10, v/v) under sonication (extraction recovery > 93%), diluted to volume (20 mL) and, after filtration, the resulting solutions were assayed for 4-MBC by HPLC. The penetration results were expressed as percentage of the applied dose.

In-vitro sun protection factor measurement

The in-vitro determination of the cream sun protection factor (SPF) was carried out according to the Diffey & Robson (1989) technique with minor modifications. The method is based on the measurement of the transmission spectrum of the UV radiation (290–400 nm) through a Transpore tape (3M Health Care, Neuss, Germany), before and after application of the sunscreen preparation. The product was applied to the tape surface at 2 mg cm^{-2} and spread uniformly. The Transpore tape was then placed into the spectrophotometer (Model V-530PC UV-VIS; Jasco, Tokyo, Japan) sample compartment, over the quartz input optics of the detector. The spectral data were processed with a personal computer and the SPF calculated according to Diffey & Robson (1989).

Statistical analysis

Statistical analyses were performed by use of Student's unpaired *t*-test, analysis of variance, non-parametric multiple comparison test (Kruskal–Wallis test) and post tests (Dunn or Tukey tests). $P < 0.05$ was considered significant. All computations were carried using the statistical software GraphPad Instat (Graphpad Software, San Diego, CA).

Results and Discussion

Lipid microparticle preparation and characterization

LMs loaded with 4-MBC were developed through a hot emulsion technique (Jaspart et al 2005; Tursilli et al 2007) using various lipid materials (tristearin, cetyl palmitate, glyceryl behenate) and emulsifiers (hydrogenated phosphatidylcholine, polysorbate 60). To evaluate the influence of the ingredient composition on the retention efficiency of the LMs, in-vitro release studies were performed using a medium (propylene glycol) in which 4-MBC was sufficiently soluble (to ensure sink conditions) whereas the lipoparticles remained intact. Marked differences in the sunscreen release profiles were observed among microparticles based on different lipids and containing phosphatidylcholine as emulsifier (Figure 1). A statistically significant decrease (Kruskal–Wallis test) in the release of 4-MBC was achieved only by the LMs prepared

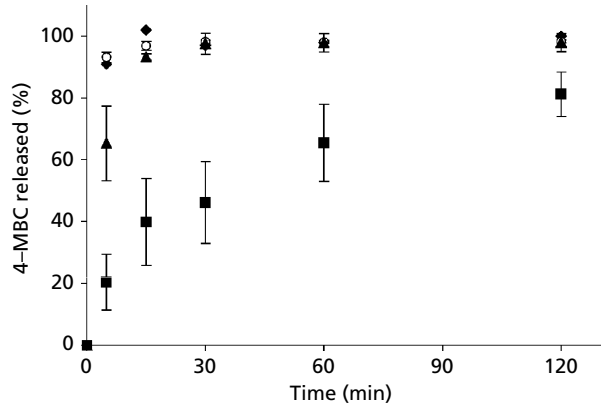

Figure 1 4-MBC dissolution (♦) and release from LMs prepared with phosphatidylcholine and cethyl palmitate (○), glyceryl behenate (▲) or tristearin (■). Values are means ± s.d., n = 3.

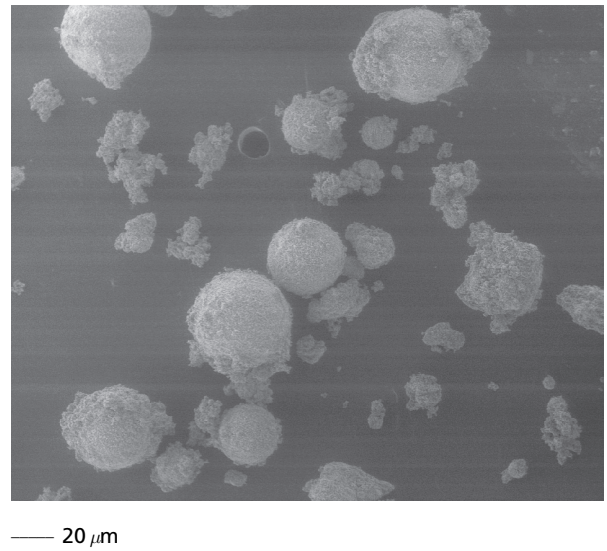

Figure 2 Scanning electron microscopy (SEM) micrographs of tristearin LMs loaded with 4-MBC.

with tristearin (Figure 1), which indicated the entrapment of the UV filter in this lipid matrix. On the other hand, the systems based on glyceryl behenate and cetyl palmitate exhibited a burst-effect phenomenon and a lack of release modulation capacity (Figure 1). These results indicate that the type of lipid material plays a major role in the microparticle incorporation process. This effect is probably due to different degree of interaction between the sunscreen and the lipid excipients. The slower release provided by the tristearin matrix can be ascribed also to the triglyceride polymorphic transformations (from stable to unstable forms) during particle preparation (Iannuccelli et al 2006). In fact, it has been shown that modifications of the crystallized lipid represent an important factor influencing the performance of LM systems (Jaspart et al 2005). Moreover, since the examined lipids have different melting points (glyceryl behenate, ca. 83°C; tristearin, ca. 65°C; cethyl palmitate, ca. 53°C), the observed release rates could be affected also by the production temperature, as higher temperatures favour burst-release effects (Müller et al 2002). No significant differences in 4-MBC release behaviour were observed when polysorbate 60 was used as emulsifier instead of phosphatidylcholine; therefore the latter was selected on the basis of better biocompatibility.

Investigation by SEM on the optimized LMs, based on tristearin and hydrogenate phosphatidylcholine, demonstrated that the lipoparticles showed spherical shape, although some irregular fragments were present (Figure 2). The particle size was between 7 and 80 μm, with the majority of the population in the 20–40 μm range, which is proper for topical application when percutaneous absorption should be prevented (Wiechers 2000; Toll et al 2004), as for sunscreen agents.

The amount of 4-MBC incorporated in the microparticles was found to be 18.2 ± 0.6% (w/w), which corresponded to an encapsulation efficiency of 72.7%.

Additional characterization of the LMs was carried out by thermal analysis (Figure 3). As previously reported (Iannuccelli et al 2006), crystallization of tristearin in lipoparticles determines a partial polymorphic modification of the stable β-form (T_{max} = 63–64°C) to unstable α-form (T_{max} about 53°C)

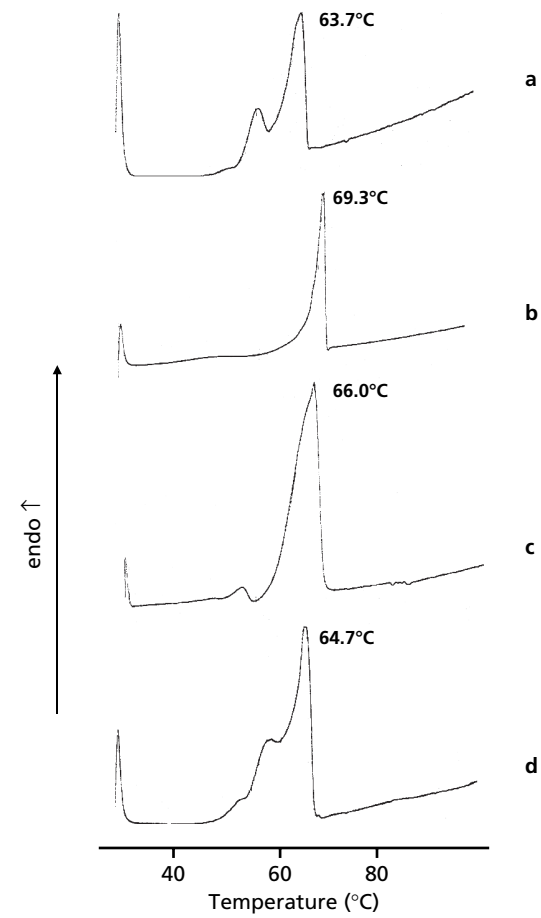

Figure 3 DSC thermograms of unloaded lipoparticles (a), 4-MBC (b), 4-MBC/unloaded lipoparticles (18/82) physical mixture (c) and lipoparticles loaded with 4-MBC (d).

and, in some cases', β-form (T_{max} about 57°C). Accordingly, the unloaded lipoparticles (Figure 3a) exhibited two thermal events attributable to the stable form of tristearin ($T_{max} = 63.7 \pm 0.6$°C) and to the overlap of the unstable forms. The DSC curve of 4-MBC (Figure 3b) showed an endothermic peak at 69.3 ± 0.2°C, corresponding to its melting point. In the thermogram of the physical mixture of 4-MBC and unloaded microparticles (Figure 3c), the sunscreen melting transition formed a broad endotherm at a lower temperature ($T_{max} = 66.0 \pm 0.3$°C), which could be reasonably related to overlapping of the 4-MBC melting peak with the thermal event of the stable form of tristearin. On the other hand, the DSC profile of the loaded lipoparticles (Figure 3d) showed a main endothermic transition at 64.7 ± 0.3°C, ascribable to the stable form of tristearin (statistical analysis by analysis of variance and Tukey's post test). This peak exhibited less band broadening than the thermal transition observed in the physical mixture (Figure 3c), indicating that the 4-MBC melting endotherm was not present. This finding suggests that the sunscreen is dispersed in an amorphous state inside the LMs.

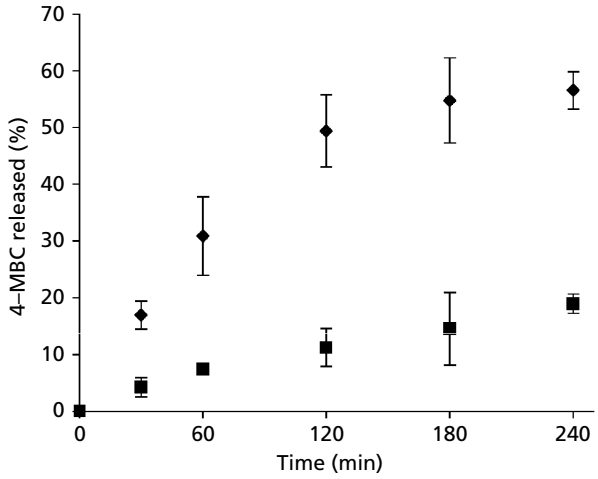

Figure 4 Franz diffusion cells: release profiles of 4-MBC from o/w emulsion (♦) and LMs in o/w emulsion (■). Values are means ± s.d., n = 5.

In-vitro penetration study

In this study the penetration of 4-MBC through a cellulose acetate membrane was investigated using creams (o/w emulsions) as topically applicable vehicles. These systems were selected as model formulations, since they represent the majority of sunscreen products (Klein & Palefsky 2005) and thus reproduce the conditions encountered in the real use by consumers. Analysis by optical microscopy (micrograph not shown) indicated that the lipoparticle structure was not compromised following incorporation into the emulsion.

The emulsion preparations containing free 4-MBC or the microencapsulated sunscreen agent were applied to the surface of the membrane mounted in the donor compartment of the Franz cells and the amount of sunscreen diffused in the receiver phase was determined by HPLC. The obtained permeation profiles (Figure 4) demonstrated that the 4-MBC release, and thereby diffusion across the cellulose acetate membrane, was significantly decreased (repeated measures analysis of variance and Tukey's post test) when the UV filter was in the LM form. Hence, the finding from the Franz-cell study correlates with the results of the release experiments (Figure 1) and indicates that, after introduction of the microparticles in the emulsion formulation, their release modulation capacity and hence the UV filter incorporation were maintained.

In-vivo penetration study

The penetration of 4-MBC into human skin was studied in-vivo using the tape-stripping method, which is a non-invasive procedure based on the progressive removal of the skin outer layers (stratum corneum) with an adhesive tape (Chatelain et al 2003; Weigmann et al 2005; Scalia et al 2007). With this technique, the corneocyte aggregates and the topically applied substances are transferred to the tape strips. The quantification of the active compound fixed to the individual tapes provides the in-vivo stratum corneum penetration profile, which is a predictor of skin absorption (Shah et al 1998).

The same formulations submitted to the in-vitro penetration experiments and containing 4-MBC free or entrapped in the optimized LMs were applied to skin areas of the human subjects' forearm and the amount of UV filter permeated into the stratum corneum was determined by extraction and HPLC analysis of the sunscreen from combined tapes (2–4, 5–7, 8–10, 11–15). The total compound recovery (sum of the 4-MBC unabsorbed and diffused into the horny layer removed by the strips) was >75.8%, which is acceptable considering that the horny layer was stripped only 15 times (Chatelain et al 2003). The quantity of 4-MBC recovered in the tape strips as a function of the strip number is shown in Figure 5. The percentage

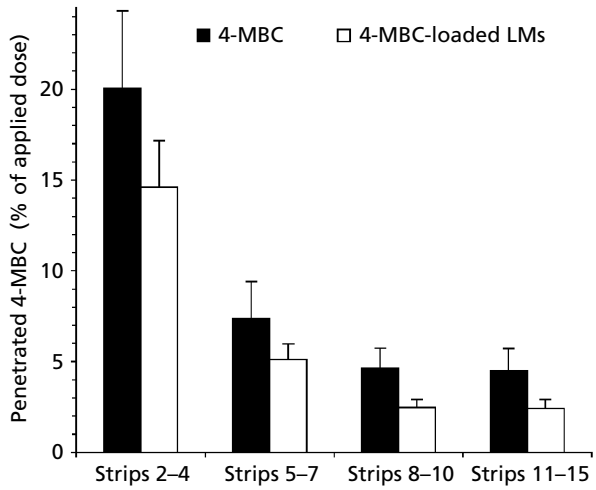

Figure 5 Concentration profiles of 4-MBC in the stratum corneum in-vivo after application of emulsions containing free 4-MBC or sunscreen-loaded lipoparticles. The UV filter amounts in strips 2–4, 5–7, 8–10 and 11–15 of the stratum corneum are shown (mean ± s.d., n = 6).

of the applied sunscreen dose penetrated into the stratum corneum was 36.55% and 24.57% for the non-encapsulated and microparticle-entrapped 4-MBC, respectively. Since the concentration of active substance that enters the stratum corneum is related to the fraction that ultimately reaches the systemic circulation (Shah et al 1998), the obtained results suggest that 4-MBC can be absorbed following topical application, in agreement with the finding by Janjua et al (2004). For both preparations, the highest proportion of the permeated UV filter (54.9–59.33%) was found within the outermost part of the horny layer (strips 2–4) and its concentration decreased with stratum corneum depth (Figure 5). The degree of distribution of 4-MBC in the horny layer, determined here, was in agreement with that reported in previous studies (Weigmann et al 2005; Scalia et al 2007). The obtained results showed that the penetration of 4-MBC into the stratum corneum was lower from the emulsion containing the sunscreen-loaded microparticles as compared with the formulation prepared with the free UV filter (Figure 5). Statistical analysis of the data (two-way analysis of variance and Tukey's post test) indicated that the differences between the formulations were significant in the upper layers (strips 2–4) of the stratum corneum, where the majority of the penetrated UV filter was localized. However, to obtain more conclusive results, a larger number of subjects should be investigated.

The hampering effect on sunscreen penetration in the stratum corneum, provided by the application of 4-MBC in microparticle form, could be traced to LMs' sustained-release characteristics. In addition, since microparticles with a diameter $>10\,\mu$m do not penetrate the horny layer (Wiechers 2000; Toll et al 2004), the LM formulation forms a film on the skin, fixing the sunscreen molecule on the cutaneous surface (Wissing & Müller 2002).

The results of the in-vivo investigation (Figure 5) show a certain degree of correlation with the in-vitro study (Figure 4), although the effect of the LMs in the Franz test was greater (the sunscreen diffusion was decreased by 66.7–77.3% in the Franz test compared with 27.3–46.9 % in the stripping test). This difference can be ascribed to the fact that human skin represents a more efficient barrier than cellulose acetate membrane. Moreover, the interaction of the microparticle lipid and surfactant with the horny layer constituents represents an additional factor that can affect the degree of cutaneous permeation (Müller et al 2002).

Since one of the most important criteria for the evaluation of a sunscreen product efficacy is its SPF (Steinberg 2005), the determination of this parameter in the examined formulations was undertaken. No significant difference ($P>0.05$, unpaired t-test) between the in-vitro SPF values of the creams containing the sunscreen free (SPF, 3.3 ± 0.6) or microencapsulated (SPF, 3.5 ± 0.4) was observed, which indicated that the incorporation of 4-MBC in the LMs has not modified its protective performance against UV radiation. Published studies have shown that incorporation of sunscreens into lipid nanoparticles enhanced the UV-B attenuation characteristics of the formulation due to scattering and absorption of the radiation (Müller et al 2002). These effects are related to the type of lipid excipient used and, especially, to the particle dimensions, since optimal UV-B scattering is observed in the nanometre-size range (Schlossman & Shao 2005).

Conclusions

From the results obtained in this study it can be deduced that the incorporation of 4-MBC in lipid microparticles decreases the percutaneous penetration of the sunscreen, thereby minimising its systemic uptake and the potential associated 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. The data reported in this investigation also show that although the in-vitro test represents a simple model for percutaneous penetration studies, in-vivo investigations are necessary for a more realistic assessment of the factors affecting the degree of skin absorption.

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