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Solid lipid microparticles for the stability enhancement of the polar drug N^6-cyclopentyladenosine

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Abstract

The objective of this study was to prepare solid lipid microparticles (SLMs) loaded with the polar adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA). The microparticles were produced by the conventional hot emulsion technique, using different lipidic carriers (tristearin, glyceryl behenate and stearic acid) and hydrogenated phosphatidylcholine as the surfactant. The controlled release of CPA was achieved only with stearic acid microparticles. This phenomenon has been attributed to direct acid–base interactions between the basic nitrogen atoms of CPA and stearic acid. These SLMs were characterized by release studies, scanning electron microscopy and powder X-ray diffraction analyses. The obtained particles showed proper features in terms of morphology and size distribution (3.2–10.3 μm), with a drug loading of $0.15 \pm 0.04\%$. The influence of the SLMs carrier system on CPA stability was investigated *in vitro* using human whole blood. The degradation kinetic of microparticle-entrapped CPA was significantly lower from that measured for the free CPA. The overall results indicate that it was possible to achieve the encapsulation and controlled release of a polar drug, such as CPA, within a lipid matrix without resorting to the complex methods generally used for the preparation of these systems.

Keywords: Controlled release; N^6-Cyclopentyladenosine; Solid lipid microparticles; Stability in blood; Stearic acid; Polar drug encapsulation

1. Introduction

N^6-Cyclopentyladenosine (CPA), an adenosine derivative (Fig. 1), is a polar drug with interesting pharmacological properties. Indeed, CPA is a potent and selective agonist of adenosine A_1 receptors (Dalpiaz and Manfredini, 2002), whose activation produces cardiac and neuronal excitability depression (Jacobson et al., 1991), inducing ischemic tolerance and protection in neuronal and cardiac tissues (Heurteaux et al., 1995; Tucker and Linde, 1993). Unfortunately, despite these encouraging results, the clinical use of CPA is hampered by several aspects: adenosine A_1 receptors are ubiquitous in the body, so their indiscriminate activation can produce dangerous unwanted effects (Dalpiaz and Manfredini, 2002). Moreover, CPA appears greatly unstable in physiological fluids, its half-life being around 7 min in conscious rats (Mathot et al., 1994) and about 20 min in fresh rat and human whole blood (Mathot et al., 1993; Pavan and Ijzerman, 1998). Finally, adenosine derivatives appear poorly adsorbed into the brain from the bloodstream (Brodie et al., 1987; Schaddelee et al., 2005).

The enhancement of CPA stability and its controlled release are, therefore, an important and challenging aspect in formulation development. Several strategies have been investigated to reduce the CPA biodegradation, including complexation with cyclodextrins (Scalia et al., 2001) and incorporation in polymeric nano- and microparticles. Accordingly, we have demonstrated that polylactic acid microparticles can control its release and improve its stability in human whole blood (Dalpiaz et al., 2001a, 2002). Similar results have been obtained with polylactic acid nanoparticles towards CPA and an alkyl-ester prodrug (Dalpiaz et al., 2005; Leo et al., 2006a,b).

The present study focuses on solid lipid microparticles (SLMs) as an alternative carrier system for CPA. These micrometer-sized particles consist of a solid fat core based on naturally occurring lipids and stabilized by a layer of surfactant molecules on the surface (Jaspart et al., 2005). SLMs combine the advantages of liposomes and polymeric microparticles, while avoiding some of their disadvantages such as instability,

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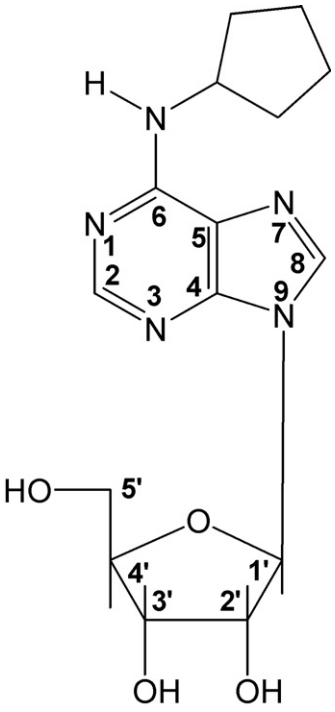

Fig. 1. Chemical formula of CPA.

toxicity, biodegradability problems, raw material and production costs (Jaspart et al., 2005; Trotta et al., 2005; Scalia et al., 2006). Due to their hydrophobic nature, SLMs attain high entrapment capacity for lipophilic compounds (Yener et al., 2003; Jaspart et al., 2005; Tursilli et al., 2007), whereas hydrophilic drugs are poorly incorporated in the lipid particles (Trotta et al., 2005; Jaspart et al., 2005; Jaspart et al., 2007). In order to overcome this disadvantage, complex production procedures (i.e., multiple emulsion techniques, two-steps manufacturing processes) (Bodmeier et al., 1992; Cortesi et al., 2002; Trotta et al., 2005; Cook et al., 2005) or the preparation of hydrophobic derivatives of the target drug prior to its encapsulation (Jaspart et al., 2007), have been described.

A different approach is proposed here which exploits direct drug-carrier interaction to overcome the challenge of encapsulating the polar drug CPA within lipid microparticles prepared by the conventional hot emulsion technique (Jaspart et al., 2005; Scalia et al., 2006). The influence of the microparticle matrix on the *in vitro* degradation of CPA in human blood was then examined.

2. Materials and methods

2.1. Materials

N^6-Cyclopentyladenosine (CPA, $\log P = 1.21$, water solubility 2.0 ± 0.1 mM = 0.67 ± 0.04 mg/ml) and N^6-cyclohexiladenosine (CHA) were obtained from Sigma–Aldrich (Milan, Italy). Glyceryl behenate (Compritol® 888 ATO) was from Gattefossé (Cedex, France). Tristearin and stearic acid were purchased from Fluka Chemie (Bucks, Switzerland). Hydrogenated soybean phosphatidylcholine was a gift by Cargill (Hamburg, Germany). Methanol, acetonitrile and water were high-performance liquid chromatography (HPLC)-grade from Merck (Darmstadt, Germany). The reversed-phase column (Hypersil BDS C-18 5U cartridge column, 150 mm × 4.6 mm i.d.) and the guard column (packed with Hypersil C-18 material) were obtained from Alltech Italia Srl BV (Milan, Italy). All other reagents and solvents were of analytical grade (Sigma).

2.2. HPLC analysis of CPA

The quantification of CPA in all samples generated from the experimental procedures was performed by HPLC. The chromatographic apparatus consisted of a modular system (Model LC-10 AD VD pump and Model SPD-10A VP variable wavelength UV–vis detector; Shimadzu, Kyoto, Japan) and an injection valve with 20 μl sample loop (Model 7725; Rheodyne, IDEX, CA, USA). The detector was set at 269 nm. Separation was performed at room temperature on a reversed-phase column (Hypersil BDS C-18 5U) equipped with a guard column. Data acquisition and processing were accomplished with a personal computer using Class-VP software (Shimadzu). The mobile phase consisted of a ternary mixture of acetonitrile, methanol and 10 mM acetate buffer (pH 4) with a ratio of 5/50/45 (v/v/v). The flow rate was 0.8 mL/min and the retention times of CPA and CHA were 4.5 and 6.4 min, respectively.

2.3. Microparticle preparation

Lipid microparticles were prepared by adding hot (70–80 °C) water (25 ml) containing 1% (w/v) of surfactant (hydrogenated phosphatidylcholine) to the melted lipid phase (1.6 g) in which CPA (0.03 g) has been dispersed. The sample was subjected to high-shear mixing (13500 rpm for 2–3 min) with an Ultra-Turrax T25 (IKA-Werk, Staufen, Germany) at 70–80 °C. The obtained emulsion was rapidly cooled at room temperature under magnetic stirring and the formed particles were recovered by centrifugation (6000 rpm for 15 min), washed with water and freeze-dried.

2.4. CPA content in the microspheres

The CPA content was determined by the following methods. The sieved (100 μm) microspheres (about 5 mg) were accurately weighed using a high precision analytical balance ($d = 0.01$ mg; Sartorius, Model CP 225D, Goettingen, Germany), and dissolved in ethanol under sonication. The sample was diluted to volume (10 ml) and 10 μl of the filtered solution (0.45 μm) were injected into the HPLC system for CPA detection. Alternatively, an accurately weighed amount of sieved microspheres (about 5 mg) were suspended into 10 ml of water. 1 ml of CH_2Cl_2 was added and the mixture was accurately vortexed to dissolve the particles in the organic phase. After centrifugation (15,000 × g for 10 min), 10 μl of the water solution were injected into the HPLC system for CPA detection. Preliminary experiments indicated that concentrations of CPA in water solutions were not altered by the presence of a 10% (v/v) of CH_2Cl_2 after vortex and centrifugation processes.

The drug loading and entrapment efficiency were calculated according to the following equations:

$$\text{Drug loading (\% w/w)} = \frac{\text{mass of drug in microparticles}}{\text{mass of microparticles recovered}} \times 100 \quad (1)$$

$$\text{Entrapment efficiency (\%)} = \frac{\text{mass of drug in microparticles}}{\text{starting mass of drug}} \times 100 \quad (2)$$

All the values obtained are the mean of four independent experiments.

2.5. "In vitro" CPA dissolution and release studies from microparticles

An accurately weighed amount of sieved (100 μm) CPA (about 0.1 mg, weighed with the analytical balance Sartorius CP 225D) or microparticles containing an equivalent quantity of drug were added to 15 ml of water or 50 mM phosphate buffer (pH 7.4). The samples were maintained at 37 °C and stirred mechanically (100 rpm) during the release experiments. Aliquots (150 μl) were withdrawn at fixed time intervals and 10 μl of filtered samples (0.45 μm) were injected into the HPLC system for CPA detection. An equal volume of fresh medium was added after each sampling to maintain sink conditions. All the values obtained are the mean of four independent experiments.

In order to clarify the mechanism of the CPA release from the particles, a simple power law expression for the analysis of controlled release data from non-swellable devices was used;

$$\frac{M_t}{M_\infty} = kt^n \quad (3)$$

where M_t/M_∞ denotes the drug fraction release at the time t, k and n being the rate constant and kinetic exponent of release, respectively. The value of the kinetic exponent n, defining the mechanism of the release process, is dependent of the geometry of the system (Ritger and Peppas, 1987).

2.6. 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 (MicrometricsTM camera 122CU and software Vision 1.0) of at least 100 particles on an optical microscope (Nikon Diaphot inverted microscope, Tokyo, Japan).

The powder X-ray diffraction patterns were recorded on a D 5000 powder diffractometer (Siemens, Munich, Germany) using a voltage of 45 kV and a current of 25 mA for the generator, with Cu anode material. The wavelength of the graphite-monocromated radiation was 1.5406 Å. The diffractograms were recorded from 3° (2θ) to 35° (2θ) at an angular speed of 1° (2θ) per minute using 1-1-1-0.15° slits.

2.7. Kinetic experiments in human whole blood

The stability of microencapsulated CPA or the free drug in the presence and in the absence of unloaded lipoparticles was investigated using the method described by Pavan and Ijzerman (1998), with minor modifications. Compounds were incubated at 37 °C in fresh whole blood obtained from healthy human volunteers. Blood was directly transferred to heparinized glass tubes. Three milliliters of whole blood were spiked with drug solutions, resulting in final concentrations of 5 μM of CPA. When present, the concentration of microspheres was 7 mg/ml. During the experiment, the samples were shaken continuously and gently in an oscillating water-bath. At regular time intervals, 100 μl of samples were taken, immediately hemolyzed in Eppendorf tubes prefilled with 500 μl of water (HPLC grade, 0 °C), and stored at −20 °C until analysis.

2.8. CPA kinetic analysis

To the blood samples 50 μl of 3 M sodium hydroxide and 50 μl of internal standard (20 μM CHA) were added. In the presence of microspheres, 200 μl of dichloromethane were included. The samples were extracted twice with 900 μl of water-saturated ethyl acetate. After centrifugation (5 min at 9000 × g), the organic layer was evaporated to dryness by N_2 flow. 100 μl of mobile phase were added and, after centrifugation, 10 μl were injected into the HPLC system for CPA and CHA detection. A preliminary analysis performed on drug-free microparticles and blood extracts showed no interference of other components on the CPA and CHA detection.

All the values obtained are the mean of three independent experiments.

2.9. Statistical analysis

The statistical analysis was performed by use of unpaired t-test, employing the computer program GraphPad Prism (GraphPad, San Diego, CA, USA). Differences were considered statistically significant when P values were less than 0.05.

3. Results and discussion

3.1. Lipid microparticle preparation and characterization

Lipid microparticles loaded with CPA were developed through a hot emulsion technique (Jaspart et al., 2005; Tursilli et al., 2007) using various lipid materials (tristearin, glyceryl behenate, stearic acid) and hydrogenated phosphatidylcholine as a biocompatible emulsifier. Exploratory experiments were performed using tristearin or glyceryl behenate as carrier, since they are commonly used excipients in SLMs (Jaspart et al., 2005). Although the obtained particles exhibited satisfactory morphological features (micrographs not shown), release studies (using water as a medium) indicated that the observed profiles were almost superimposible with the dissolution curve of pure CPA (Fig. 2), suffering from high burst effect phenomena (>90% released after 5 min) and a lack of release modulation.

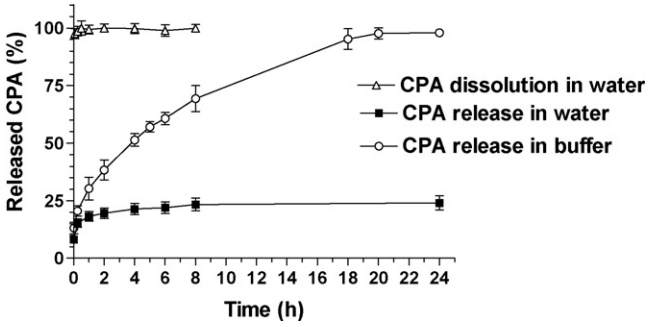

Fig. 2. Dissolution profile in water of free CPA and release profile in water and in phosphate buffer of CPA loaded in stearic acid microparticles. All dissolution and release studies were performed at 37 °C. Data are reported as the mean ± S.E. of four independent experiments.

These data indicate that the polar and slightly hydrophilic CPA ($\log P = 1.21$, Dalpiaz et al., 2001b) diffuses out of the lipid matrix during formation of the microparticles and it remains adsorbed on their external surface, probably by weak interactions with the polar functional groups of the surfactant on the particle outer shell. Indeed, the phosphatidylcholine employed for microparticle preparation, should be orientated with the lipophilic portion into the hydrophobic core of the microparticles and with the polar head facing outward. In addition, the low drug loading values (<0.2%, Table 1) obtained can be traced to the partitioning of CPA from the lipid droplets into the external water phase of the hot emulsion preparation. These results were expected since moderately water-soluble drugs, as CPA (aqueous solubility 0.67 ± 0.04 mg/ml) are not efficiently incorporated into SLMs with usual production methods (Cortesi et al., 2002; Jaspart et al., 2005, Trotta et al., 2005). Several strategies have been developed in order to overcome this limitation including the preparation of multiple emulsions (Bodmeier et al., 1992; Cortesi et al., 2002), the use of the solvent-in-water emulsion–diffusion technique (Trotta et al., 2005), the incorporation of hydrophylic nanoparticles in lipid microspheres (Cook et al., 2005) or the synthesis of hydrophobic derivatives (Jaspart et al., 2007).

However, the complexity of these procedures and the additional requirement of stabilizers (Cortesi et al., 2002) or organic solvents (Trotta et al., 2005) are disadvantages.

In the present study a different approach was explored which takes advantage of the basic nitrogen atoms in the adenine moiety of CPA (Fig. 1). Accordingly, it seemed reasonable to assume that by using an acidic fat excipient, entrapment of CPA in the lipid matrix would be enhanced by acid–base interaction. To demonstrate this working hypothesis, stearic acid was examined as lipid material for the preparation of microparticles. As illustrated in Fig. 2, a reduced release for CPA was achieved in water by the SLMs based on stearic acid which indicated the entrapment of CPA in this lipid matrix. Indeed, the obtained profile in water showed an initial burst of about 20% of the incorporated CPA and a continuous slow release thereafter. A high percentage (76%) of the encapsulated CPA was not released (Fig. 2) during the monitored time period (24 h), as confirmed by determination of the CPA microparticle content after the release experiment. Replacing water as release medium with phosphate buffer (pH 7.4) produced a significant enhancement of the release rate, with more than 65% CPA released at 8 h. In particular the amounts of CPA detected in buffer solution were significantly higher ($p < 0.05$) than those in water after 2 h of release.

Table 1
Effect of the lipidic excipient on the CPA content of the microspheres

Microparticle lipidic phase	Drug loading (%)	Entrapment efficiency (%)
Tristearin	0.08 ± 0.01	2.1 ± 0.4
Glyceryl behenate	0.18 ± 0.06	6.4 ± 1.8
Stearic acid	0.15 ± 0.04	4.4 ± 1.2

All microparticles were obtained in the presence of phosphatidylcoline. Data are reported as mean ± S.D. of four independent measurements.

This observation suggests that the entrapment of CPA in the lipid matrix involves acid–base interaction. Indeed, the pH value of the water medium was found to range between 5.4 and 5.6, allowing an acid–base interaction of stearic acid with the adenine moiety of CPA. On the other hand, at the pH (7.4) of the phosphate buffer medium, the basic nitrogen atoms of adenine moiety become uncharged (Bloomfield et al., 2000), stearic acid is converted to the ionised form and, consequently, their interaction is strongly weakened. Moreover, the ionisation of stearic acid favours water diffusion in the lipoparticles.

The semiempirical equation (3) was applied on the drug release data in the buffer solution. The value of the release exponent n (0.37 ± 0.01, $r = 0.996$) suggests a diffusion-type kinetic. In fact, the value of n is similar to the value of the kinetic exponent of release (0.43 ± 0.01), indicating a Fickian-type release from a spherical device (Ritger and Peppas, 1987).

The amount of CPA incorporated in the microparticles based on stearic acid was found to be 0.15% ± 0.04 (w/w), which corresponded to an encapsulation efficiency of 4.4 ± 1.2% (Table 1). The results obtained on the same samples by applying the two different analysis methods (Section 2.3) appeared statistically equivalent. The very low drug loading can be traced to the amphyphilic characteristics of this drug. In fact CPA presents remarkable challenges for sustained release delivery systems, as confirmed by the drug loading values (0.06–0.12%) reported in previous studies on the encapsulation of CPA in polymeric microparticles (Dalpiaz et al., 2002). However, owing to its high activity, the relatively low amount of encapsulated CPA can be considered to be sufficient to achieve a pharmacological effect, as previously demonstrated with polymeric microparticles (Dalpiaz et al., 2002).

Investigation by SEM on the optimized SLMs, based on stearic acid and phosphatidylcholine, revealed a spherical shape with a quite smooth surface, although irregular fragments were also present (Fig. 3). The particle size was between 3.2 and 10.3 μm (mean diameter, 5.5 ± 1.5 μm).

Additional information on the solid state of the SLMs was obtained by powder X-ray diffraction. As illustrated in Fig. 4, pure CPA (a) exhibited distinct diffraction peaks. The diffractogram of the physical mixture of the drug with blank microparticles (b) displayed the characteristic crystalline peak of CPA at 4.5° and peaks due to stearic acid (20.3°, 21.2°, 23.8°). These signals were also detectable, though with a lower intensity,

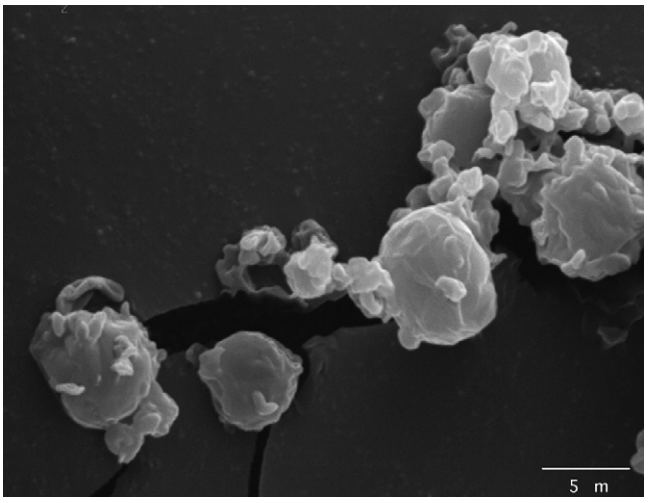

Fig. 3. Scanning electron microscopy (SEM) micrograph of stearic acid SLMs loaded with CPA.

in the diffraction pattern of the loaded lipoparticles (c), suggesting the presence of CPA at least partially in the crystalline state in the SLMs.

3.2. Evaluation of CPA degradation kinetics

The kinetics of CPA degradation are represented in Fig. 5, where three degradation-time relationships are shown: free CPA (open circles), CPA encapsulated in the microparticles (solid circles) and free CPA in the presence of unloaded microspheres (open squares, inset). The degradation rate of free CPA was not altered by the presence of unloaded microspheres. The corresponding half-life (t) values are, in fact, 22.0 ± 1.8 and 21.4 ± 1.9 min in the absence and in the presence, respectively, of unloaded microspheres. These data are in good agreement with those obtained by previous studies on CPA pharmacokinetics (Dalpiaz et al., 2001a). On the other hand, it is interesting to observe that the degradation of CPA encapsulated in microspheres was significantly reduced with respect to that of free CPA. Indeed, after 1 h the extent of degradation of free and encapsulated CPA was 80 and 30%, respectively. After 3 h the

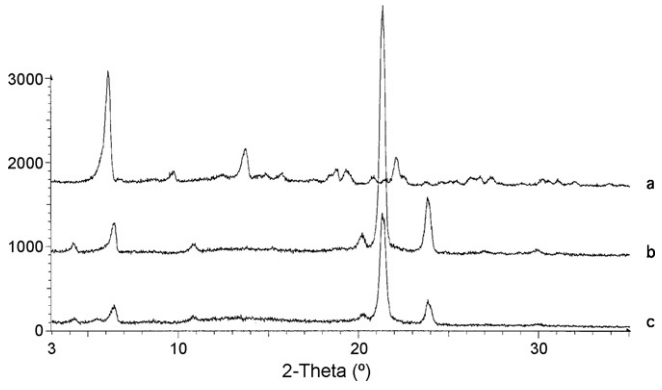

Fig. 4. Powder X-ray diffraction patterns of CPA (a), CPA-unloaded lipoparticles physical mixture (b) and CPA-loaded lipoparticles (c).

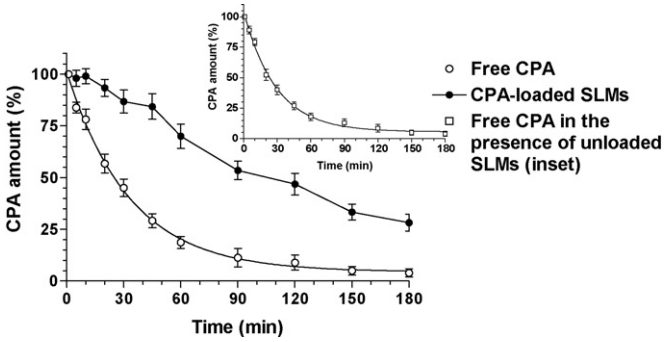

Fig. 5. Degradation kinetics in human whole blood of free or encapsulated CPA in stearic acid SLMs. The inset reports the degradation of free CPA in the presence of unloaded microparticles. Data are reported as the mean ± S.E. of three independent experiments.

degradation of free CPA was completed, whereas 30% of the encapsulated drug appeared not yet degraded.

Two aspects can be highlighted by the analysis of these results: (i) the prepared microparticles do not interfere with the enzymatic systems involved in CPA degradation, suggesting a good biocompatibility of this system; (ii) the CPA release profile in phosphate buffer indicate the possibility to achieve a CPA stabilisation in physiological fluids. However the protective effect in whole blood was lower than that expected by the analysis of the release profile of encapsulated CPA in buffer solution (Fig. 2). This difference can be ascribed to the biodegradation of the lipid matrix in the blood.

In conclusion, although the encapsulation of CPA in polymeric micro-and nanoparticles has been described (Dalpiaz et al., 2001a, 2002, 2005; Leo et al., 2006a,b), this is the first report on the incorporation of the polar CPA in lipid particles. The latter are particularly suitable for modern strategies of particle technology, since the non-polymeric natural excipients employed are more suitable for registration as their clearance is favourable.

This microparticulate system, able to control the release of CPA, can be useful for the topical application of this drug to enhance wound healing and hair growth (Sun et al., 1999).

Owing to micronic size of the particles and their optimal biocompatibility for the mucosal tissues, SLMs could also be nasally administered. It could be interesting to investigate if these systems may exploit the direct CPA transport pathway from nose to brain via the olfactory region (Illum, 2003, 2004; Vyas et al., 2005). In this case, further investigations are necessary in order to enhance the microparticle drug loading.

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