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RESEARCH ARTICLE

In vitro evaluation of idebenone-loaded solid lipid nanoparticles for drug delivery to the brain

Lucia Montenegro[1], Agata Campisi[2], Maria Grazia Sarpietro[3], Claudia Carbone[1], Rosaria Acquaviva[2], Giuseppina Raciti[2], and Giovanni Puglisi[1]

[1]Department of Pharmaceutical Sciences, University of Catania, Catania, Italy, [2]Department of Medicinal Chemistry and Biochemistry, University of Catania, Catania, Italy, and [3]Department of Chemical Sciences, University of Catania, Catania, Italy

Abstract

Context: Solid lipid nanoparticles (SLN) are regarded as interesting drug delivery systems and their preparation techniques have gained a great deal of attention.

Objective: To evaluate the feasibility of preparing idebenone (IDE) loaded SLN from O/W microemulsions by the phase-inversion temperature (PIT) method. Since SLN have been proposed to improve drug delivery to the brain, IDE was chosen as model drug due to its activity in the treatment of neurodegenerative diseases.

Materials and Methods: Cetyl palmitate was used as solid lipid to prepare SLN containing two surfactant/cosurfactant mixtures, isoceteth-20/glyceryl oleate (SLN A) and ceteth-20/glyceryl oleate (SLN B) by the PIT method.

Results and discussion: All the formulations tested showed a mean particle diameter ranging from 30 to 95 nm and a single peak in size distribution. Stability tests showed that SLN B were more stable than SLN A. IDE release was dependent both on the type of primary surfactant used and the amount of loaded drug. IDE-loaded SLN were effective in inhibiting 2,2′-azobis-(2-amidinopropane)dihydrochloride (APPH)-induced lactic dehydrogenase (LDH) release and reactive oxygen species (ROS) production in primary cultures of astrocytes obtained from rat cerebral cortex. It is noteworthy that SLN B2 (containing ceteth-20 as primary surfactant and 0.7% w/w IDE) were able to prevent entirely both the LDH release and ROS production induced by APPH.

Conclusion: The PIT method provided SLN with good technological properties. The tested SLN could be regarded as interesting carriers to overcome the blood brain barrier and increase the efficacy of the loaded drug.

Keywords: *In vitro* release, blood brain barrier, drug delivery, *in vitro* antioxidant activity, colloidal carrier

Introduction

In recent years, solid lipid nanoparticles (SLN) have been proposed as drug delivery systems for topical and systemic therapy, due to their advantages compared to other colloidal carriers such as good tolerability, improved drug stability, drug targeting, increased bioavailability, ability to incorporate drugs with different physico-chemical properties, wide potential application spectrum (topical, oral, intravenous administration; Müller et al., 2000; Mehnert and Mäder, 2001). Different methods have been developed to prepare SLN but none of them is devoid of drawbacks. High-pressure (hot or cold) homogenization is regarded as a reliable and useful technique for the preparation of SLN (Mehnert and Mäder, 2001; Muchow et al., 2008).

Hot homogenization is performed at temperatures above the melting point of the lipid and it allows to obtain particles with very small sizes. However, high temperatures may increase the degradation rate of the drug and the carrier. Furthermore, the lipid crystallization may be retarded due to the formation of a supercooled melt (Bunjes et al., 1998). High-pressure cold homogenization results in larger particle sizes and broader size distribution compared to hot homogenization (Mehnert and

Address for Correspondence: Lucia Montenegro, Department of Pharmaceutical Sciences, University of Catania, V.le A. Doria, 6, 95125 Catania, Italy. E-mail: lmontene@unict.it

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Mäder, 2001). The solvent emulsification/evaporation technique provides SLN with very small particle sizes avoiding any thermal stress (Siekmann and Westesen, 1996). However, the need of using organic solvents is a clear disadvantage of this technique. In 1993, Gasco developed an SLN preparation technique based on dilution in cold water of oil-in-water microemulsions (MEs). Although this method allows to overcome some of the problems connected to other techniques, the need of using high surfactant concentrations to prepare the initial ME could represent an important limit to its pharmaceutical application.

Recently, the feasibility of using the phase-inversion temperature (PIT) method to obtain MEs with low surfactant content has been reported (Diec et al., 2001; Montenegro et al., 2006).

In this study, we investigated the relevance of the PIT method as a new technique for the preparation of SLN from O/W MEs to overcome the disadvantages of other preparation techniques and to allow large scale production. With this aim, cetyl palmitate (CP) was selected as lipid solid at room temperature to prepare SLN containing low amounts of two surfactant/cosurfactant mixtures, isoceteth-20/glyceryl oleate and ceteth-20/glyceryl oleate. CP was chosen as solid lipid because its use in SLN intended for systemic administration has been widely investigated and it has been reported to be a safe ingredient (Lukowski et al., 2000; Wang et al., 2009; Yang et al., 2010). The emulsifying systems were selected from previous studies performed on MEs obtained using the PIT method (Montenegro et al., 2006). Non-ionic surfactants were used because of their low toxicity compared to ionic ones. Since SLN have been regarded as a promising alternative to other colloidal drug carriers to improve drug delivery to the brain (Wissing et al., 2004a), we chose idebenone (IDE) as model drug. IDE has been proposed for the treatment of neurodegenerative diseases such as Parkinson's and Alzheimer diseases due to its antioxidant properties (Schöls et al., 2004). Therefore, the influence of different surfactant's structure and drug content on size, stability, and drug release profile of IDE-loaded SLN was investigated. *In vitro* biological tests were performed in rat primary cultures of astrocytes in order to evaluate the antioxidant properties of IDE incorporated in SLN prepared using the PIT method.

Materials and methods

Materials

CP (Cutina CP) was purchased from Cognis S.p.a. Care Chemicals (Como, Italy). Polyoxyethylene-20-cetyl ether (Brij 58) was supplied by Fluka (Milan, Italy). Polyoxyethylene-20-isohexadecyl ether (Arlasolve 200 L) was a kind gift of Bregaglio (Milan, Italy). Glyceryl oleate (Tegin O) was obtained from Th. Goldschmidt Ag (Milan, Italy). IDE was a kind gift of Wyeth Lederle (Catania, Italy). Nicotinamide-adenine dinucleotide, reduced form disodium salt, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2′,7′-dichlorofluorescein diacetate were obtained from Sigma Aldrich (Milan, Italy). 2,2′-azobis-(2-amidinopropane)dihydrochloride (APPH) was obtained from Trimital (Magenta, Italy). Cell-culture medium and sera were obtained from Invitrogen (Milan, Italy). Methylchloroisothiazolinone and methylisothiazolinone (Kathon CG®), and imidazolidinyl urea were kindly supplied by Sinerga (Milan, Italy). Regenerated cellulose membranes (Spectra/Por CE; Mol. Wet. Cut off 3000) were supplied by Spectrum (Los Angeles, CA). Methanol and water used in the high-performance liquid chromatography (HPLC) procedures were of LC grade and were bought from Merck (Darmstadt, Germany). All other reagents were of analytical grade and they were used as supplied.

Preparation of SLN

SLN, whose composition is reported in Table 1, were prepared using the phase-inversion temperature (PIT) method (Montenegro et al., 2006). The aqueous phase was a phosphate buffer (pH 7.4) consisting of: (A) NaCl 4.60 g/L, phosphate monobasic 0.64 g/L and phosphate dibasic 5.0 g/L for the preparation of SLN A (containing isoceteth-20/glyceryl oleate); (B) NaCl 3.0 g/L, phosphate monobasic 0.60 g/L and phosphate dibasic 6.40 g/L for the preparation of SLN B (containing ceteth-20/glyceryl oleate). Both buffers contained 0.35% w/w imidazolidinyl urea and 0.05% w/w methylchloroisothiazolinone and methylisothiazolinone as preservatives.

The aqueous phase and the oil phase (CP and the selected emulsifiers) were separately heated at ~90°C; then the aqueous phase was added drop by drop, at constant temperature and under agitation, to the oil phase.

Table 1. Composition (% w/w) of SLN formulations.

SLN	Ceteth-20	Isoceteth-20	Glyceryl oleate	Cetyl palmitate	Idebenone	Buffer*
A	/	10.6	3.5	8.0	/	q b 100
A 1	/	10.6	3.5	8.0	0.5	q b 100
A 2	/	10.6	3.5	8.0	0.7	q b 100
B	8.7	/	4.4	8.0	/	q b 100
B 1	8.7	/	4.4	8.0	0.5	q b 100
B 2	8.7	/	4.4	8.0	0.7	q b 100
B 3	8.7	/	4.4	8.0	1.1	q b 100

*Phosphate buffer containing 0.35% w/w imidazolidinyl urea and 0.05% w/w Kathon CG.
SLN, solid lipid nanoparticles.

The mixture was then cooled to room temperature under slow and continuous stirring.

At the PIT, the turbid mixture turned clear. The PIT temperature was determined using a conductivity meter mod. 525 (Crison, Modena, Italy) which measured an electric conductivity change when the inversion from W/O to O/W system occurred. To prepare SLN containing the active ingredient different amounts of IDE (0.5–1.1% w/w) were added to the oil phase. A thin-layer chromatography analysis confirmed that no degradation of IDE occurred under these conditions.

Determination of IDE solubility

The solubility of IDE in water was determined in triplicate by stirring an excess of drug in 2 mL of solvent with a magnetic stirrer for 24 h at room temperature. The experiments were performed avoiding light exposure to prevent IDE photodegradation. Thereafter, the mixture was filtered and IDE concentration in its saturated solution was determined by the HPLC method described below.

Transmission electron microscopy

For negative-staining electron microscopy, 5 μL of SLN dispersions were placed on a 200-mesh formvar copper grid (TAAB Laboratories Equipment, Berks, UK), and allowed to be adsorbed. Then the surplus was removed by filter paper. A drop of 2% (w/v) aqueous solution of uranyl acetate was added over 2 min. After the removal of the surplus, the sample was dried at room condition before imaging the SLN with a transmission electron microscope (model JEM 2010, Jeol, Peabody, MA) operating at an acceleration voltage of 200 KV.

Photon correlation spectroscopy

The particle sizes of the SLN tested were determined using a Zetamaster S (Malvern Instruments, Malvern, UK), at 20°C, by scattering light at 90°. The instrument performed particle sizing by means of a 4 mW laser diode operating at 670 nm. The values of the mean diameter and polydispersity index were the averages of results obtained for three replicates of two separate preparations.

Differential scanning calorimetry analyses

Differential scanning calorimetry (DSC) studies were performed using a Mettler TA STAR^e System equipped with a DSC 822^e cell and a Mettler STAR^e version 8.10 software (Mettler Toledo, Milan, Italy). The reference pan was filled with 100 μL of the same phosphate buffer used to prepare the SLN under investigation. The calorimetric system was calibrated, in transition temperature and enthalpy changes, by using indium and palmitic acid (purity ≥ 99.95% and ≥ 99.5%, respectively; Fluka, Switzerland) and following the procedure of the Mettler STAR^e software. 100 μL of each sample was transferred into a 160 μL calorimetric pan, hermetically sealed and submitted to DSC analysis as follows: (A) a heating scan from 5 to 65°C, at the rate of 2°C/min; (B) a cooling scan from 65 to 5°C, at the rate of 4°C/min, for at least three times.

Each experiment was carried out in triplicate.

Stability tests

Samples of SLN were stored in airtight jars, and then kept in the dark at room temperature and at 37°C for 2 months.

Droplet size, polydispersity index, pH, and osmolarity of the samples were measured at fixed time intervals (24 h, 1 week, 2 weeks, 3 weeks, 1 month, and 2 months) after their preparation. A pH-meter CRISON, mod. Basic 20 (Milan, Italy) was used to measure pH values. Osmolarity was determined using an osmometer Osmomat, mod. 030-D (Gonotec, Berlin, Germany). The instrument was previously calibrated with a sample of saline whose osmolarity value was 300 mosm/kg.

In vitro release experiments

IDE release rates from the prepared SLN were measured through regenerated cellulose membranes using Franz-type diffusion cells (LGA, Berkeley, CA). This technique has been previously reported in the literature as a suitable method for evaluating drug release from pharmaceutical formulations (Shah et al., 1989).

The cellulose membranes were moistened by immersion in water for 1 h at room temperature before being mounted in Franz-type diffusion cells. Diffusion surface area and receiving chamber volume of the cells were, respectively 0.75 cm^2 and 4.5 mL. The receptor was filled with water/ethanol (50/50 v/v) for ensuring pseudo-sink conditions by increasing active compound solubility in the receiving phase (Touitou and Fabin, 1988; Montenegro et al., 2006). A receiving phase consisting of water/ethanol 50/50 has already been used by other authors (Stancampiano et al., 2006) who, studying *in vitro* release of IDE from SLN, reported that the presence of ethanol did not compromise the nanoparticle integrity. The receiving solution fluid was constantly stirred (700 rpm) and thermostated at 37°C. 500 μL of each formulation were applied on the membrane surface and the experiments were run for 24 h. Due to IDE photoinstability, all the release experiments were carried out avoiding light exposure. At intervals, 200 μL of the receptor phase were withdrawn and replaced with an equal volume of receiving solution equilibrated to 37°C. The receptor phase samples were analyzed by the HPLC method described below to determine the active compound content. At the end of the experiments, samples of the SLN applied on the membrane surface were withdrawn and analyzed to determine particle sizes and polydispersity indexes. Each experiment was performed in triplicate.

In vitro biological tests

Cell culture

Primary cultures of astrocytes were prepared from cerebral cortex of newborn albino rats (1–2-day-old Wistar strain) as previously reported (Booher and Sensenbrenner, 1972). Briefly, after dissection and mechanical dissociation of cerebral tissues, isolated

cells were suspended in Dulbecco's Modified Eagle's Medium supplemented with 20% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, streptomycin (50 μg/mL), and penicillin (50 U/mL). Cells were plated in 2.5 mm of diameter Falcon Petri dishes at a density of 0.6 × 106 cells and maintained at 37°C in a 5% CO_2/95% air humidified atmosphere for 2 weeks. The medium of cell cultures was replaced twice a week. Astroglial cell purity was performed by immunofluorescence staining for glial fibrillary acidic protein (GFAP) as previously reported (Campisi et al., 2003).

Treatment

Astrocytes at 13 days *in vitro* (DIV) were treated with 20 mM 2,2'-azobis-(2-amidinopropane)dihydrochloride (APPH), a hydrosoluble peroxy-radical generator, and the SLN formulations being tested. The effects on cell cultures were evaluated by the MTT assay, lactic dehydrogenase (LDH) release, and reactive oxygen species (ROS) production. Astrocyte cultures were incubated for 12 h at 37°C with APPH and the SLN formulations A, A1, A2, B, B1, and B2 at an equivalent drug concentration of 6.25 or 12.50 μM for SLN A1 and B1 and 8.75 or 17.5 μM for SLN A2 and B2. The formulation SLN B3 was not tested due to its low IDE release. Three measurements for each samples were performed.

MTT bioassay

To monitor cell viability, the astrocytes were set up 60×10^4 cells per well of a 96-multiwell flat-bottomed 200 μL microplate (Acquaviva et al., 2004). Cells were incubated at 37°C in a humidified 5% CO2/95% air mixture. At the end of treatment time, 20 μL of 0.5% MTT in phosphate buffer saline were added to each microwell. After 1 h of incubation with the reagent, the supernatant was removed and replaced with 200 μL of dimethylsulfoxide. The optical density of each well sample was measured with a microplate spectrophotometer reader (Titertek Multiskan, Flow Laboratories, Helsinki, Finland) at 570 nm.

LDH release

LDH release was measured to evaluate the presence of cell necrosis as a result of cell disruption subsequent to membrane rupture. LDH activity was measured spectrophotometrically in the culture medium and in the cellular lysates at $\lambda = 340$ nm by analyzing NADH reduction during pyruvatelactate transformation (Murphy and Baraban, 1990). The amount of LDH released was calculated as percentage of the total amount, considered as the sum of the enzymatic activity present in the cellular lysate and that in the culture medium. A Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) was used.

ROS formation

ROS determination was performed by using the fluorescent 2′,7′-dichlorofluorescein diacetate (DCFH-DA) probe; 100 μM DCHF-DA, dissolved in 100% methanol was added to the cellular medium and the cells were incubated at 37°C for 30 min. Under these conditions, the acetate group is not hydrolyzed (Hempel et al., 1999). After incubation, astrocytes were lysated and centrifuged at 10 000 *g* for 10 min. The fluorescence [corresponding to the radical species-oxidized 2′,7′-dichloro-fluorescein (DCF)] was monitored spectrofluorometrically using a Hitachi F-2000 instrument (Hitachi, Tokyo, Japan): excitation 488 nm, emission 525. The values were expressed as fluorescence intensity/mg protein. Protein concentration was measured as previously reported (Bradford, 1976).

HPLC analysis

The HPLC apparatus consisted of a Hewlett–Packard model 1050 liquid chromatograph (Hewlett–Packard, Milan, Italy), equipped with a 20 μL Rheodyne model 7125 injection valve (Rheodyne, Cotati, CA) and an UV-VIS detector (Hewlett–Packard, Milan, Italy).

The chromatographic analyses were performed using a Simmetry, 4.6 × 15 cm reverse phase column (C_{18}; Waters, Milan, Italy) at room temperature and a mobile phase consisting of a methanol/water mixture (80:20 v/v). The column effluent (flow rate 1 mL/min) was monitored continuously at 280 nm to detect IDE. Quantifying IDE was performed by measuring the peak areas in relation to those of a standard calibration curve that was built up by relating known concentrations of IDE with the respective peak areas. No interference of the other formulation components was observed. The sensitivity of the HPLC method was 0.1 μg/mL.

Data analysis

The transfer rate constants (k) of the active compound between the particles and the aqueous phase were calculated according to a model that considers the rate of release to be limited by an interfacial barrier (Guy et al., 1982; Montenegro et al., 2006) using the following equation:

$$\ln\left(1-\frac{Mt}{M_0}\right) = -\frac{-3kt}{r^2},$$

where Mt/M_0 is the fraction of released drug at time t and r is the particle radius. Plotting the natural logarithm of the fraction of released drug against time, release curves whose slope was $-3k/r^2$ were obtained.

IDE apparent diffusion coefficients (D_{app}) through cellulose membranes were calculated according to the relation:

$$D_{app} = \frac{h^2}{6t_L},$$

where h is the thickness of the membrane (25 nm) and t_L is the lag time. The lag time was calculated by plotting the cumulative amount of IDE released against time and dividing the slope of the steady-state portion of the graph by the area through which diffusion took place. The lag time was determined from the x-intercept values of the regression line.

Results are expressed as mean values ± standard deviation. Statistical data analysis was performed using a one-way analysis of variance with a posteriori Bonferroni's t-test.

Results and discussion

SLN characterization and stability

The percentages of surfactants required to obtain SLN containing 8% w/w of the lipid solid at room temperature were determined from preliminary experiments showing that the lowest amount of emulsifying systems needed were: 10.6% of isoceteth-20 and 3.5% of glyceryl oleate (SLN A), and 8.7% of ceteth-20 and 4.4% of glyceryl oleate (SLN B).

Transmission electron microscopy (TEM) analyses of SLN A and B (empty or loaded) showed spherical particles with no evident sign of aggregation (Figure 1).

Many authors reported that DSC analysis can be used to determine the physical state of the core lipid in SLN (Müller et al., 2000; Mehnert and Mäder, 2001). In general, the melting peak of the lipid core of the SLN is observed at a lower temperature than that of bulk lipid, mainly due to the nanocrystalline size of the lipids in the SLN (Westesen and Bunjees, 1995). As shown in Figure 2, the calorimetric curve of CP bulk was characterized by a broad peak at about 39°C and a main peak centered at about 50.5°C. The calorimetric curve of SLN B exhibited a well defined peak at about 38°C and a shoulder at 42°C. Similar behaviors were observed for SLN A and IDE-loaded SLN (data not shown). The melting peak of loaded or unloaded SLN was observed at a temperature about 12–14°C lower than the bulk CP, suggesting that CP located in the core of the SLN had been successfully solidified by the PIT method we used to prepare SLN. As previously reported by others (Lee et al., 2007), these results confirmed that SLN were prepared.

As shown in Table 1, IDE *loading capacity* was different for SLN A and B (0.7 and 1.1% w/w, respectively). This behavior could be due to the different structure of the acyl chain of the primary surfactant: the isopropylic group of isoceteth-20 acyl chain could determine a steric hindrance that prevented the incorporation of higher amounts of active compound.

All the formulations tested showed physiological pH (7.2–7.3) and osmolarity (289–316 mosm) values, a mean particle diameter in the range of 30–95 nm, and a single peak in size distribution (Table 2). Incorporation of IDE, in the lipid particles containing isoceteth-20 as primary surfactant, resulted in a decrease of particle sizes since the control (SLN A) had a mean size greater than the corresponding IDE-loaded SLN containing 0.5% (SLN A1) and 0.7% w/w (SLN A2) of IDE (Table 2). Analogous behavior was observed for polydispersity indexes, with a better homogeneity for SLN containing the active compound with respect to control. Formulations containing ceteth-20/glyceryl oleate as emulsifying system showed different results about size and polydispersity values. The control (SLN B) had a small particle size (30 nm) that increased by addition of 0.5 or 0.7% w/w of IDE with simultaneous decrease of homogeneity. A further increase of IDE incorporation to 1.1% w/w had as a consequence the reduction of particle size with a better homogeneity, so that SLN B3, containing the highest IDE concentration (1.1% w/w), had a mean particle size analogous to that of the corresponding control. Wissing et al. (2004b), reported that coenzyme Q_{10}, that is structurally related to IDE, was in part homogenously dispersed within the SLN matrix and in part assembled in separate nanoaggregates. The different status of IDE into the solid

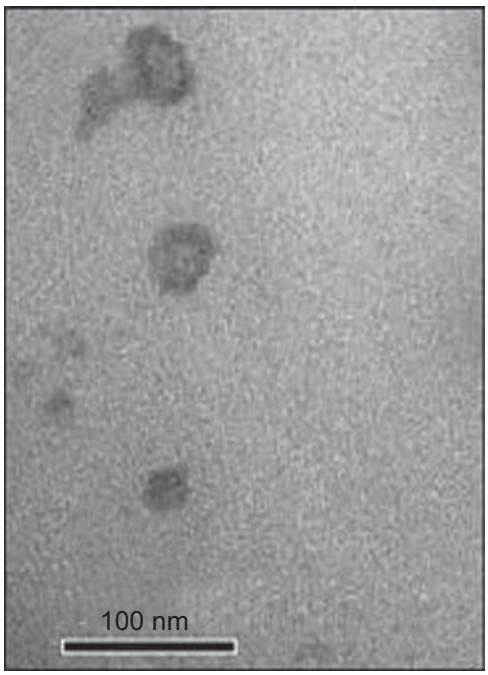

Figure 1. Transmission electron microscopy picture of solid lipid nanoparticles prepared by the phase-inversion temperature method.

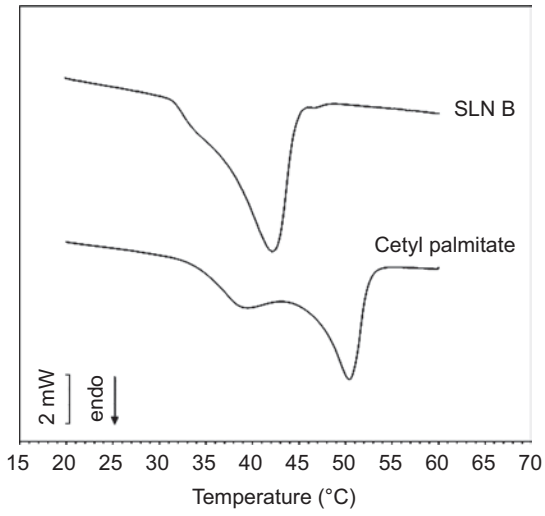

Figure 2. Differential scanning calorimetry curves of solid lipid nanoparticles (SLN) B and cetyl palmitate.

Table 2. Characterization of SLN formulations: PIT, pH, Osm size ± SD, and polydispersity indexes ± SD (poly ± SD) 24 h after their preparation.

SLN	PIT (°C)	pH	Osm (mosm)	Size ± SD (nm)	Poly ± SD
A	80	7.26	298	95.3 ± 1.9	0.504 ± 0.098
A 1	80	7.21	307	42.5 ± 0.6	0.391 ± 0.011
A 2	80	7.24	308	45.4 ± 2.0	0.433 ± 0.027
B	80	7.20	289	30.3 ± 0.3	0.217 ± 0.045
B 1	80	7.30	316	48.7 ± 0.9	0.523 ± 0.019
B 2	78	7.31	305	45.3 ± 1.1	0.489 ± 0.084
B 3	81	7.30	307	29.9 ± 0.2	0.156 ± 0.017

Osm, osmolarity; PIT, phase-inversion temperature; SD, standard deviation; SLN, solid lipid nanoparticles.

lipid matrix could account for the changes of particle size observed by loading different amount of IDE into SLN. In order to elucidate the above findings, further studies are ongoing to asses the state of dispersion of IDE within the lipid matrix.

As reported in the literature (Izquierdo et al., 2005), the phase-inversion temperature of emulsion-based systems is a parameter that could allow to predict the formulation stability. Since PIT values were similar for all the formulations tested (Table 2), the same stability would have been expected for SLN A and B. Stability tests, performed at room temperature and 37°C, did not show any significant variation of pH values (data not shown). On the contrary, an increase of osmolarity values was observed for SLN A stored at room temperature while SLN B did not show any significant variation. (Table 3). Furthermore, particle size analyses of formulations stored for 2 months at room temperature showed a different behavior for SLN A and SLN B since formulations with ceteth-20 showed a better stability than those prepared using isoceteth-20 (Figure 3). The increase of particle sizes observed could suggest the presence of aggregation phenomena between nanoparticles, so to produce the growing of mean size. Experimental data showed less stability both in terms of osmolarity and particle size for all the formulations when stored at 37°C (data not shown). As reported in the literature (Mehnert and Mäder, 2001), less stability at higher temperature could be due to the introduction of energy into the system, that leads to particle growth and subsequent aggregation. Our results suggest that the different structure of the acyl chain of the primary surfactants could determine different packing of the surfactant and cosurfactant molecules at the interface, leading to particles with different curvature radius and stability properties.

In vitro drug release

In this work we assessed *in vitro* drug release from SLN prepared by the PIT method using the infinite dose technique, i.e. applying a large amount of formulation (500 µL) on the membrane surface. The use of an infinite dosing in *in vitro* release studies avoids compound depletion from the donor compartment during the experiment, thus ensuring a constant driving force for the release process and allowing the achievement of steady-state conditions.

IDE was supposed to be released only from the lipid phase of SLN dispersion because of its very poor water solubility (3 µg/mL) that prevented its solution in water, as reported for other lipophilic drugs loaded into SLN, such as vitamin A (Jenning et al., 2000). Furthermore, all the formulations tested were clear with no sign of drug precipitation. Plotting the cumulative amount of active compound released during 24 h from SLN A and B as a function of time, different release profiles depending on type of surfactant and drug content were obtained (Figures 4 and 5). An initial slow release followed by a faster release of the active compound was observed for all SLN under investigation. This behavior could be explained taking into account the experimental conditions used in this study. Since *in vitro* release experiments were performed leaving the donor phase open to the air (non-occlusion conditions), water evaporated from the SLN formulations so that during 24 h the liquid dispersion turned slowly into a semisolid gel. As reported in the literature (Jenning et al., 2000), gel formation of SLN can be correlated with polymorphic transitions of the lipid matrix. Different polymorphic form of the lipid can affect drug release from SLN due to the different ability to include host molecule, so that drug expulsion from the solid matrix is a consequence of the transition. Since IDE is poorly soluble in water, an increase of its release from SLN results in an increase of its thermodynamic activity that, in turn, increases the diffusion rate from the donor phase.

The lower apparent diffusion coefficient of IDE from SLN B compared to SLN A could be due to the different lipophilicity of the emulsifiers used. Log P values of ceteth-20 and isoceteth-20 (calculated by ACD/LogPDB software ver. 11.01) were 2.48 and 2.33, respectively. The higher lipophilicity of ceteth-20 (SLN B) could reduce IDE thermodynamic activity in the surface layers of the particles and this could result in an unfavorable effect on drug release dynamics. Data reported in Table 4 show that SLN B2, containing ceteth-20 as primary surfactant and IDE 0.7% w/w, provided the highest cumulative amount of drug released after 24 h (Q_{24}). No significant difference was found between SLN A1 and B1 carrying the lowest IDE amount (0.5% w/w). The lowest Q_{24} value was observed for system B3, characterized by the couple of surfactants ceteth-20/glyceryl oleate and the highest drug loading (1.1% w/w).

To explain the observed release profiles, transfer rate constants (K) from SLN and D_{app} through cellulose

Table 3. Osmolarity values of SLN A and B during storage at RT for 2 months.

	Osmolarity						
SLN	T_0	24 h	7 Days	14 Days	21 Days	30 Days	60 Days
A	298	307	306	306	317	352	404
A 1	307	315	312	317	381	403	403
A 2	308	312	310	325	384	409	412
B	289	289	289	286	286	293	348
B 1	316	313	312	315	315	316	303
B 2	305	290	291	300	312	315	353
B 3	307	295	316	309	313	317	356

RT, room temperature; SLN, solid lipid nanoparticles.

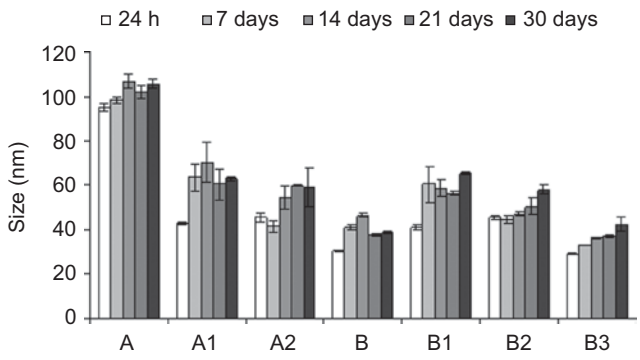

Figure 3. Mean particle size during storage at room temperature for 2 months.

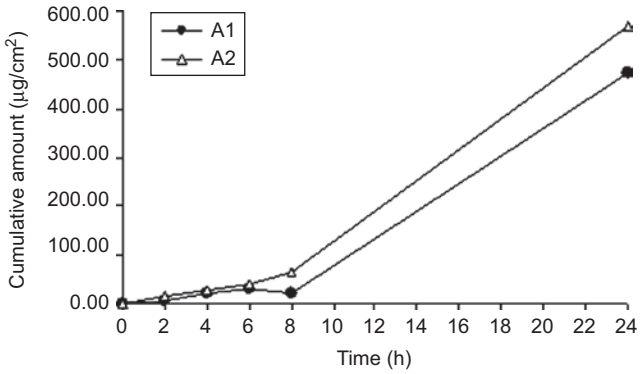

Figure 4. *In vitro* release of idebenone through cellulose membranes from solid lipid nanoparticles A1–A2.

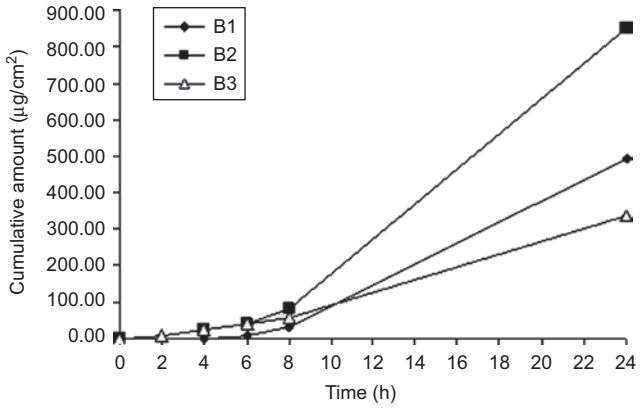

Figure 5. *In vitro* release of idebenone through cellulose membranes from solid lipid nanoparticles B1–B3.

membranes were calculated (Table 4). D_{app} values were in the same order of magnitude for all the tested SLN and were higher than the corresponding transfer rate constants. These results show that the rate-limiting step for *in vitro* IDE release from the SLN under investigations was drug diffusion out of the lipid nanoparticles rather than drug diffusion through the cellulose acetate membrane. The observed transfer rate constants were consistent with an interfacial barrier being the rate-limiting step for drug release. The important role of interface structure in determining the barrier properties to drug diffusion out of O/W micelles has already been reported (Siekmann and Westesen, 1996; Trotta, 1999). As shown in Figures 6 and 7, the release of IDE out of the particles of SLN A and B was similar although different interactions between drug and vehicle components may occur since the addition of 1.1% of drug into formulation A did not allow to obtain a stable system. Q_{24} and K values of SLN B3 were found to be lower than those obtained for the other SLN, although a greater amount of drug was loaded. The higher concentration of IDE into lipid nanoparticles could probably lead to changes in the packing of the surfactant molecules at the interface. A different organization of surfactants could occur with a consequent smaller curvature radius, demonstrated by the smaller mean particle diameter of SLN B3 (30 nm), similar to those obtained for the corresponding unloaded SLN.

In vitro biological test

Our experimental model employed primary cultures of astrocytes obtained from rat cerebral cortex and maintained for 13 (DIV). After 13 days, cell cultures were enriched in differentiated astrocytes with a GFAP-positive staining of 90% of cells and high levels of glutamine synthetase activity (Campisi et al., 2003). Cultures of newborn rat astrocytes were exposed to IDE-loaded SLN at different drug concentrations. To evaluate the antioxidant activity, an oxidative stress was induced in cells by a peroxyl radical produced by thermolysis of APPH; thereafter, each formulation was tested for protective effects against the APPH-induced cell damage.

MTT assay was performed to monitor cell viability, measuring the conversion of tetrazolium salt to yield colored formazan, the amount of which is proportional to the number of living cells. Results did not show any significant modification in succinate dehydrogenase

Table 4. Fluxes ± SD, lag time, cumulative amount released after 24 h (Q_{24} ± SD), transfer rate constants (K) from particles and apparent diffusion coefficients (D_{app}) through cellulose membrane from SLN A and B.

SLN	% IDE	Flux ± SD (μg/cm²/h)	Lag time (h)	Q_{24} ± SD (μg/cm²)	$K \times 10^2$ (nm² s⁻¹)	$D_{app} \times 10^2$ (nm² s⁻¹)
A1	0.5	27.73 ± 2.51	2.88	473.95 ± 32.01	31.30	361.7
A2	0.7	33.13 ± 2.04	2.51	567.64 ± 21.72	42.67	415.0
B1	0.5	29.29 ± 3.05	3.25	492.71 ± 28.05	43.42	320.5
B2	0.7	51.16 ± 4.81	2.87	849.44 ± 64.79	65.44	362.9
B3	1.1	19.72 ± 1.57	2.36	338.22 ± 27.93	10.94	441.4

IDE, idebenone; SD, standard deviation; SLN, solid lipid nanoparticles.

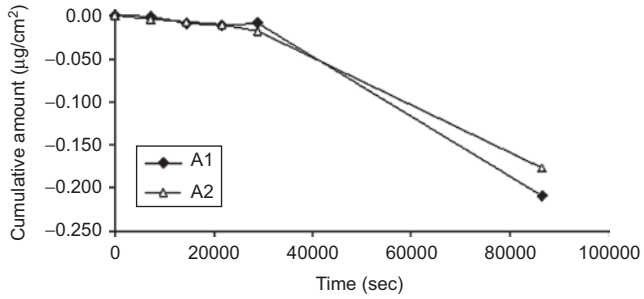

Figure 6. Natural logarithm of the amount of idebenone remaining in the particles against time during *in vitro* release experiments from solid lipid nanoparticles A1–A2.

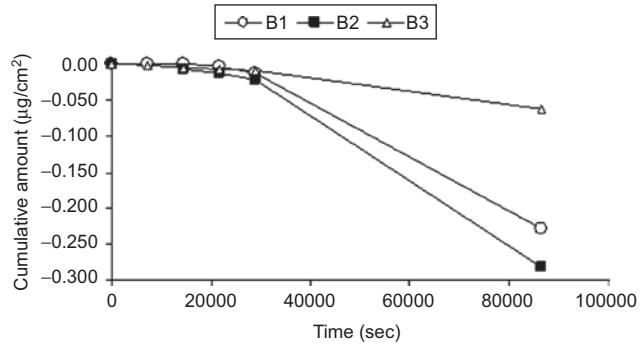

Figure 7. Natural logarithm of the amount of idebenone remaining in the particles against time during *in vitro* release experiments from solid lipid nanoparticles B1–B3.

activity, the major mitochondrial enzyme responsible for the MTT formazan conversion. Therefore, all the formulations at different concentrations, added to the cultures, did not cause any cytotoxic effect compared to the controls (data not shown).

The cellular necrosis of tissues induced by APPH is associated with the release of LDH; therefore, by measuring the percentage of LDH released in the medium it is possible to verify the integrity of cytoplasm membrane. Addition of APPH caused a strong release of LDH compared to control cells.

Exposure of cells to empty SLN A and B did not induce any significant increase of LDH release with respect to controls, thus confirming the absence of cytotoxicity of the SLN under investigation (data not shown). As expected, empty nanoparticles were not able to antagonize the toxicity of APPH (Figure 8). IDE-loaded SLN A and B were effective in contrasting the cell damaging effects of APPH although no clear dose-dependent profile was observed. At the highest equivalent drug dose (17.5 μM) SLN B2 were able to prevent entirely the LDH release induced by the treatment with APPH since LDH release values were equal to those obtained for the controls.

ROS were determined using a fluorescent probe, DCFH-DA. The probe diffuses into the cells, intracellular esterases hydrolyze the acetate groups and the resulting DCFH then reacts with intracellular oxidants resulting in the observed fluorescence that is proportional to the levels of intracellular oxidant species. A shown in Figure 9, IDE-loaded SLN exerted a strong protective effect against APPH-induced ROS production. An activity profile similar to that observed in *in vitro* LDH release experiments was obtained with no clear dose-effect relationship.

Other authors (Stancampiano et al., 2006) reported that IDE loaded into SLN was more effective in inhibiting APPH-induced LDH release in primary cultures of astrocytes compared to free IDE, even at higher concentrations (25 μM), but it was less effective in antagonizing ROS production. In our study, IDE-loaded SLN showed the same antioxidant effect against LDH release and ROS production although SLN B1 and B2, at the equivalent drug dose tested, were more effective that SLN A1 and A2. These results suggest that the type of emulsifying system used to prepared SLN may strongly affect not only SLN technological properties but also their biological activity.

Conclusion

The use of the PIT method provided SLN that showed good properties in terms of mean size and stability upon storage at room temperature, avoiding the use of the great amount of surfactants required by conventional micro-emulsion procedures. IDE was loaded into SLN prepared by the PIT method using two different emulsifying systems: isoceteth-20/glyceryl oleate (SLN A) and ceteth-20/ glyceryl oleate (SLN B). IDE release from the tested SLN was dependent both on the type of primary surfactant used and the amount of loaded drug. IDE-loaded SLN showed a strong activity in inhibiting APPH-induced LDH release and ROS production in primary cultures of astrocytes obtained from rat cerebral cortex. It is interesting to note that SLN B2 (containing ceteth-20 as primary surfactant and 0.7% w/w IDE) were able to prevent entirely both the LDH release and ROS production induced by the treatment with APPH. Therefore, the tested SLN could be regarded as interesting carriers to

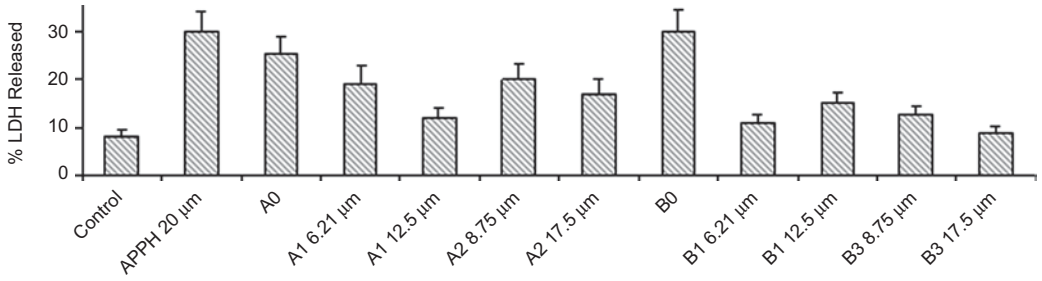

Figure 8. Inhibition of 2,2′-azobis-(2-amidinopropane)dihydrochloride (APPH)-induced lactate dehydrogenase release in astrocyte cultures by different concentrations of idebenone-loaded solid lipid nanoparticles.

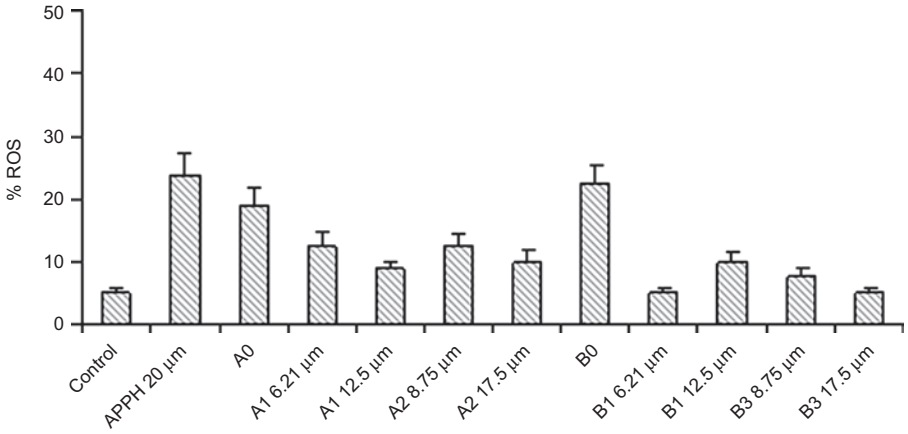

Figure 9. Inhibition of 2,2′-azobis-(2-amidinopropane)dihydrochloride (APPH)-induced oxidative damage in astrocyte cultures by different concentrations of idebenone-loaded solid lipid nanoparticles. ROS, reactive oxygen species.

overcome the blood brain barrier and increase the efficacy of the loaded drug. Further studies are planned to evaluate the antioxidant activity of IDE-loaded SLN in suitable *in vitro* and *vivo* models.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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