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Nanocapsules, nanoemulsion and nanodispersion containing melatonin: preparation, characterization and stability evaluation

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In a previous work, we have demonstrated that melatonin-loaded polymeric nanoparticles provided an important increase in the antioxidant effect of melatonin against lipid peroxidation. Hence, in this work, the aim was to study the stability of nanocapsules containing melatonin (1.5 mg/mL) prepared by interfacial deposition, using different polymers. For comparison, the stability of the nanoemulsion and nanodispersion was also evaluated. These nanoparticulated systems had diameters between 134 and 325 nm. The associated melatonin concentrations ranged from 29% to 50%, depending on the composition of the nanocarriers. The stability evaluation of formulations was preformed at room temperature and protected from or exposed to the natural light or at 50 °C and protected from the light. The stability of the nanocarriers was evaluated in terms of the macroscopic aspects, the total contents of melatonin, associated melatonin concentrations, pH and sizes of particles. The compositions of the nanocarriers and the condition of storage influenced the stability of melatonin.

1. Introduction

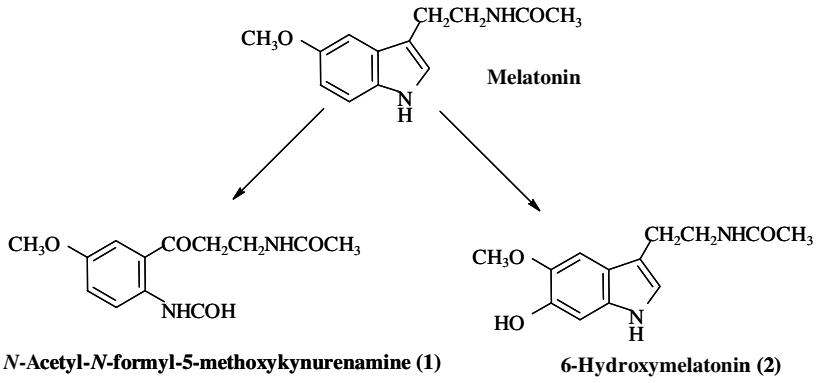
Melatonin (*N*-acetyl-5methoxytryptamine) is a lipophilic hormone mainly produced and secreted by the pineal gland (Barrenetxe et al. 2004). It was characterized after isolation from bovine pineal tissue about 50 years ago. Melatonin is synthesized and secreted during the dark phase of the day (Reiter 2003), and plays an important role in the biologic regulation of circadian rhythms, sleep, mood, aging, reproduction (Epstein 1997) and has an immunoenhancing effect (Esquifino et al. 2004). Furthermore, it is also a potent antioxidant, a free radical scavenger, a protector of nuclear DNA and membrane lipids against oxidative damage (lipid peroxidation), altering the activities of antioxidant enzymes and improving the total antioxidant defense capacity of the organism (Reiter et al. 1997; Reiter 2003). Hence, the pharmacological effects of melatonin have been investigated in sleep disorders (Siegrist et al. 2001), Alzheimer disease (Matsubara et al. 2003), alleviation of stress (Kirby et al. 1999), diabetes (Nishida et al. 2002), ischemia/reperfusion injury (Cuzzocrea and Reiter 2001), epilepsy (Gupta et al. 2004), cancer (Reiter 2003) and radiation protection (Vijayalaxmi et al. 2004).

Melatonin has a very short half-life, low and variable bioavailability presumably due to an extensive first-pass metabolism and/or a variable absorption, when orally administered (Lee et al. 1999; El-Gibaly et al. 2003). In addition, melatonin is a poorly aqueous soluble hormone and presents slow dissolution characteristics (Kumar et al. 2003). For these reasons melatonin is not a good candidate for conventional oral immediate-release formulations (Lee et al. 1999; El-Gibaly et al. 2003). So, sustained release formulations containing melatonin have been developed for oral (Lee et al. 1995; Lee and Min 1996a; Lee et al. 1996b, 1998; El-Gibaly et al. 2002, 2003; Kumar et al. 2003), intranasal (Mao et al. 2004), transdermal, transmucosal (Bénès et al. 1997) and transepidermal (Kandimalla et al. 1999) administration. Modified release bi-layered tablets containing melatonin using β-cyclodextrin (Kumar et al. 2003), microparticles (Lee et al. 1998; El-Gibaly 2002; Mao et al. 2004) and hydroxypropylmethylcellulose matrix tablets (Lee et al. 1999) have been successful prepared.

Polymeric nanoparticles, submicronic systems, have been extensively studied as drug carriers. Nanocapsules are a particular type of nanoparticles composed by an oily core surrounded by a polymeric wall, stabilized by surfactants at the particle/water interface (Legrand et al. 1999; Couvreur et al. 2002; Schaffazick et al. 2003a). The potential uses of nanocapsules include the protection of drugs against inativation in the gastrointestinal tract and the improvement of bioavailability of drugs (Allémann et al. 1998), the protection of gastrointestinal mucosa from toxicity of drugs (Guterres et al. 1995a, 2001; Müller et al. 2001; Schaffazick et al. 2003b), the improvement of therapeutic index (Couvreur et al. 2002), the controlled release of drugs (Ferranti et al. 1999; Couvreur et al. 2002), the delivery of poorly water-soluble compounds (Legrand et al. 1999; Teixeira et al. 2005), the reduction of systemic side effects (Losa et al. 1993) and the improvement of antioxidant effect (Palumbo et al. 2002).

Recently, we have developed new melatonin delivery systems, based on its association in polymeric nanoparticles (Schaffazick et al. 2005, 2006). The melatonin-loaded Eu-

Scheme



N-Acetyl-*N*-formyl-5-methoxykynurenamine (1) 6-Hydroxymelatonin (2)

dragit S100®-nanoparticle suspensions provided an important increase in its antioxidant effect against the lipid peroxidation of the phosphatidylcoline liposomes and liver microssomes (Schaffazick et al. 2005).

However, the nanoparticle aqueous suspensions present physico-chemical instability during the storage due to the aggregation of particles (Molpeceres et al. 1997), the degradation of components such as the polymer (Guterres et al. 1995b; Calvo et al. 1996; Abdelwahed et al. 2006) or the decrease of the associated drug content (Lacoulonche et al. 1999; Abdelwahed et al. 2006).

Concerning the stability of melatonin, there is a lack of information related to its storage at high temperatures. Cavallo and Hassan (1995) studied melatonin stability in aqueous solution (1.0–113.0 μg/mL) stored in sterile and pyrogen-free glass vacuum vials at −70 °C, at 4 °C and at room temperature. They verified that there was no loss of potency of melatonin up to 6 months. According Daya et al. (2001) the melatonin content in aqueous solution (50 μg/mL) stored at a pH range from 1.2 to 12, at room temperature or 37 °C, declined by less than 30% after 21 days. After UV irradiation, the major degradants of melatonin (Scheme) were identified as N^1-acetyl-N^2-formyl-5-methoxykynurenamine (**1**) and 6-hydroxymelatonin (**2**) (Maharaj et al. 2002). Those products retained the antioxidant capacity against lipid peroxidation in rat brain homogenate caused by quinolinic acid. According to Andrisano et al. (2000), the presence of oxygen is essential for the melatonin photodegradation and it is fast at pH 9, decreasing with the decrease in the pH of the solution. The possible mechanism for this degradation involves the photo-oxidation of the indole ring giving an endoperoxide, which rapidly rearranges to the stable product (**1**).

Taking into account all this, the aim of the present work was to prepare nanocapsules containing melatonin (1.5 mg/mL) using as polymer the poly(ε-caprolactone) and the polymethacrylates Eudragit S100® or Eudragit RS100®, as well as to evaluate the stability of those systems at room temperature and protected from or exposed to the natural light or at 50 °C and protected from light. In order to compare the influence of the presence of the polymer in the formulations, the stability of melatonin was also determined for drug-loaded nanoemulsion and drug-loaded nanodispersion, which nanocarriers correspond to a submicronic emulsion and to a submicronic dispersion of surfactants, respectively. The formulations were evaluated considering the total concentrations of melatonin, the associated drug concentrations, the pH values, the macroscopic aspects and the particle mean diameters.

2. Investigations, results and discussion

2.1. Characterization of nanocapsule suspensions

All formulations appeared macroscopically homogeneous and had acid pH values. The formulations containing oil core (nanocapsules and nanoemulsion) looked like a milky white bluish opalescent fluid (Tyndall effect), while the nanodispersion of the surfactants presented a bluish few opalescent aspect (almost transparent). The nanocarrier sizes were lower than 350 nm (Table), in agreement with the diameters usually found for nanocapsule formulations (Legrand et al. 1999). The total contents of melatonin (98% to 105% m/v) were close to the theoretical concentration (1.5 mg/mL) for all formulations (Table).

Regarding the associated melatonin with nanocapsules, the values ranged from 38% to 50% (m/v) depending on the type of polymer employed. In our previous work (Schaffazick et al. 2006), the melatonin-loaded nanocapsules (0.5 mg/mL), prepared using polymethacrylates, presented higher values of associated melatonin (45% for Eudragit RS100® and 56% for Eudragit S100®) if compared to the polyesters [around 37% for both poly(ε-caprolactone) and poly(lactide)]. The increase in the concentration of melatonin from 0.5 mg/mL (Schaffazick et al. 2006) to 1.5 mg/mL did not result in an important change in the drug associated concentration (Table). On the other hand, the presence of the polymer in the nanocapsules promoted a sig-

Table: Physicochemical characteristics of the nanocapsule suspensions, nanoemulsion and nanodispersion containing melatonin, after preparation (n = 3)

Formulations	Total content of melatonin (mg/mL)	Associated melatonin (%)	Size (nm)	pH
Eudragit S100®-nanocapsules	1.51 ± 0.05	50 ± 2	229 ± 56	4.4 ± 0.5
Eudragit RS100®-nanocapsules	1.48 ± 0.01	43 ± 2	135 ± 38	5.0 ± 0.1
Poly(ε-caprolactone)-nanocapsules	1.47 ± 0.05	38 ± 1	245 ± 56	6.0 ± 0.0
Nanoemulsion	1.58 ± 0.02	33 ± 2	325 ± 18	5.9 ± 0.3
Nanodispersion	1.56 ± 0.05	29 ± 2	134 ± 3	5.5 ± 0.3

nificant increase in the drug associated concentration with the nanocarriers if compared to the nanoemulsion or nanodispersion, especially for the formulations prepared with the polymethacrylates.

2.2. Stability study

The melatonin-loaded nanodispersion stored at 50 °C and under natural light was slightly yellowish after 1.5 month, probably due to the degradation of the melatonin. After 1.5 month, Eudragit RS100®-nanocapsules stored under natural light also looked similar. The other nanocapsule suspensions appeared milky white opalescent during the whole period, rendering difficult any visualization of the yellowish colour. In addition, no formulation showed any precipitate after 3 months of storage.

The total contents of melatonin remained constant after 1 month of storage for all nanocapsule suspensions under all experimental conditions (Figs. 1A, 1B, 1C). However, after 2 months a significant decrease of the drug recovery was verified, except in the case of poly(ε-caprolactone) formulation stored at room temperature and exposed to natural light. After this period, the decrease in the total content of drug ranged from $2 \pm 2\%$ to $8 \pm 3\%$ (room temperature), from $5 \pm 3\%$ to $13 \pm 3\%$ (natural light) and from $7 \pm 3\%$ to $13 \pm 3\%$ (50 °C). After 3 months, significant decreases in the total content of drug were observed: from $12 \pm 2\%$ to $21 \pm 2\%$ (room temperature), from $15 \pm 1\%$ to $22 \pm 5\%$ (natural light) and from $17 \pm 2\%$ to $21 \pm 2\%$ (50 °C). In the case of the nanoemulsion, a significant decrease in the drug content has already been observed after 1 month for formulations stored under natural light ($8 \pm 3\%$) and at 50 °C ($8 \pm 4\%$) (Fig. 1D). On the other side, for the nanoemulsion stored at room temperature, a significant decrease in melatonin content was observed exclusively after 2 months ($7 \pm 5\%$). In the case of the nanodispersion (Fig. 1E), decreases of $9 \pm 6\%$ and $7 \pm 4\%$ in the melatonin contents were also observed after storage under natural light and at 50 °C after 1 month, respectively. For the nanodispersion stored at room temperature, the melatonin content significantly decreased after 2 months of storage ($11 \pm 3\%$). After 3 months, the decreases in the content of melatonin for the nanodispersion ($15 \pm 5\%$ to $19 \pm 3\%$) and for the nanoemulsion ($16 \pm 1\%$ to $19 \pm 3\%$) were similar to those observed for nanocapsules.

Based on these results, it was verified that the decreases in the content of melatonin were slightly accelerated by the natural light and by the increase in the temperature.

Despite the partial encapsulation of melatonin (Table), the presence of the polymer (Eudragit RS100® and Eudragit S100®) in the nanocapsule formulations retarded in 1 month the decrease in the total content of the drug in comparison with the nanodispersion and the nanoemulsion (Fig. 1), for formulations stored under natural light or at 50 °C. For the poly(ε-caprolactone) formulation, a significant decrease in drug content was observed exclusively after 3 months of storage at room temperature and at natural light, retarding the degradation of melatonin in 2 months as compared to the other nanocarrier systems. Tursilli et al. (2006) have also verified that the composition of the formulation influenced the photostability of melatonin associated with lipid microparticle carrier systems, observing that only the cream containing melatonin-loaded tristearin-phosphatidylcholine particles showed to improve the photostability of melatonin.

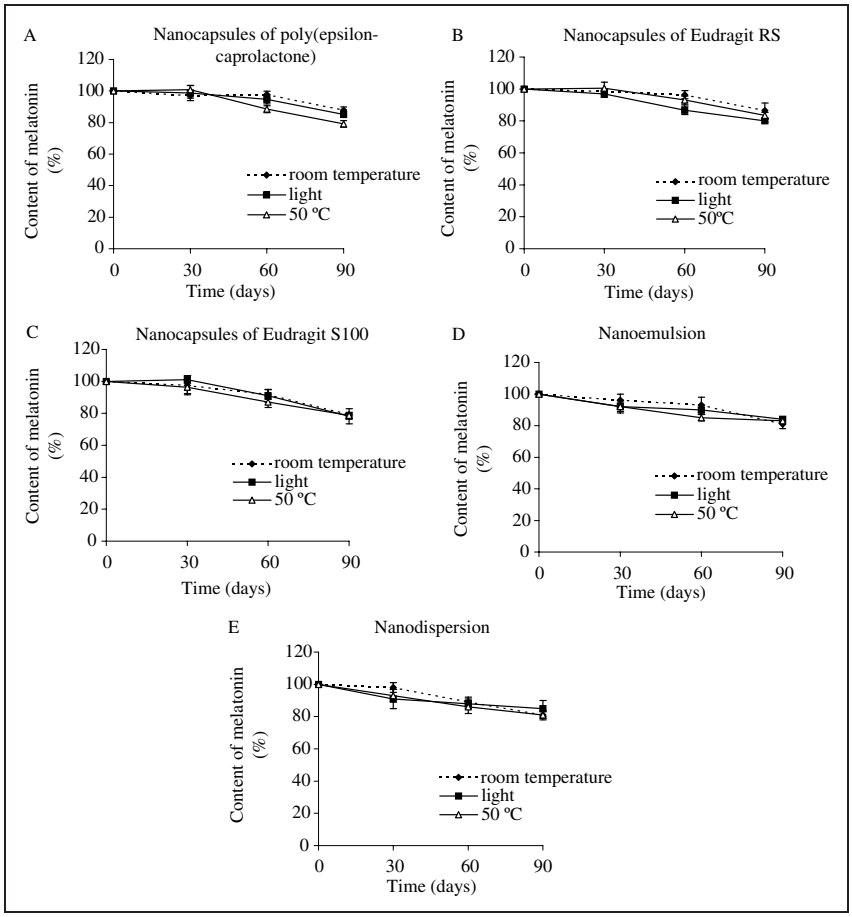


Fig. 1:
Content of melatonin associated with nanocarrier systems stored at room temperature, at natural light and at 50 °C

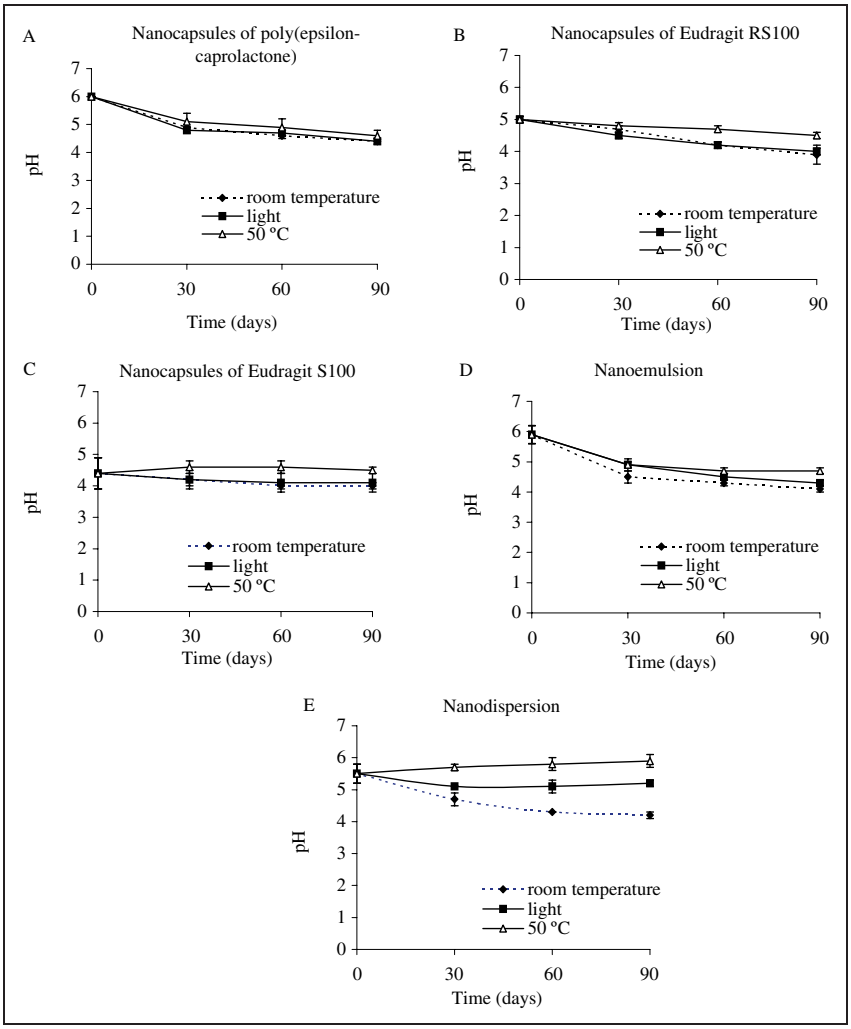


Fig. 2:
pH of the nanocarrier systems containing melatonin in function of time

For the formulations prepared with poly(ε-caprolactone) or Eudragit RS100®, the pH values significantly decreased after 30 days of the storage under all conditions (Fig. 2A, 2B). For Eudragit S100®, the formulations did not show significant changes in the pH values, after 90 days of storage (Fig. 2C). In the case of nanodispersion and nanoemulsion (Fig. 2D, 2E) the pH significantly decreased after 30 days of the storage under all conditions, except for the nanodispersion stored at 50 °C that did not show significant changes after 90 days. The changes in the pH values of the formulations were probably due to the degradation of the formulation components in function of the time. Calvo et al. (1996) explained that the decreases in the pH values of the poly(ε-caprolactone)-nanoparticle suspensions were due to the possible hydrolysis of the triglyceride (oil), leading to the release of free fatty acids, besides the production of free ε-hydroxycaproic acid as a result of polymer degradation.

Fig. 3 shows the associated melatonin concentrations during three months. For the formulations stored at room temperature, the associated melatonin concentrations with nanocapsules ranged from 49% to 54%, from 40% to 45%, and from 38% to 45% for Eudragit S100®, Eudragit RS100® and poly(ε-caprolactone), respectively (Fig. 3A, 3B, 3C). Therefore, for the formulations prepared with Eudragit S100® or Eudragit RS100® the associated drug did not present a major variation after 90 days of storage at room temperature, while the associated melatonin with poly(ε-caprolactone)-nanocapsules presented the tendency to increase in function of time, probably due to the adsorption of the drug on the polymer (Fig. 3A). In the case of nanoemulsion and nanodispersion stored at room temperature (Fig. 3D, 3E), the associated drug ranged from 36% to 27% and 31 to 22%, respectively, showing a decrease in the associated melatonin with these nanocarriers in function of time. These results indicated that the polymeric wall had an important role in stabilizing the associated melatonin concentrations during the storage.

The formulations prepared with Eudragit S100®or Eudragit RS100® (Fig. 3B, 3C) presented decreases of the associated melatonin after 2 months of storage under natural light and after 1 month of storage at 50 °C for Eudragit RS100®. The nanoemulsion and the nanodispersion (Fig. 3D, 3E) had already showed a partial loss of the associated melatonin after 1 month of storage under natural light and at 50 °C.

Comparing the particles sizes after preparation and after 90 days of storage, the nanocapsules and nanoemulsion did not present a significant variation the mean sizes (Figs. 4A, 4B, 4C, 4D). These results showed that the nanocapsules or nanoemulsion did not agglomerate during this period independently of the condition of the storage, showing physical stability. However, for the nanodispersion (Table, 134 ± 3 nm), the nanocarriers had already presented significant increase in the mean diameter after 2 months, when stored under natural light (178 ± 4 nm) and at 50 °C (183 ± 9 nm). Furthermore, a significant increase of the mean size of the nanodispersion stored at room temperature (188 ± 8 nm) occurred after 3 months (Fig. 4E).

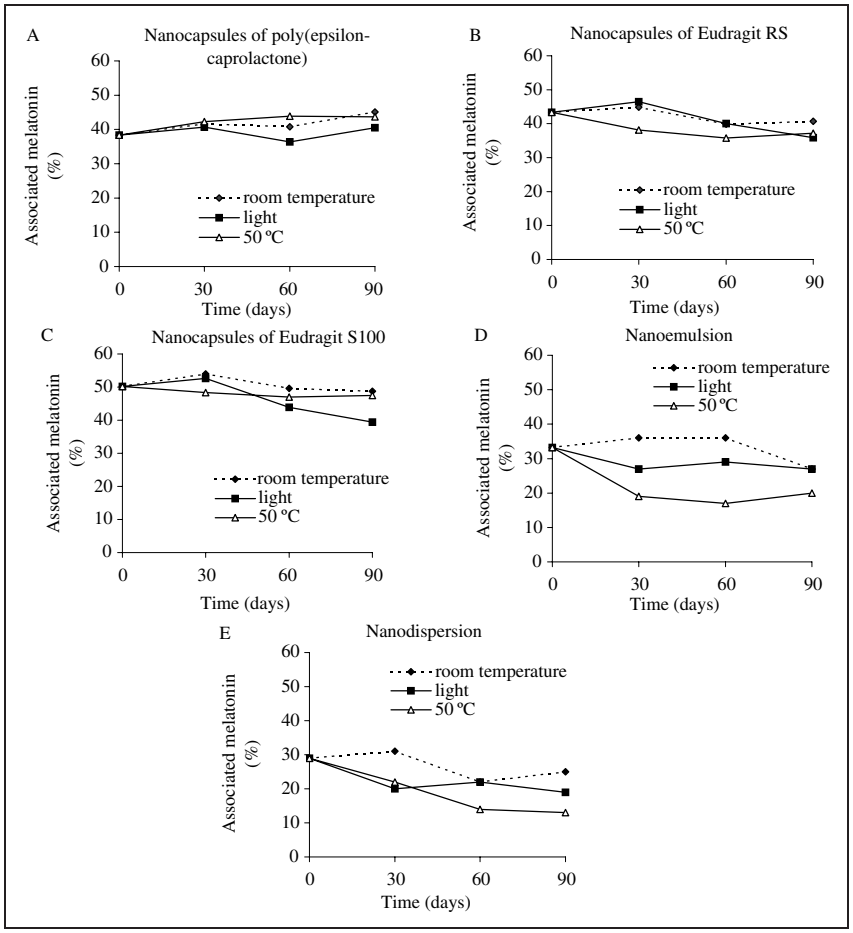


Fig. 3:
Melatonin associated with the nanocarrier systems in function of the time

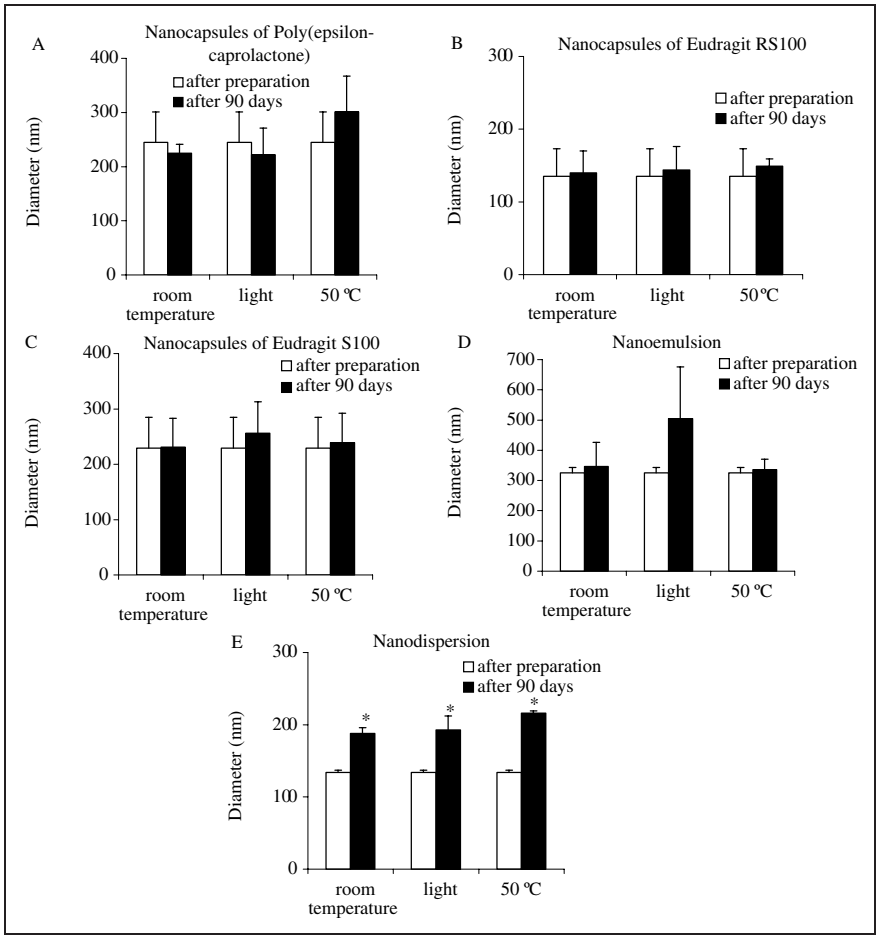


Fig. 4:
Diameters of the nanocarrier systems containing melatonin, after preparation and after 90 days

In conclusion, it was possible to obtain relatively stable nanocapsule suspensions containing 1.5 mg/mL of melatonin, using poly(ε-caprolactone), Eudragit S100® or Eudragit RS100® as polymers. The associated drug with nanocarriers, the total drug contents and the size of the particles were stable up to 30 days for all melatonin loaded-nanocapsule suspensions. After 60 days, a significant decrease in total content of melatonin in nanocapsule suspensions (Eudragit RS100® and Eudragit S100®) occurred, except for the poly(ε-caprolactone) nanocapsules. The melatonin-loaded nanoemulsion and nanodispersion presented significant decreases of the drug content already after 1 month, when stored in the light and at 50 °C. The degradation of melatonin was slightly accelerated by the natural light and the temperature. The presence of the polymers (nanocapsules) increased the associated melatonin concentrations with the nanocarriers and the chemical stability of melatonin during storage, as well as for maintaining the balance between free and associated melatonin concentrations similar to those obtained after preparation.

3. Experimental

3.1. Materials

Melatonin was obtained from Acros Organics (Belgic). Poly(ε-caprolactone) (MW = 65,000 g/mol) was supplied by Aldrich (Strasbourg, France). Eudragit S100® and Eudragit RS100® were obtained from Almapal (São Paulo, Brazil). Caprylic/capric triglyceride was obtained from Brasquim (Porto Alegre, Brazil) and sorbitan monooleate was supplied by Sigma (St. Louis, USA). Polysorbate 80 (Tween 80®) was acquired from Delaware (Porto Alegre, Brazil). All other chemicals and solvents used were of pharmaceutical grade. All reagents were used as received.

3.2. Preparation of the nanocapsule suspensions, nanoemulsion and nanodispersion

Nanocapsules containing melatonin (1.5 mg/mL) were prepared by interfacial deposition of preformed polymers, as described by Fessi et al. (1989). Briefly, the lipophilic solution consisted of 0.8 mL of oil (caprylic/capric triglyceride), 250 mg of polymer [poly(ε-caprolactone), Eudragit S100® or Eudragit RS100®], 37.5 mg of melatonin, 192 mg of Span 80® (surfactant) and 67 mL of acetone. This organic phase was added under magnetic stirring into an aqueous solution (133 mL) containing 192 mg of surfactant (Tween 80®). The acetone was removed and the water was concentrated by evaporation under reduced pressure and the final volume of suspension was adjusted to 25 mL. The formulations were prepared in triplicate. The nanoemulsion and the nanodispersion (1.5 mg/mL) were prepared as described above omitting the polymer for the former and omitting both the polymer and the oil for the latter.

3.3. Determination of particle sizes and pH

The particle sizes were measured by photon correlation spectroscopy (PCS) (Pohlmann et al. 2002). For PCS measurements, 20 μL of each formulation were diluted to 10 mL with water (MilliQ®). Measurements were made at room temperature (20 °C) using a Brookheaven Instruments standard setup (BI-200M goniometer, BI-9000AT digital correlator and a BI9863 detection system). A Spectra Physics He−Ne laser (model 127, $\lambda_0 = 632{,}8$ nm) was used as light source. The scattered light was observed at a fixed angle of 90°.

The pH values of formulations were determined using a potentiometer B474 (Micronal, Brazil), after calibration with both 4.0 and 7.0 standard pH solution.

3.4. Determination of drug content

Melatonin was assayed by liquid chromatography (HPLC: Waters, USA; pump 600 controller; 2487 Dual λ absorbance detector; 717 plus autosampler), using a Merck column LiChrospher® RP-18 (5 μm; 250 × 4 mm, Germany). The mobile phase consisted of acetonitrile/water (55 : 45 v/v). The total sample volume injected was 20 μL and melatonin was detected at 229 nm. Free melatonin (non-associated) was determined in the ultrafiltrate after separation of the nanocarriers by ultrafiltration-centrifugation technique (Ultrafree-MC® 10,000 MW, Millipore), at 12,000 rpm for 10 min. Total content of melatonin was determined using HPLC after dissolution of the components by acetonitrile. The associated melatonin with the nanocarriers was calculated from the difference between the total and free drug concentrations determined in the formulations and in the ultrafiltrates. The analytical method was validated (linearity: $R^2 = 0.9998$ for 2.5 to 17.5 μg/mL; accuracy = 101 ± 1%; precision: relative standard deviation = 1.4%–1.8% for repeatability and relative standard deviation = 3.1% for intermediate precision).

3.5. Stability study

The melatonin-loaded formulations (1.5 mg/mL: nanocapsule suspensions, nanoemulsion or nanodispersion) were stored up to 90 days under different experimental conditions: a) at room temperature protected from the natural light; b) at room temperature exposed to natural light; and c) at 50 ± 1 °C protected from the natural light. The experiments were carried out in triplicate. Samples were withdrawn at predetermined time intervals (0, 30, 60 and 90 days) and analyzed in order to determine the particle sizes, pH, drug content and associated melatonin with the nanocarriers.

3.6. Statistical analyses

The statistical analyses of the data was carried out by the Student's t-test and significance was taken as $p < 0.05$.

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