Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 289 (2005) 209–213
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Protective properties of melatonin-loaded nanoparticles against lipid peroxidation

S.R. Schaffazick[a], A.R. Pohlmann[a,b], C.A.S. de Cordova[c], T.B. Creczynski-Pasa[c], S.S. Guterres[a,*]

[a] *Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752, 90610-000 Porto Alegre, RS, Brazil*
[b] *Departamento de Química Orgânica, Instituto de Química, UFRGS, CP15003, 91501-970 Porto Alegre, RS, Brazil*
[c] *Departamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Federal de Santa Catarina, CP476, 88049-900 Florianópolis, SC, Brazil*

Received 17 March 2004; received in revised form 4 August 2004; accepted 3 November 2004
Available online 19 December 2004

Abstract

The aim of this study was to prepare melatonin-loaded nanoparticles (nanocapsules and nanospheres) by nanoprecipitation, using Eudragit S100® as polymer. The potential of these systems to protect lipids against peroxidation was evaluated in comparison to melatonin in aqueous solution and nanoemulsion. Liposomes and microsomes were used as model of a lipid membrane and lipid peroxidation was induced by free radical ascorbyl. Nanocapsule and nanosphere suspensions presented total recoveries of melatonin near 100% and associated drug around 55%. The zeta potential values were negative and the hydrodynamic diameter of particles were lower than 255 nm. The results demonstrate that the lipids were protected against peroxidation from 8 to 51% due to the presence of the melatonin and that this effect depended on the drug dose, the type of the lipid substrate and the type of colloid, in which melatonin was incorporated. Nanocapsules and nanospheres provided an important increase in the antioxidant effect of melatonin against lipid peroxidation.

Keywords: Melatonin; Nanoparticles; Lipid peroxidation

Melatonin (*N*-acetyl-5-methoxytryptamine) is a hormone principally produced and released by the pineal gland under influence of the environmental light/dark cycle. It participates in several important physiological functions, including biologic regulation of circadian rhythms, sleep, mood, reproduction and neuroimmunomodulation. Also, anti-tumor effect of melatonin on various cancers has been reported (Vijayalaxmi et al., 2002). Exogenous melatonin has been used for treating a variety of circadian rhythm disorders,

* Corresponding author. Tel.: + 55 21 51 33165411; fax: +55 21 33165437.
E-mail address: nanoc@farmacia.ufrgs.br (S.S. Guterres).

0378-5173/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2004.11.003

including jet–lag and insomnia. Recently, melatonin was reported to be an effective free radical scavenger (Epstein, 1997; Beyer et al., 1998) and it has been found to protect lipid peroxidation in many experimental models, as liposomes and microsomes (Leaden et al., 2002; Saija et al., 2002; Teixeira et al., 2003). Different mechanisms are combined to explain the melatonin protective effect: (a) a direct antioxidant action inhibiting the lipid peroxidation, (b) inducing changes on lipidic bilayers fluidity, (c) protecting intracellular components against peroxidative damage (Saija et al., 2002). The potential clinical benefit of the use of melatonin as an antioxidant could include the treatment of many diseases, as cancers and neurodegenerative diseases such as Alzheimer (Beyer et al., 1998).

Exogenous controlled-release melatonin formulations have been reported to be clinically more useful for sleep maintenance than immediate release melatonin formulations (Jan et al., 2000). Furthermore melatonin-loaded controlled-release dosage forms have been investigated, due to the short half-life of melatonin (Lee et al., 1998; El-Gibaly et al., 2003).

Polymeric nanoparticulated systems (nanocapsules and nanospheres) for controlled release of drugs have been intensively investigated in the past years in the pharmaceutical field (Couvreur et al., 2002). The nanocapsule is a carrier composed by an oil core surrounded by a polymeric wall, whereas the nanosphere consists of a matrix of polymer. Both colloids, as well as nanoemulsions, are stabilized by surfactants at the interface particle/water, preventing particle agglomeration and/or drug leakage. These systems can be prepared by different methods based on polymerization of dispersed monomers or precipitation of preformed polymers (Schaffazick et al., 2003). The associated drug could be adsorbed on nanoparticles, or dissolved and entrapped in colloids (Pohlmann et al., 2004). For i.v. route, nanoparticle suspensions have been developed as drug targeting delivery systems, improving the therapeutic index of drugs (Couvreur et al., 2002), whereas for oral administration, polymeric nanoparticles have been studied as carriers for drug sustained-release (Verger et al., 1998), mucosa protection from the toxicity of drugs (Guterres et al., 2001), as well as to improve the bioavailability of drugs (Allémann et al., 1998).

Up to now, melatonin has not been associated with nanoparticles. Consequently, the potentiality of these systems to improve the antioxidant effect of this drug was not yet evaluated. Hence, the aim of the present work was to prepare melatonin-loaded nanocapsules, nanospheres and nanoemulsion, and to evaluate the protective properties of these formulations against lipid peroxidation induced by ascorbyl free radical.

Nanocapsules containing melatonin were prepared as described by Fessi et al. (1989). Briefly, the acetone solution (67 mL) of melatonin (0.0125 g), octanoic/decanoic triglyceride mixture (0.8 mL), Eudragit S100® (0.25 g), sorbitan monooleate (0.1915 g) was added into an aqueous solution (133 mL) of polysorbate 80 (0.1915 g). The solvents were evaporated to eliminate the acetone and to concentrate water to a final volume of 25 mL (0.5 mg/mL melatonin). Nanospheres and nanoemulsion were also prepared, omitting the triglyceride or the polymer, respectively. Control formulations were prepared omitting the drug.

The pH values of suspensions were determined and the particle sizes were measured by photon correlation spectroscopy according to Pohlmann et al. (2002) (Brookheaven Instruments BI-200M, Spectra Physics He–Ne, $\lambda_0 = 632.8$ nm). The zeta potential (Zetasizer®, Malvern) of suspensions were determined after dilution of samples in 1 mM NaCl.

Melatonin was assayed by HPLC. The system consisted of a UV–vis detector, pump and auto-injector S200 Perkin-Elmer and Nova-Pak® C_{18} Waters column. The mobile phase consisted of acetonitrile/water (55:45, v/v). Free melatonin was separated from colloids by ultrafiltration–centrifugation (Ultrafree-MC 10,000 MW, Millipore). Total drug was measured, at 229 nm, after dissolution of colloids with acetonitrile. The associated melatonin with the nanocapsules, nanospheres and nanoemulsion was calculated from the difference between the total and the free drug concentrations.

All formulations presented macroscopic homogeneous aspect and total recoveries of melatonin were close to 100%. Nanocapsule and nanosphere suspensions presented similar amounts of associated drug (55.77 and 54.35%, respectively) (Table 1). On the other hand, the nanoemulsion showed a lower value (32.65%) than the polymeric formulations. Nanospheres presented particle sizes of 192 and 126 nm for unloaded and melatonin-loaded formulations, respectively. Besides, nanocapsules and nanoemulsion presented similar diameters (254 and

Table 1
Physico-chemical properties of drug-loaded and drug-unloaded colloids: nanospheres (NS), nanocapsules (NC) and nanoemulsion (NE)

Formulations	Associated drug (%)	Size (nm)	Zeta potential (mV)	pH
NS–melatonin	54.35	126 ± 12	−36.6 ± 1.9	3.88
NS	–	192 ± 46	−35.4 ± 2.3	4.11
NC–melatonin	55.77	236 ± 20	−43.5 ± 0.8	3.97
NC	–	254 ± 52	−45.0 ± 1.5	4.81
NE–melatonin	32.65	246 ± 4	−33.8 ± 0.5	5.15
NE	–	230 ± 48	−38.0 ± 0.5	5.01

230 nm) and the presence of melatonin did not influence this parameter (236 and 246 nm). These results showed that the presence of the oil phase caused an increase in size of colloids. The zeta potential values were −37 and −35 mV for nanospheres containing or not melatonin and −34 and −38 mV for nanoemulsions containing or not drug, respectively. For nanocapsules, the absolute values were higher (−43 and −45 mV containing or not melatonin) than those observed for the other formulations. In all cases, these values correspond to stable formulations (Verger et al., 1998). The pH values for all formulations were in the range of 3.88–5.15.

Liposomes and microsomes were used as substrates for lipid peroxidation to evaluate the protective effect of melatonin associated with colloids. Bilayer liposomes (277 ± 23 nm) were prepared by dialysis as described previously (Creczynski-Pasa and Gräber, 1994). Briefly, the method consisted of the dissolution of 50 mg/mL phospholipids in buffer containing 10 mM tricine, 20 g/L sodium cholate, 10 g/L deoxicholate at pH 8.0 followed by a dialysis procedure at 30 °C for 5 h. Microsomes were obtained from liver of Wistar rats by differential centrifugation with calcium aggregation and stored at −70 °C. The lipid peroxidation was induced by the addition of 25 μM $FeSO_4$ and 500 μM ascorbate in a reaction medium containing 1 mg/mL microsomal protein (or 12.5 mg/mL phosphatidylcoline liposome), 0.1 M Tris–HCl pH 7.4 (Cordova et al., 2002) and 200 μM (or 400 μM) melatonin aqueous solution (0.5 mg/mL drug containing 1% ethanol) or drug-loaded colloids. The controls of the

Table 2
Effect of melatonin encapsulation on lipid peroxidation

Formulations	Dose of melatonin μM	Lipid peroxidation[a] ± S.D.[b] (nmol TBARS/mL)		Protection of melatonin against lipid peroxidation[c] ± S.D. (%)	
		Liposomes	Microsomes	Liposomes	Microsomes
Control	–	291 ± 21	3885 ± 109	–	–
Aqueous solution	200	228 ± 12*	3415 ± 378	21.8 ± 1.9	12.1 ± 1.4
	400	200 ± 22*	3250 ± 202*	31.3 ± 4.1	16.4 ± 1.1
Control	–	324 ± 43	2512 ± 500	–	–
Nanocapsules	200	216 ± 29*	2288 ± 138	33.3 ± 6.3	8.9 ± 1.9
Control	–	489 ± 61	2318 ± 83	–	–
Nanocapsules	400	239 ± 6*	1492 ± 132*	51.1 ± 6.5**	35.6 ± 3.4**
Control	–	405 ± 30	2631 ± 146	–	–
Nanospheres	200	301 ± 23*	2408 ± 180	25.8 ± 2.7	8.5 ± 0.8
Control	–	469 ± 30	2294 ± 79	–	–
Nanospheres	400	271 ± 28*	1374 ± 230*	42.2 ± 5.1**	40.1 ± 6.8**
Control	–	304 ± 7	2847 ± 156	–	–
Nanoemulsion	200	250 ± 9*	2547 ± 210	17.9 ± 0.8	10.5 ± 1.0
Control	–	410 ± 56	2835 ± 223	–	–
Nanoemulsion	400	213 ± 21*	2310 ± 155*	48.0 ± 8.1**	18.5 ± 1.9

[a] TBARS = thiobarbituric acid reactive substances.
[b] S.D., standard deviation, calculated from triplicate of experiments.
[c] This protection is relative to the formulations prepared without melatonin (controls).
* Statistical differences between each formulation and its respective control established using *t*-test ($p < 0.05$).
** Statistical differences between the solution (400 μM of melatonin) and the formulations established using *t*-test ($p < 0.05$).

assay consisted of each colloid prepared without drug. The samples were incubated for 30 min at 37 °C and the extent of lipid peroxidation was determined by the thiobarbituric acid method (Bird and Draper, 1984). The amount of lipid peroxidation was determined using the molar extinction coefficient of 1.56×10^5 (M^{-1} cm^{-1}) and expressed as thiobarbituric acid reactive substances (TBARS). The experiments were carried out in triplicate and the *t*-test ($p < 0.05$) was conducted for statistical analysis.

Table 2 shows the protective properties of melatonin against lipid peroxidation depending on the drug dose and on the type of colloid, in which melatonin was incorporated. Comparing the drug-loaded formulations (200 or 400 μM) with the controls, the results demonstrated that the lipids were protected against peroxidation from 8 to 51%, due to the presence of the drug. As it can be observed in Table 2, the presence of the melatonin has decreased significantly the lipid peroxidation, except in the case of the microsomes and melatonin at 200 μM.

Additionally, the effect was dependent on the type of the lipid substrate (microsomes or liposomes). It was observed that the antioxidant properties of melatonin can also vary depending on the lipid membrane composition (Teixeira et al., 2003). For the dose of 400 μM, comparing melatonin solution and drug-loaded colloids, the antioxidant properties of melatonin have been significantly improved when it was associated with colloids and liposomes were used as substrate. On the other hand, in the case of microsomes, only the formulations containing polymer (nanocapsules and nanospheres) showed a significant increase in the antioxidative effect of melatonin (Table 2). According to Palumbo et al. (2002), the polymeric nanostructures could adhere to the cells improving the antioxidant action of a lipophilic drug associated with nanocapsules of poly(ethyl 2-cyanoacrilate). In the present study, the polymer in nanocapsule and nanosphere formulations caused higher encapsulation rates of melatonin (55%) than in nanoemulsion (33%), improving the antioxidative effect of the drug.

In conclusion, our study showed that nanoparticles containing melatonin can be prepared from pre-formed polymer using the nanoprecipitation technique. Additionally, this nanoparticles provided an important increase in the antioxidative effect of melatonin against lipid peroxidation.

Acknowledgement

FAPERGS, FUNCITEC, CAPES, Rede Nanobiotecnologia-CNPq/MCT-Brazil.

References

Allémann, E., Leroux, J.-C., Gurny, R., 1998. Polymeric nano- and microparticles for the oral delivery of peptides and peptidomimetics. Adv. Drug Deliv. Rev. 34, 171–189.

Beyer, C.E., Steketee, J.D., Saphier, D., 1998. Antioxidant properties of melatonin–an emerging mystery. Biochem. Pharmacol. 56, 1265–1272.

Bird, R.P., Draper, A.H., 1984. Comparative studies on different methods of malondyaldehyde determination. Meth. Enzymol. 90, 105–110.

Cordova, C.A.S., Siqueira, I.R., Netto, C.A., Yunes, R.A., Volpato, A.M., Filho, V.C., Curi-Pedrosa, R., Creczynski-Pasa, T.B., 2002. Protective properties of butanolic extract of the Calendula officinalis L.(marigold) against lipid peroxidation of rat liver microssomes and action as free radical scavenger. Redox Rep. 7, 95–102.

Couvreur, P., Barrat, G., Fattal, E., Legrand, P., Vauthier, C., 2002. Nanocapsule technology: a review. Crit. Rev. Ther. Drug Carrier Syst. 19, 99–134.

Creczynski-Pasa, T.B., Gräber, P., 1994. ADP binding and ATP synthesis by reconstituted H^1–ATPase from chloroplasts. FEBS Lett. 350, 195–198.

El-Gibaly, I., Meki, A.M.A., Abdel-Ghaffar, S.K., 2003. Novel B melatonin-loaded chitosan microcapsules: in vitro characterization and antiapoptosis efficacy for aflatoxin B1-induced apoptosis in rat liver. Int. J. Pharm. 260, 5–22.

Epstein, F.H., 1997. Mechanisms of disease–melatonin in humans. New Engl. J. Med. 336, 186–195.

Fessi, H., Puisieux, F., Devissaguet, J.-Ph., Ammoury, N., Benita, S., 1989. Nanocapsule formation by interfacial polymer deposition following solvent displacement. Int J. Pharm. 55, r1–r4.

Guterres, S.S., Müller, C.R., Michalowski, C.B., Pohlmann, A.R., Dalla-Costa, T., 2001. Gastro-intestinal tolerance following oral administration of spray-dried diclofenac-loaded nanocapsules and nanospheres. S.T.P. Pharma Sci. 11, 229–233.

Jan, J.E., Hamilton, D., Seward, N., Fast, D.K., Freeman, R.D., Laudon, M., 2000. Clinical trials of controlled-release melatonin in children with sleep-wake cycle disorders. J. Pineal Res. 29, 34–39.

Leaden, P., Barrionuevo, J., Catalá, A., 2002. The protection of long chain polyunsaturated fatty acids by melatonin during nonenzymatic lipid peroxidation of rat liver microsomes. J. Pineal Res. 32, 129–134.

Lee, B.-J., Choe, J.S., Kim, C.-K., 1998. Preparation and characterization of melatonin-loaded stearyl alcohol microspheres. J. Microencapsul. 15, 775–787.

Palumbo, M., Russo, A., Cardile, V., Renis, M., Paolino, D., Puglisi, G., Fresta, M., 2002. Improved antioxidant effect of

idebenone-loaded polyethyl-2-cyanoacrylate nanocapsules tested on human fibroblasts. Pharm. Res. 19, 71–78.

Pohlmann, A.R., Weiss, V., Mertins, O., Pesce da Silveira, N., Guterres, S.S., 2002. Spray-dried indomethacin-loaded polyester nanocapsules and nanospheres: development, stability evaluation and nanostructure models. Eur. J. Pharm. Sci. 16, 305–312.

Pohlmann, A.R., Soares, L.U., Cruz, L., Pesce da Silveira, N., Guterres, S.S., 2004. Alkaline hydrolysis as a tool to determine the association form of indomethacin in nanocapsules prepared with poly(ε-caprolactone). Curr. Drug Del. 1, 103–110.

Saija, A., Tomaino, A., Trombetta, D., Pellegrino, M.L., Tita, B., Caruso, S., Castelli, F., 2002. Interaction of melatonin with model membranes and possible implications in its photoprotective activity. Eur. J. Pharm. Biopharm. 53, 209–215.

Schaffazick, S.R., Guterres, S.S., Lucca-Freitas, L., Pohlmann, A.R., 2003. Caracterização e estabilidade físico-química de sistemas poliméricos nanoparticulados para administração de fármacos. Quim. Nova. 26, 726–737.

Teixeira, A., Morfim, M.P., Cordova, C.A.S., Charão, C.C.T., Lima, V.R., Creczynski-Pasa, T.B., 2003. Melatonin protects against pro-oxidant enzymes and reduces lipid peroxidation in distinct membranes induced by the hydroxyl and ascorbyl radicals and by peroxinitrite. J. Pineal Res. 35, 262–268.

Verger, M.L.-Le., Fluckiger, L., Kim, Y.-I.I., Hoffman, M., Maincent, P., 1998. Preparation and characterization of nanoparticles containing an antihypertensive agent. Eur. J. Pharm. Biopharm. 46, 137–143.

Vijayalaxmi, C.R.T., Reiter, R.J., Herman, T.S., 2002. Melatonin: from basic research to cancer treatment clinics. J. Clin Oncol. 20, 2575–2601.