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Research paper

Preparation and characterization of nanoparticles containing an antihypertensive agent

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Abstract

Isradipine, an antihypertensive agent, was encapsulated by the nanoprecipitation method using polymers including poly(epsilon-caprolactone), poly(d,l-lactide) and poly(d,l-lactide-co-glycolide). In vitro scanning electron microscopy and differential scanning calorimetry were used to characterize the nanoparticles. The average diameters of the nanoparticles ranged from 110 nm to 208 nm. PCL nanoparticles were larger than nanoparticles prepared with the other polymers. The zeta potential of the nanoparticles was negative, with values of about –25 mV which promoted good stabilization of the particles. The amorphous state of PLA and PLAGA non-loaded nanoparticles and the semi-crystalline state of PCL were demonstrated with X-ray diffraction and differential scanning calorimetry. For all nanoparticles, isradipine was found to be totally amorphous in the polymer which suggested that the drug was molecularly dispersed in the matrix. The colloidal suspensions displayed a sustained release profile in comparison with the drug release profile of isradipine in a PEG solution. Results from this investigation suggest that these nanospheres will be a good candidate delivery system for oral administration, to reduce the initial hypotensive peak and to prolong the antihypertensive effect of the drug.

Keywords: Nanoparticles; Poly(epsilon-caprolactone); Poly(d,l-lactide); Poly(d,l-lactide-co-glycolide); Drug release; Antihypertensive agent; Isradipine

1. Introduction

Poly(epsilon-caprolactone) (PCL), poly(d,l-lactide) (PLA) and poly(d,l-lactide-co-glycolide) (PLAGA) have gained attention for the preparation of a wide variety of delivery systems (blends, films, matrices, microspheres, nanospheres, pellets, etc.) containing several drugs (anaesthetics, antibiotics, anti-tumoural drugs, enzymes, hormones, proteins, etc.) [1,2] due to their biodegradable and biocompatible properties [3,4]. The degradation of these aliphatic polyesters is known to be affected in vitro by the preparation methods of the delivery systems, as well as the properties of the polymer which include initial molecular weight, d,-lactide ratio (PLA, PLAGA), glycolate-lactate ratio (PLAGA), and the pH of the environment.

Isradipine is a calcium antagonist which selectively inhibits the transmembrane influx of calcium ions into vascular smooth muscle cells. Since the contractile process depends on the movement of extracellular calcium into the cells, administration of isradipine results in the dilatation of the vascular bed. Peripheral resistance is thus reduced and blood pressure is lowered [5]. However, therapeutic use of dihydropyridine calcium entry blockers, such as isradipine, are hampered by the rapidity, intensity and brevity of their vasodilation effect. Hubert et al. [6] have shown that in awake, renovascular hypertensive rats, darodipine in a poly(isobutylcyanoacryate) nanocapsular form lowered blood pressure when given orally. The initial fall in blood

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pressure was less marked than that observed when the same dose of darodipine was administrated in a polyethylene glycol 400 (PEG) solution. In addition, Damge et al. [7] showed that nanocapsules were able to preserve and prolong the biological effect of insulin after oral administration. However, dosage forms based on poly(alkylcyanoacrylate) polymers have yet to be approved. In addition, poly(alkylcyanoacrylates) have displayed some toxicity in culture cells which could impair their pharmaceutical development [8]. Therefore, the incorporation of a dihydropyridine calcium entry blocker in colloidal carriers made with biodegradable and well-known polymers would be more appropriate for human therapy. Recently, a well-known antihypertensive drug, nifedipine has been incorporated into PCL and PLAGA nanoparticles [9]. We therefore selected PCL, PLA and PLAGA due to their biodegradability and low toxicity [10].

The aim of this work was to produce and characterize isradipine-loaded nanoparticles with a view to selecting the most suitable polymer(s) for oral administration based on both a slow initial release in order to reduce the initial hypotensive peak and a prolongation of the antihypertensive effect of the molecule.

2. Materials and methods

2.1. Materials

Poly(epsilon-caprolactone) (PCL) was supplied by Aldrich Chemical, France and poly(d,l-lactide) (PLA 100) and poly(d,l-lactide-co-glycolide) (PLAGA) were supplied by Vetisorb, Technologies International L.P. c/o Dupont International S.A., Genève, Switzerland. Two different PLAGA polymers were used: PLA85GA15, which contains 85% of the lactate unit and 15% of the the glycolate unit, and PLA50GA50, which contains 50% of the lactate unit and 50% of the glycolate unit. Isradipine was a gift from Sandoz, Paris, France. Pluronic F68, furnished by BASF (Ludwigshafen, Germany), was used as a non-ionic stabilizer. Polyethylene glycol 400 (PEG 400) was purchased from Cooper (Melun, France). All reagents used were of analytical grade.

For the in vitro release studies, dialysis tubes (Spectra/Por 6, diameter 21.6 mm, M_w cutoff 50 000) and appropriate closures were purchased from Spectrum (Los Angeles, CA, USA).

2.2. Methods

2.2.1. Preparation of nanosuspensions

Nanoparticles were prepared according to the nanoprecipitation method published by Fessi et al. [11]. Briefly, 0.125 g of polymer was dissolved in 20 ml of acetone. The solubility of isradipine in aqueous solution is less than 0.001%, but the drug was soluble in the polymer/acetone solution. Two mg of isradipine were added to the polymer/acetone solution. This organic phase was then added to 50 ml of an aqueous solution containing 0.250 g of Pluronic F68.

Acetone was eliminated by evaporation under reduced pressure and the final volume of the suspension was adjusted to 10 ml. The final concentration of isradipine in the colloidal suspension of nanoparticles was 0.2 mg/ml.

2.2.2. Physicochemical characterization of the suspensions

Diameter and zeta potential. The zeta potential was determined by laser doppler velocimetry (Zetamaster, Malvern Instruments, Orsay, France). All preparations were diluted at 1/100 with a 10^{-3} M NaCl solution to maintain a constant ionic strength and an adequate concentration of particles [12].

pH. pH values of the nanoparticles aqueous suspensions were measured with a pH meter (pH meter P500, Consort) by simply plunging the electrode into the nanosuspensions.

Particle size. Particles diameters were determined by photon correlation spectroscopy. The nanoparticles were coated with gold-palladium and their surface morphologies were observed through a Jeol JSM-840 scanning electron microscope at 20 kV.

X-Ray diffraction and differential scanning calorimetry. The crystallinity of the polymer was examined by differential scanning calorimetry and X-ray diffraction. The glass transition temperature (T_g) and the melting temperature (T_m) were measured with a Mettler 30 differential scanning calorimeter (DSC). The samples (5–10 mg) were scanned from −100°C to 200°C at a scan rate of 10°C/min. The X-ray powder diffraction diagrams were measured using a Siemens D5000. Bragg's angle was obtained from the diffraction pattern [13].

Size exclusion chromatography. The average molecular weights and polydispersity of the polymers were determined by size exclusion chromatography (SEC). The chromatographic system included the following: a pump (LC-10 AS, Shimadzu), a pre-column and two columns (PL gel 5 μm mixed-D, 30 cm, Shimadzu). Tetrahydofuran was used as the mobile phase at 0.8 ml/min and toluene as the internal standard. Samples were freeze-dried and solid residues were then dissolved in tetrahydrofuran containing toluene. The injection volume was 20 μl. The SEC software was calibrated using polystyrene standards (Polymer Laboratories) with a molecular weight ranging from M_w = 11 948 to M_w = 160 680. The weight average molecular weight, number average molecular weight and polydispersity were calculated using SEC software (P.L. caliber GPC/SEC Software version 5.1) from the elution curve using a series of polystyrene standards. The method was validated and the variation coefficient was 3.83% (M_w). Polymer molecular weights were obtained from one sample of nanoparticles.

Determination of percent of theoretical encapsulation. The non-entrapped drug was separated from the carriers by gel filtration chromatography [14]. Sepharose CL4B (Sigma, St Quentin Fallavier, France) gel was filled into

the column, avoiding bubbles and cracks. The column had a length of 45 cm, an inner diameter of 2 cm and contained about 125 ml of gel. In order to separate the free drug from nanoparticles, the solvent used was first water and then a solution of ethanol/water (50:50 v/v) in which the drug was freely soluble. The flow rate of both solvents was adjusted to 1 ml/min with a peristaltic pump. The volume of the samples was fixed to 1 ml. Nanoparticles appeared within about 45 min. Water was then replaced by the mixture of ethanol/water and the free drug appeared about 30 min later. Isradipine was measured by UV spectrophotometry at 326 nm. The percent of theoretical encapsulation into nanoparticles was obtained with the following formula: $((D_{total} - D_{eluant})/D_{total}) \times 100$, where D_{total} is the total drug amount and D_{eluant} is the amount of drug found in the eluant aqueous phase.

In vitro release kinetic experiments. The dialysis bag diffusion technique was used [15]. Four millilitres of the nanosuspensions were placed in the dialysis bag (i.d. 22 mm, M_w cutoff 50 000, Spectrum Medical Industries, Houston, TX, USA), hermetically sealed and dropped into 400 ml of a aqueous environment. Two receptor media were used: phosphate buffer (pH 6.8) and hydrochloric acid medium (pH 1.3) under sink conditions. The entire system was kept at 37°C with continuous magnetic stirring at 200 rev./min. Samples (1 ml) were withdrawn from the receptor compartment at predetermined time intervals and replaced by fresh buffer. The amount of drug dissolved was determined with UV spectrophotometry at 326 nm. Since isradipine is insoluble in water, an isradipine solution (0.2 mg/ml) in 50% of polyethylene glycol 400 and 50% of water was used as a control for the in vitro studies.

Statistical analysis. Results are given as mean ± standard error of the mean (SEM) or ± standard error (SE). Means were compared with the Student's *t*-test. Differences are considered significant at a level of $P < 0.05$.

3. Results and discussion

3.1. Particle size and surface potential characterization

Nanoparticles are characterized by their mean particle diameter and their size distribution. The average diameters, zeta potentials, pH values of empty nanoparticles and isradipine-loaded nanoparticles are listed in Table 1. The average diameters of nanoparticles from the different preparations ranged from 110 to 208 nm. PCL nanoparticles were larger than nanoparticles prepared with the other polymers. There was no significant difference (*t*-test, $P < 0.05$) between diameters of both empty and charged nanoparticles. Although it is now well known that the in vivo fate of nanoparticles is primarily a function of their size, the shape of the nanoparticles may also determine their toxicity.

These measures are confirmed by scanning electron microscopy that allowed both the determination of the particle size and the shape of the particles (Fig. 1). These average diameters were similar to those that were observed with particles reported in previous studies with the nanoprecipitation method [16]. The particles were sufficiently small to avoid sedimentation.

The zeta potential of the nanoparticles was negative due to the presence of terminal carboxylic groups.

High potential values should be achieved in order to ensure a high-energy barrier [17] and favour a good stability. Muller [12] considered that a zeta potential of about −25 mV allows an ideal stabilization of nanoparticles because the repulsive forces prevent aggregation upon ageing. Accordingly, −35 mV, which is the value obtained with PLA50GA50 isradipine loaded nanoparticles, favour the best stability among the designed nanoparticles.

The gel filtration method allows the separation of both the free drug and the loaded nanoparticles. It was found that a high entrapment efficiency of isradipine was obtained with all forms. The percent of theoretical encapsulation was 74.2%, 76.2% and 87.4% for PLA100, PLA85GA15 and PCL, respectively. PLA50GA50 nanoparticles displayed the highest percent of theoretical encapsulation (97.4%).

3.2. Characterization of the physicochemical state of the drug and the polymer in nanoparticles

The physical state of both the drug and the polymer were determined since this will have an influence on the in vitro and in vivo release characteristics of the drug. Different drug/polymer combinations may coexist in the polymeric carriers, such as (i) amorphous drug in either an amorphous or a crystalline polymer, and (ii) crystalline drug in either an amorphous or a crystalline polymer [18]. Also, a drug may

Table 1
Mean diameters (nm), zeta potentials (mV), and pH values of empty nanoparticles and isradipine loaded nanoparticles

Nanoparticles	Empty nanoparticles			Isradipine loaded nanoparticles		
	Diameters (nm)	Zeta potentials (mV)	pH values	Diameters (nm)	Zeta potentials (mV)	pH values
PCL	208.4 ± 2.9*	−29.0 ± 1.1	6.5	197.5 ± 7.2*	−25.5 ± 0.9*	6.3
PLA85GA15 (d,l)	122.7 ± 4.6	−24.7 ± 1.1*	2.9	126.5 ± 4.7	−24.5 ± 1.0*	3.1
PLA50GA50 (d,l)	125.1 ± 2.1	−32.4 ± 1.4	3.9	126.3 ± 2.2	−34.8 ± 0.3	4.0
PLA100 (d,l)	109.9 ± 1.0	−20.3 ± 0.7*	2.8	118.8 ± 0.8	−19.0 ± 0.9*	2.8

Data shown are means ± SE, $n = 4$.
*Significant versus NS PLA50GA50 ($P < 0.05$).

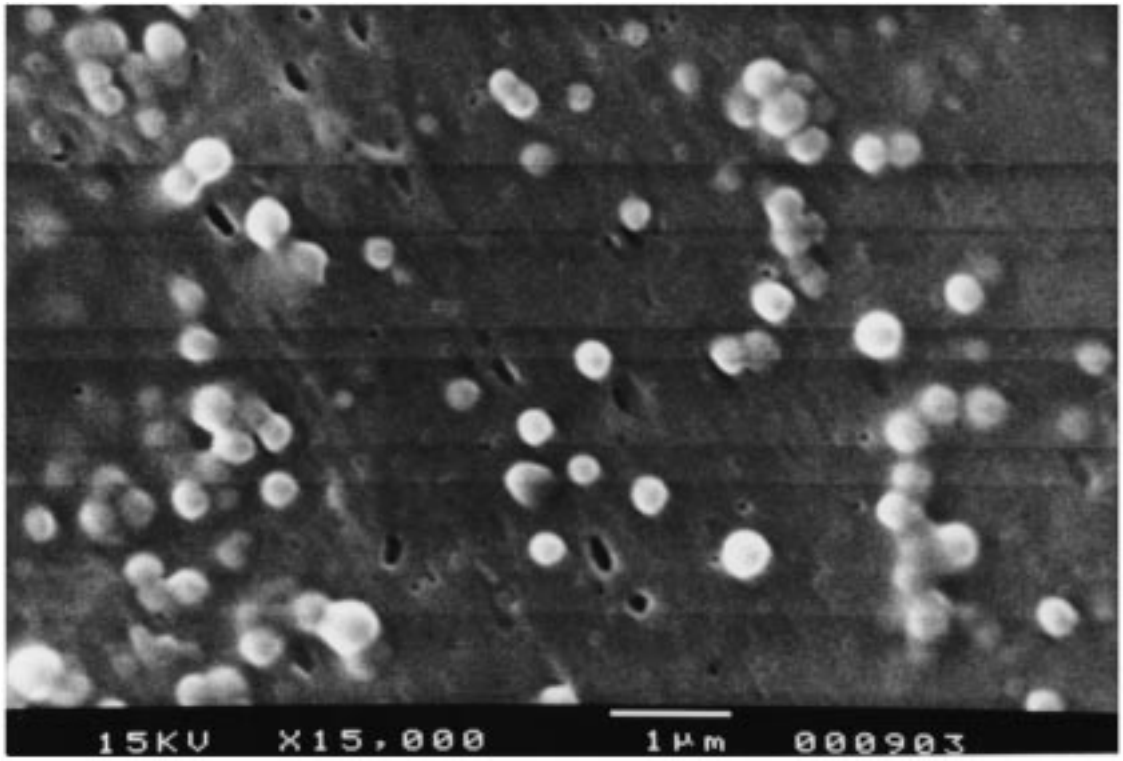

Fig. 1. Scanning electron micrograph of isradipine-poly(epsilon-caprolactone) prepared by nanoprecipitation method.

be present either as a solid solution or a solid dispersion in an amorphous or crystalline polymer.

The physical state of the components of the nanoparticles were determined before preparation of the nanospheres.

The analysis by X-ray diffraction technique demonstrated the presence of many diffraction bands in the isradipine powder. The diffractogram was characteristic of a crystalline material. We observed diffraction bands in Pluronic F68 and PCL polymers that showed crystalline domains in these polymers (Table 2). A more regular baseline was observed in PLA50GA50, PLA85GA15 and PLA100, which verified the amorphous character of these polymers.

We have confirmed these results by DSC studies (Table 2). Isradipine has presented a melting temperature of 169°C that confirmed a crystalline structure. PLA50GA50, PLA85GA15 and PLA100 have shown glass transition temperatures of +40, +51 and +48°C, respectively, which are characteristic of amorphous polymers. For PCL and Pluronic F68, amorphous as well as crystalline domains have been found and so the degradation process, both in vitro and in vivo, will be different for the two domains.

Empty nanoparticles were also characterized with DSC (Table 3). The diffractograms of PLA50GA50, PLA85GA15 and PLA100 nanoparticles have shown diffraction peaks that were not due to a change in the physical state but due to the presence of Pluronic F68. Nanoparticles with PCL have presented diffraction bands due to PCL and Pluronic F68. The results of the DSC studies have confirmed these data with no change in melting temperature and glass transition temperature when compared to polymers before nanoparticles were prepared (Table 3).

The diffraction patterns of all types of nanoparticles incorporating isradipine have shown that some distinctive isradipine diffraction peaks observed in isradipine powder disappeared (Table 4). PCL maintained its semi-crystalline characteristics in PCL nanoparticles prepared by the precipitation method.

The precipitation method implies a re-precipitation of

Table 2

Interreticulary distance (d) determined by X-ray diffraction and main thermal events (°C) observed in the bulk compounds (e.g. melting temperature (T_m) and glass transition temperature (T_g)) before preparation of nanoparticles

	Isradipine (main peaks)	Pluronic F68	PCL	PLA50GA50	PLA85GA15	PLA100
d	9.26	4.59	4.15	–	–	–
	7.43	3.78	3.75			
	3.81 and many more	3.39	3.39			
T_m (°C)	+169.4	+50.1	+65.6	–	–	–
T_g (°C)	–	–63.4	–65.1	+40.2	+50.6	+48.4

Table 3

Interreticulary distance (d) determined by X-ray diffraction, melting point temperature (T_m) and glass temperature (T_g) of PCL, PLA50GA50, PLA85GA15, PLA100 of empty nanoparticles stabilized by Pluronic F68

Empty NP	PCL	PLA50GA50	PLA85GA15	PLA100
d	4.60	4.60	4.63	4.64
	4.16	3.78	3.81	3.81
	3.73	3.39	3.37	34.0
	3.40			
T_m (°C)	+51.4	–	–	–
T_g (°C)	–65.0	+48.7	+48.6	+48.7

both the drug and the polymer from the previously solubilized state in acetone. Because of the low concentration of the drug in the nanoparticles, it cannot really be discriminated between a molecular dispersion of the drug and amorphous solid drug in the polymers. However, in both cases, isradipine will dissolve faster which may change the in vivo behaviour of this poorly soluble drug. In addition, the semicrystalline character of PCL was not modified except for a slight shift in the glass transition and melting temperatures of the polymer.

DSC and X-ray diffraction techniques are often combined together to give useful information on the structural characteristics of both polymers and drugs in the field of microencapsulation. For nanoparticle characterization, it was also beneficial to use both techniques. There are only a few reports on the X-ray diffraction pattern of nanoparticles in the literature. Polyacrylic nanoparticles and poly(butylcyanoacrylate) nanoparticles had amorphous profiles using X-ray diffraction and showed no signs of crystallinity; however, the diffraction pattern of poly(butylcyanoacrylate) containing pilocarpine was different from the empty particles and that of the drug [19]. In this case too, the formation of a molecular dispersion of the drug in the polymer matrix is suggested.

The molecular weight characterization of the bulk polymers is summarized in Table 5. Almost all synthetic polymers are heterodispersed because they have different chain lengths and a range of molecular weights. The polymer weight is a very important parameter, since it will influence

Table 4

Interreticulary distance (d) determined by X-ray diffraction, glass temperature (T_g) and melting point temperature (T_m) of PCL, PLA50GA50, PLA85GA15, PLA100, nanoparticles incorporating isradipine stabilized by Pluronic F68

Isradipine loaded NP	PCL	PLA50GA50	PLA85GA15	PLA100
d	4.79	4.60	4.63	4.63
	4.16	3.78	3.80	3.81
	3.73	3.39	3.37	3.35
	3.39			
T_m (°C)	+50.8	–	–	–
T_g (°C)	–62.2	+41.0	+47.7	+48.5

Table 5

Characterization of polymers used in the study

Polymers	PCL	PLA50GA50	PLA85GA15	PLA
M_w	54624 ± 620	51475 ± 526	112573 ± 346	93149 ± 476
I	1.15	1.10	1.33	1.29

Data shown are means ± SE, $n = 3$.
M_w was determined by SEC as described in Section 2.
I, polymolecularity index.

physicochemical properties such as the diffusion rate of a drug or the degradation rate of the polymer. In our study, the molecular weight was expressed by the weight average molecular weight (M_w), and the ratio M_w/M_n (M_n was the number average molecular weight) was a measure of the polydispersity of a polymer sample. PCL and PLA50GA50 have similar molecular weights of 54 624 and 51 475, respectively. For PLA85GA15 and PLA 100, the molecular weights were higher at 112 573 and 93 149, respectively.

3.3. Drug release studies

It needs to be verified in vitro that nanoparticles are able to release incorporated drugs in order to achieve a biological effect. Membrane diffusion techniques are the most widely used experimental methods for the study of the in vitro release profiles of drugs incorporated in nanoparticles. The release profile of isradipine at pH 1.3 is presented in Fig. 2, which shows a very rapid diffusion of isradipine with the PEG solution: 50% of isradipine is released after 2 h and 90% after 4 h. This release profile show a biphasic phenomenon.

The colloidal suspensions display a sustained release profile. The profiles were similar for all nanoparticles during the first 14 h. After this time period, only about 40% of isradipine was released. During the first 24 h, the release profiles were not significantly different and the percentage of isradipine released was about 50%.

After 72 h of dialysis at pH 1.3, the percentages of isradipine released were 60, 80 and 85% for PLA50GA50, PLA85GA15 and PLA100 nanoparticles, respectively. At pH 6.8, the percentage of isradipine released was 100% for PLA85GA15 and PLA100 nanoparticles and only 90% for PLA50GA50.

The profiles obtained at pH 6.8 (not shown) are very similar and also display a biphasic phenomenon over a 24-h period. Consequently, the effect of acidic and neutral pH conditions on drug release seems minor.

In both pH media, there was no burst effect and consequently it is believed that the initial hypotensive initial peak observed with solutions should be reduced or absent, as observed in previous studies [9].

The selection and optimization of drug release studies is a problem with colloidal drug delivery systems. Other solid dosage forms (e.g. tablets, capsules, microspheres) can be

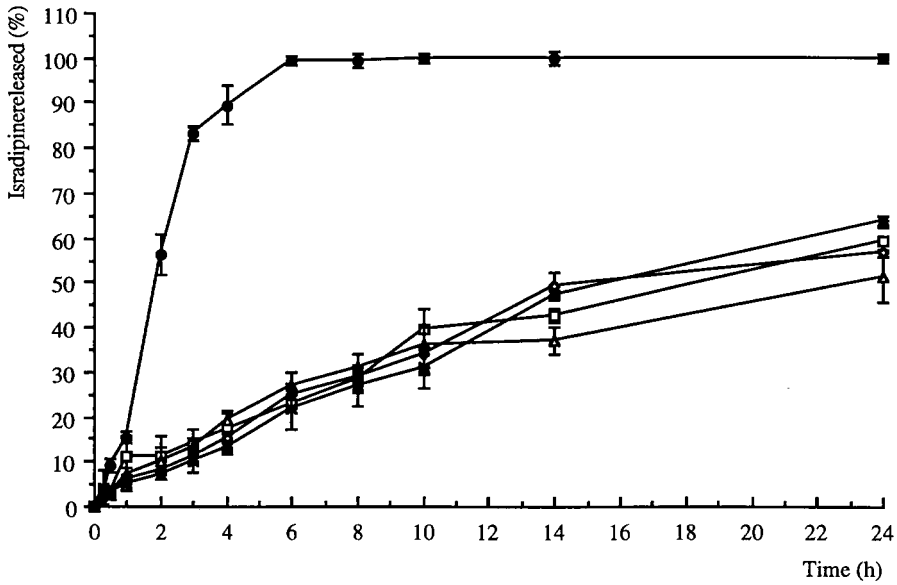

Fig. 2. Cumulative percentage of isradipine released from polyethylene glycol 400 solution and nanoparticles (n = 3) at pH 1.3. (●) PEG solution; (□) PCL nanoparticles; (△) PLA50GA50 nanoparticles; (◇) PLA85GA15 nanoparticles; (■) PLA100 nanoparticles. Each point represents the mean and vertical lines show SEM.

readily separated from the surrounding bulk medium. This is not the case for colloidal polymeric dispersions and it is generally necessary, as for the determination of the percent of theoretical encapsulation of the drug, to separate the release medium from the polymeric suspension by means of either ultracentrifugation or separation membrane.

The dialysis technique is a very popular method to study the release of drugs from colloidal suspensions [20]. The release studies were carried out in an aqueous environment with pH 1.3 and pH 6.8 to mimic the in vivo condition, e.g. gastric pH and intestinal pH, since the aim of the study was to administer the nanoparticles by the oral route. The limits of this dialysis technique have been discussed by Washington [20,21], who reported that the colloidal dispersions were not diluted inside the bag. The release rate of the drug and its appearance in the dissolution medium is governed by the partition coefficient of the drug between the polymeric phase and the aqueous environment in the dialysis bag and by the diffusion of the drug across the membrane as well. This technique allows the comparison of different formulations.

To explain the difference in the percentages of isradipine released, the relative loss in the weight molecular weight for each polymer was calculated. The parameter was calculated by the formula:

$$\frac{\Delta M_w}{M_{w_i}} \times 100$$

where

$$\Delta M_w = Mw_i - Mw_f$$

Mw_i was the weight molecular weight before dialysis

Mw_f was the weight molecular weight after dialysis

Table 6

Relative loss of molecular weight for polymer (n = 3 samples) after dialysis in phosphate buffer at pH 6.8

	Nanoparticles			
	PLA50GA50	PLA85GA15	PLA100	PCL
$(\Delta M_w/M_{w_i}) \times 100$ (%)	2.26 ± 0.49	10.37 ± 0.25	9.87 ± 4.48	2.93 ± 0.75

PLA85GA15 and PLA100 nanoparticles showed a slight loss in molecular weight (10%), which was significantly higher than the loss for PLA50GA50 and PCL (2 and 3%, respectively). PLA85GA15 and PLA100 were more susceptible to hydrolysis. The drug release was a product of diffusion and bioerosion mechanisms that were less significant with PLA50GA50 and PCL (Table 6).

In summary, nanoparticles were produced containing isradipine with a high entrapment efficiency of isradipine. Nanoparticles prepared with PLA50GA50 presented the best entrapment efficiency (97%). X-Ray diffraction and differential scanning calorimetry have shown the amorphous state of PLA 100 and PLAGA nanoparticles and the semi-crystalline state of PCL. In all types of nanoparticles, non-crystalline isradipine was found in the polymer which suggests a molecular dispersion of the drug in the matrix. The four colloidal suspensions all displayed a sustained release profile and an absence of a burst effect when compared to the release profile of isradipine in the PEG solution. All formulations are good candidates for our future studies of oral administration of the nanoparticles, and our goal will be to reduce the initial hypotensive peak and to prolong the antihypertensive effect of the drug.

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