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Development and Characterization of Cefoxitin Loaded D,L-PLA Nanoparticles

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ABSTRACT

Nanoparticles containing cefoxitin (CEF) made of D,L-PLA (PLA) were designed by a multiple emulsion/solvent evaporation method. The particles were extensively evaluated in relation to morphology, encapsulation efficiency, drug-polymer interactions as well as *in vitro* drug release kinetics. Nanoparticles were spherical in shape and isolated, with a mean diameter of about 600 nm. The thermal behaviour (DSC) of CEF-containing nanoparticles suggested that the drug was dispersed at a molecular level within the system. The drug encapsulation efficiency in the system for a CEF concentration of 30 mg/mL was 5.5%, as assessed after the drug extraction, by a validated HPLC method. This low encapsulation efficiency is understandable, since CEF is highly hydrophilic. The *in vitro* assays showed a strong sustained drug release profile from the nanoparticles with kinetics following pure Fickian diffusion.

Keywords: Nanoparticles. Controlled release. D,L-PLA. Cefoxitin. *In vitro* release.

INTRODUCTION

Many researches have been carried out based on drug release from biodegradable polymeric devices. Among different biodegradable polymer classes, aliphatic poly(esters), such as the polymers of lactic and glycolic acids and the correspondent copolymer, poly(lactic-co-glycolic) acid (PLGA) have generate great interest due to their favourable characteristics of biodegradability and resorbability through natural pathways (Rajeev, 2000; Panyam & Labhasetwar 2003). Recently, an important study was performed in order to evaluate the *in vivo* distribution and safety of PLGA nanoparticles as drug delivery systems. The results showed that biodegradable and biocompatible PLGA nanoparticles are non toxic in cell culture and when orally administered to Balb/c mice. Besides, the biodistribution data show that nanoscale drug delivery systems will be suitable to improve the permeability, and thus the bioavailability of therapeutic compounds (Semete et al*.,* 2010).

In addition to drugs of different classes, various antibiotics, such as gentamicin (Huang & Chung 2001), vancomycin (Cevher et al*.,* 2006), ciprofloxacin (Orhan et al. 2006), ampicilin (Giunchedi et al., 1998), tetracyclin (Liu et al*.,* 2004) and rifampicin (Durán et al., 2008) have been microencapsulated in order to optimise the treatment of local infections, but no results about micro-nanoencapsulation of CEF could be found in the literature.

The majority of intracellular infections is parasitic and difficult to eradicate because the bacteria, since located in fagosomes, stay protected against the antibiotic action. In these cases, the infected cells constitute real reservoirs of microorganisms being released in a stepwise fashion, causing the systemic infections to be recurrent (Couvreur et al., 1991). As a strategy to increase the intracellular antibiotic efficiency, the dosage form can be modified in order to achieve the drug controlled release and its targeting to specific sites. Particulate systems are well known for being able to deliver drugs with high efficiency and fewer adverse side effects, possibly because the endocytosis of the drug carriers (Vieira & Carmona-Ribeiro, 2008). Associations of antibiotics and colloidal particles allow the development of endocytable formulations (Couvreur et al., 1991; Prior et al*.,* 2000; Wu et al., 2004) and it is well known that nanoparticles present several advantages over liposomal preparations concerning the ease of preparation and stability issues (Benita, 2006).

Micro- nanoencapsulation presents a promising delivery system of controlled and targeted release (Ravichandran, 2009). It has been widely used in the drug and dairy industries as well as in agriculture. This technology can increase the drug stability, eliminate incompatibilities, mask the undesirable organoleptic aspects and, additionally, allows the development of dosage forms

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able to control or prolong the drug release (Schnieders et al., 2006). This control over the drug release is specially represented by the ability of the micro- nanoparticles to provide a targeted drug release, not only because of their very small size (passive targeting) as well as by guiding the systems to specific tissues, thanks to special substances that can be incorporated onto their surfaces (active targeting) (Jyothi et al., 2010). Several methods are described in the literature for the preparation of micro- nanoparticles, but the appropriated technique is mainly governed by the solubility of both the polymer and the drug. Due to its preferential solubility in volatile organic solvents, the classical method for preparing PLA or PLGA micro- or nanoparticles is the emulsion-solvent evaporation (Yeh et al., 2004, Pinto-Reis et al., 2006; Uchegbu & Schatzlein, 2006). However, this method yields poor encapsulation efficiency for hydrophilic substances, because the great amount of water of the emulsion external phase causes the drug being encapsulated to diffuse into it, resulting in low encapsulation rates. In order to solve this problem, Nihant et al., (1994) developed the multiple emulsion/solvent evaporation method. Subsequently, several hydrophilic drugs have been successfully encapsulated by this method (Freitas et al., 2005; Pinto-Reis et al., 2006, 2006a).

Cefoxitin (CEF) belongs to the cephamycin group, b-lactam antibiotics with narrow chemical relativity with cephalosporins (Robbers et al., 1997). It is very soluble in water (Farmacopeia Portuguesa, 2002; USP, 2005). Since b-lactam antibiotics act in the process of synthesis of bacterial cellular wall (peptidoglycan), they are less toxic to the human organism than antibiotics of other classes (Trabulsi & Toledo, 2002). Such drugs inhibit in an irreversible way the transpeptidation enzyme, which acts on the crosslinking of peptide chains bonded to the peptidoglycan main axis. The final bactericidal action consists in the inactivation of an inhibitor of auto-catalytic enzymes at the cellular wall, resulting in the bacteria lysis. It is prescribed in severe infectious processes caused by Gram-negative bacilli and by anaerobic germs (Rang & Dale, 2007). It shows *in vitro* activity against *Mycobacterium fortuitum* and has been used for the treatment of pulmonary and non-pulmonary infections caused by fast-growing *Mycobacteria* (Cynamon & Klemens, 1994).

Since CEF is useful for treating intracellular bacterial infections and no work about nanoencapsulation of CEF is to date described in the literature, the aim of this work was to prepare CEF loaded PLA nanoparticles by means of multiple emulsion/solvent evaporation method and to characterize them in order to foresee possibilities to further design an endocytable controlled drug release system.

MATERIALS AND METHODS

Materials

The biodegradable polymer studied was D,L-polylactic acid with an average molecular weight of 19,000 Da (Purac, Holland). As surface-active agent, polyvinyl alcohol (PVA, Mallinckrodt, France) was used. Cefoxitin Sodium (CEF) standard (Sigma, USA) and commercially available Cefoxitin Sodium (Mefoxin® Merck, Sharp & Dohme) were used to analyse and to prepare drug-containing nanoparticles. Purified water of Milli-Q quality was used to prepare the solutions as well as the aqueous phases of the emulsions. All other reagents were of analytical or HPLC grade.

METHODS

Nanoparticles preparation

The nanoparticles were prepared following the double emulsion/solvent evaporation method described by Zambaux et al., (1998) with some adaptations. Although the W/O/W encapsulation process can be considered relatively simple to be carried out, there are several parameters affecting the properties of microparticles such as particle size, morphology and release characteristics, which must be taken into account. Some of these variables were evaluated in the present work.

Approximately 25 mg of PLA were dissolved into 4 mL of methylene chloride. This organic phase was emulsified with 200 µL of sodium phosphate buffer pH 7.0 by sonication (Ultrasonic Process XL Heat Systems) or high-speed homogenisation (Ultraturrax homogenizer, mod T25, Ika-Labortechnik) during 30 min in a sonication tube. This first W/O emulsion was re-emulsified in 4 mL of aqueous 5.0% PVA solution by sonication or high-speed homogenisation (Ultraturrax homogenizer, mod T25, Ika-Labortechnik) for 10 min, resulting the W/O/W multiple emulsion. The whole sonication process was conducted on an ice bath. The multiple emulsion system was dispersed into further 40 mL of aqueous 0.1% PVA solution under stirring by vortex. Solvent removal was carried out in a rotate evaporator (Tecnal, mod. TE 120) under partial vacuum for 20 min. The precipitated nanoparticles were separated by centrifugation at 20,000 rpm for 15 min (Avanti, mod. 30) and washed three times with purified water. Finally, the samples were freeze-dried (Edwards, mod. Pirangi 10) for 24h.

Morphology

The morphology of the nanoparticles produced was investigated by scanning electron microscopy (JEOL, JSM, mod. 330T). The nanoparticles were fixed on adequate supports and coated with gold under an argon atmosphere using a gold sputter module in a high-vacuum evaporator. Observations under different magnifications were performed at 20 kV.

Particles size and size distribution measurements

The size and size distribution of the nanoparticles were analysed by photon correlation spectroscopy (PCS) using a particle size analyser (Brookhaven Instruments Corp.). Particles diameters were assessed at scattering angle of 90° and at a temperature of 25°C for two different

dilutions of particles in water, i.e. 1:1 and 1:4. Dilution was necessary, because the particles can develop a slow Brownian motion in concentrated environments, which results in diameters bigger than the actual. The samples and respective dilutions were kept in scintillation vials free of dust. Ten determinations for diameter and for polydispersity index were taken for each preparation and the standard deviation and the variation coefficient were calculated. All samples were considered as monodisperse systems.

DSC analysis

The physical state of CEF entrapped in the nanoparticles, as well as the polymer, the black nanoparticles and the physical mixture of two substances (cefoxitin and blank nanoparticles) were characterised by differential scanning calorimetry thermogram analysis (Shimadzu DSC 50). The samples (~2 mg) were weighed and sealed in aluminium pans and heated under nitrogen by a heating rate of 10°C/min, the heat flow being recorded from 30 to 200°C. Indium was used as standard reference material to calibrate the temperature and energy scales of the DSC instrument. The data were analysed by the TAS-Thermal Analysis Workstation software.

Determination of CEF loading

About 10 mg of nanoparticles containing cefoxitin were carefully weighed and 5 mL of chloroform were added to dissolve the polymer matrix. Then, 20 mL of sodium phosphate buffer pH 7.0 were added to precipitate the polymer and extract the drug. This mixture was homogenized and centrifuged at 12,000 rpm for 15 min, separating the dissolved drug from the polymer matrix. The amount of entrapped drug was quantified by HPLC (Shimadzu, Japan) in triplicate with the UV detection set at 254 nm (USP, 1990). A reversed phase Lichrospher C18 (Merck, USA) column (240x4mm i.d., pore size 5 µm) was used. The mobile phase consisted of a mixture of water:acetonitrile:glacial acetic acid (84:16:1 v/v) and the flow rate was set at 1.0 $mL.min^{-1}$.

***In vitro* release profiles**

The diffusion cell model adapted to the spectrophotometer cubet, with 1 cm of optic way and 2.5 mL of volume (Mainardes & Evangelista, 2005), was used for the *in vitro* release of CEF. About 10 mg of nanoparticles containing drug were dispersed into 1.0 mL of sodium phosphate pH 7.0. A volume of 100 µL of this dispersion was placed onto the cellulose acetate membrane attached in the diffusion cell. The following conditions were used in the assay: Temperature of 37 ± 2ºC; cellulose acetate membrane for retention of macromolecules with molecular weight over 12,000 Da (Sigma-Chemical, EUA); volume of receptor solution (phosphate buffer pH 7.0) of 2.5 mL placed in the spectrophotometer cubet; constant magnetic stirring; analysis time of 15 min, the absorbances at 254 nm been assessed each 5 min.

Several mathematical models describing the release kinetics were fited to the CEF dissolution curve in order to try to explain the release mechanism. For that, the plot equation tool of the SigmaPlot 2004 for Windows Version 9.0 from Systat Software, Inc. was used. The release mechanism was obtained from the best fits based on the adjusted R^2 values. Adjusted R^2 was used because it is more meaningful when models with different number of parameters are being compared (Costa & Lobo 2001; Sonnengaard, 2006).

The model developed by Higuchi for both water soluble and low soluble drugs being released from semi-solid or solid matrices relates the release rate with the cubic square root of time. It can be expressed by the simple equation (Costa & Lobo, 2001):

$$Q_t = K_H \sqrt{t}$$

where Q_t is the amount of drug dissolved at time t and K_H is the Higuchi's constant. This relative simple mathematical model describes a typically Fickian drug diffusion from a matrix system (Siepmann & Peppas, 2001).

Baker and Lonsdale developed a mathematical model derived from that of Higuchi. This is useful for describing the controlled drug release from spherical matrices. The following equation expresses the model, since the matrix is homogeneous and presents no fractures and/or capillaries contributing effects on the drug release process (Costa & Lobo, 2001):

$$(3/2)\left[1-\left(-1(Q_t/Q_\infty)\right)^{2/3}\right]-(Q_t/Q_\infty)=K_t$$

where Q_∞ is the total (or initial) drug amount and K_t is the equation coefficient.

The Peppas model, sometimes called Korsmeyer-Peppas model, is an adaptation of the Power Law for drug release purposes. Although it is more frequently used with swellable systems, if applied to the first 60% of cumulative amount of drug released from systems presenting unknown release mechanism, the corresponding values of the exponent n can differentiate, in a satisfactory manner, the release mechanism (Brazel & Peppas, 2000; Costa & Lobo, 2001).

$$Q_t/Q_\infty = Kt^n$$

Since sigmoid curves represent very frequently the drug release profiles, the general empirical Weibull equation was adapted to release processes and has been successfully used to explain the kinectic behaviour of drug dissolution (Papadopoulou et al., 2006). Its expression is:

$$Q_t/Q_\infty = 1-\exp\left(-at^b\right)$$

where a is a scale parameter related with the time and b is a shape parameter of the dissolution curve.

The best fits, on which adjusted R^2 were above 0.99, corresponded to the models: Higuchi (0.9910), Baker & Lonsdale (0.9916), Korsmeyer & Peppas (0.9951) and Weibull (0.9954).

RESULTS

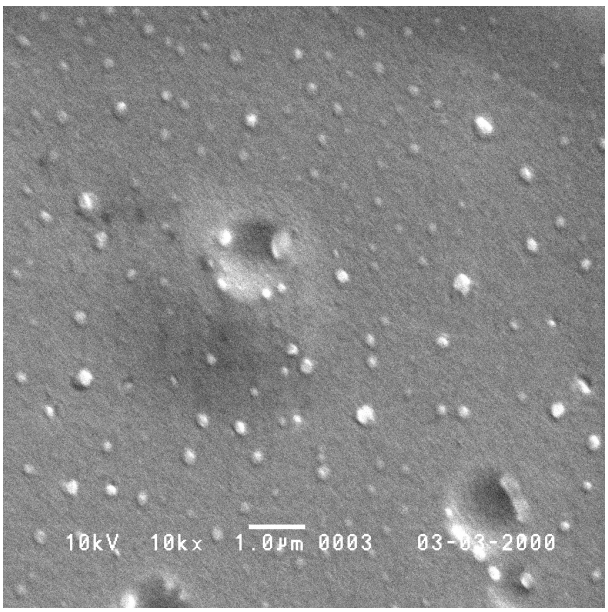
Size, size distribution and morphology

The influence of the process' parameters in size, size distribution and morphology is demonstrated in Table I and Figure 1. Each formulation was analysed in triplicate and the data represent mean values with the standard deviation.

Table I: Influence of methodological parameters on D,L-PLA nanoparticles granulometry.

Internal Phase (μL)	Effective mean diameter (nm)	Granulometric distribution (%)
100	176.2 ± 2.353	89% (125-200 nm)
500	181.5 ± 3.681	100% (172-200 nm)
PVA (%)		
3.0	271.8 ± 4.634	100% (210-224 nm)
5.0	180.3 ± 1.098	100% (100-240 nm)
8.0	169.3 ± 2.030	85% (130-160 nm)
Evaporation by:		
Magnetic stirring	210.6 ± 0,987	96% (157-258 nm)
Rotate evaporator	176.2 ±1,467	89% (125-200 nm)
Sonication time:		
10 min	345 ± 1,843	60% (250-460 nm)
30 min	180.3 ± 2,973	100% (100-240 nm)
Homogenisation:		
Sonication (10 min)	345 ± 3,462	100% (100-240 nm)
Homogeniser (16.000 rpm)	800 ± 3,654	70 % (700-1000 nm)

After careful analysis of all parameters tested, the drug containing D,L-PLA nanoparticles were prepared under the following conditions: a) Volume of the internal aqueous phase W_1 = 200 μL; b) 5.0% PVA as stabilizer; c) removal of organic solvent from multiple emulsions ($W_1/O/W_2$) by rotate evaporator and d) homogenisation by sonication for 10 min. D,L-PLA nanoparticles containing CEF (30 and 40 mg.mL^{-1}) presented mean diameter of 600 and 527 nm, respectively.



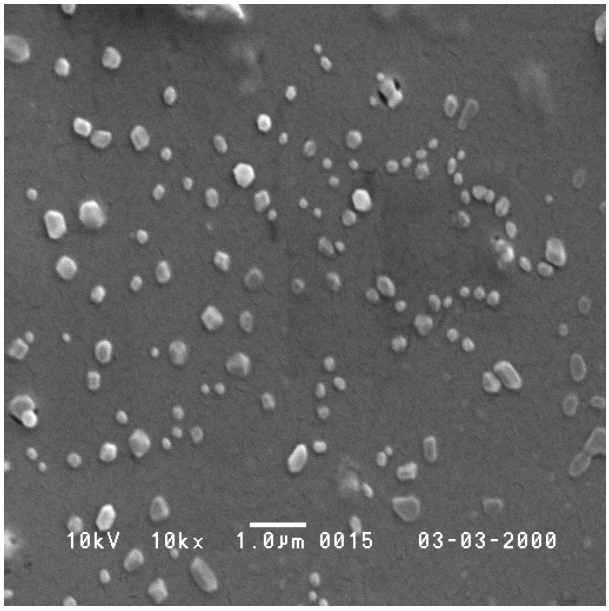


Figure 1: SEM images of D,L-PLA nanoparticles prepared by multiple-emulsion/solvent evaporation with a) homogenisation by sonication during 10 min and b) homogenisation by high-speed homogeniser (16,000 rpm) during 10 min.

Morphological analysis of the D,L-PLA nanoparticles was carried out by scanning electron microscopy and showed regular and isolated particles (Figure 1).

Determination of encapsulation efficiency

As one of this work's goals was to quantify the encapsulated drug, it was necessary to develop and validate a HPLC analytical methodology, which should be sensitive and reproducible for dosing CEF in the preparations obtained. Application of linear regression on statistical data of the analytical curve of CEF in water, relating peak areas with CEF concentration, shows linearity for the range from 20 to 200 μg.mL^{-1} described by y = 22087x + 34199, with r = 0.9994. Method's precision and accuracy were confirmed (RDS < 5.0%) by the results obtained from the analysis of CEF standard solutions prepared in three different concentrations and in triplicate.

It was also necessary to standardise and validate a method for the extraction of CEF after the degradation of polymer matrix. The results showed that this method was exact and accurate, presenting 91.3% of recovery. Each formulation was made in triplicate and the data represented mean values with the standard deviation.

Table II: Encapsulation efficiency for CEF in PLA nanoparticles.

Theoretical drug concentration (mg.mL^{-1})	Actual drug concentration (μg.mL^{-1})	Actual drug concentration (%)
30	129.60 ± 4.22	5.4
40	96.06 ± 1.74	3.0

The results of the encapsulation efficiency (Table II) showed that the increasing of drug concentration did not cause an increase in the encapsulation rate of the system.

DSC studies

Figure 2 and Table III show the values for DSC data of pure D,L-PLA, of nanoparticles with and without drug and the melting point of CEF and physical mixture blank nanoparticles and CEF.

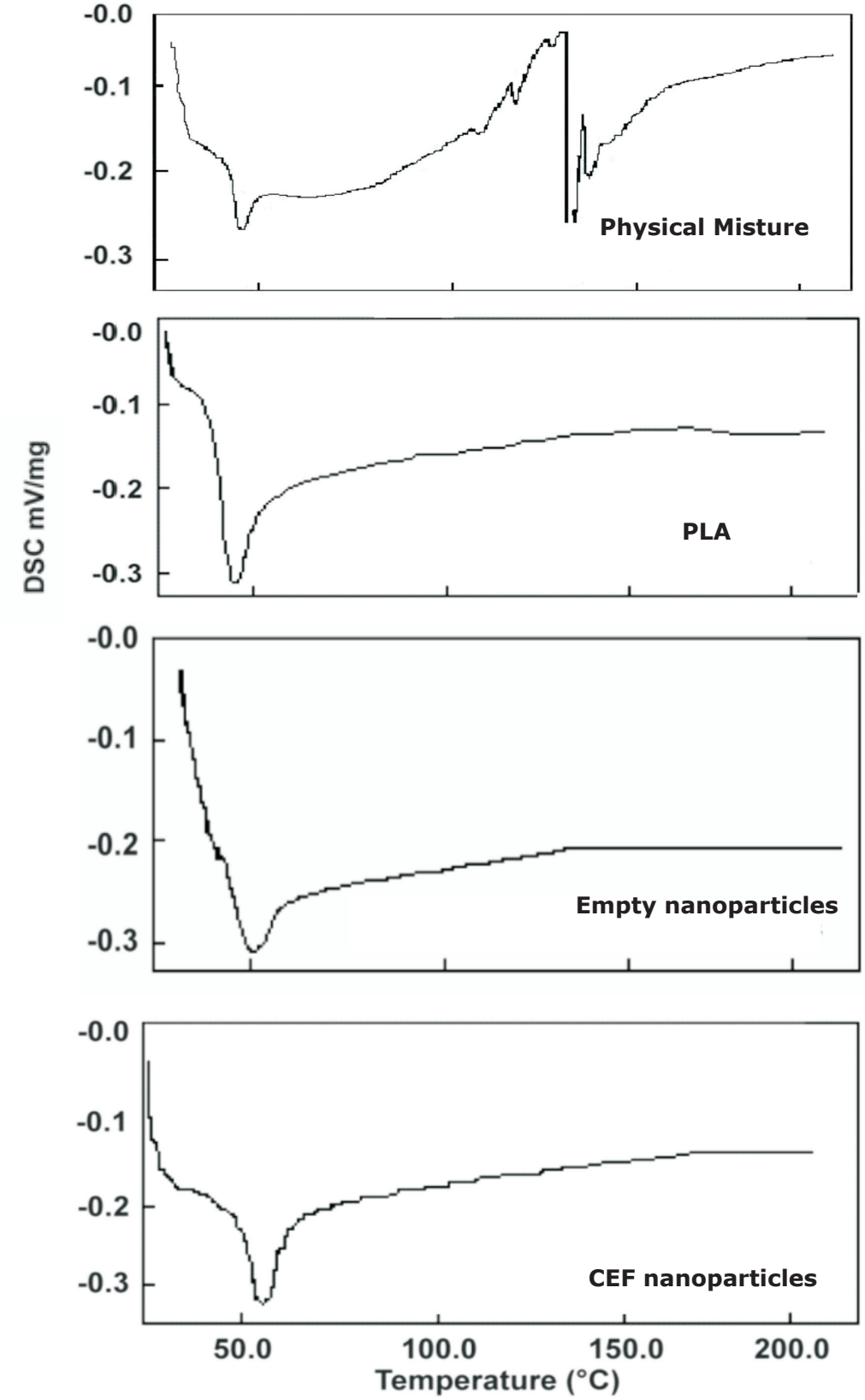


Figure 2: DSC data of physical misture (empth nanoparticles + cefoxitin), pure D,L-PLA and of nanoparticles with and without CEF.

Table III: The glass transition temperature (Tg) obtained from DSC analysis of the D,L-PLA, CEF, Inert nanoparticles and CEF containing nanoparticles (5.4%).

Materials	Temperature (°C)
D,L-PLA	46
Inert nanoparticles	50
CEF-containing nanoparticles	52
CEF (melting point)	135
Physical mixture	50/ 145

The results showed an increase in glass transition temperature of the polymer submitted to the nanoencapsulation process. This was already expected, since there is a structural polymer rearrangement prior to the particles formation. Another interesting detail observed was the absence of the peak corresponding to the melting of CEF around 135 ºC on the isotherm of the drug-containing nanoparticles.

In vitro CEF release

In order to quantify the concentration of drug released in the receptor millieu (sodium phosphate buffer pH 7.0) it was necessary to validate an analytical curve for CEF at 225 nm, since the device used in the release studies was attached to the apparatus allowing to direct measurements of the concentration of released drug. The results of the application of linear regression on the standard curve showed a linear relationship between absorbances and drug concentration in the range from 0.25 to 92 µg/mL according to the equation y=0.0184x – 0.0159 with r = 0.9993. In order to assess precision and accuracy of the method, solutions of CEF standard in three different concentrations and in triplicate were prepared and analyzed. The results showed that the method is precise and accurate (RDS < 5.0%).

Figure 3 shows the *in vitro* release profile of CEF from PLA nanoparticles (n=6) compared to an aqueous solution of the drug.

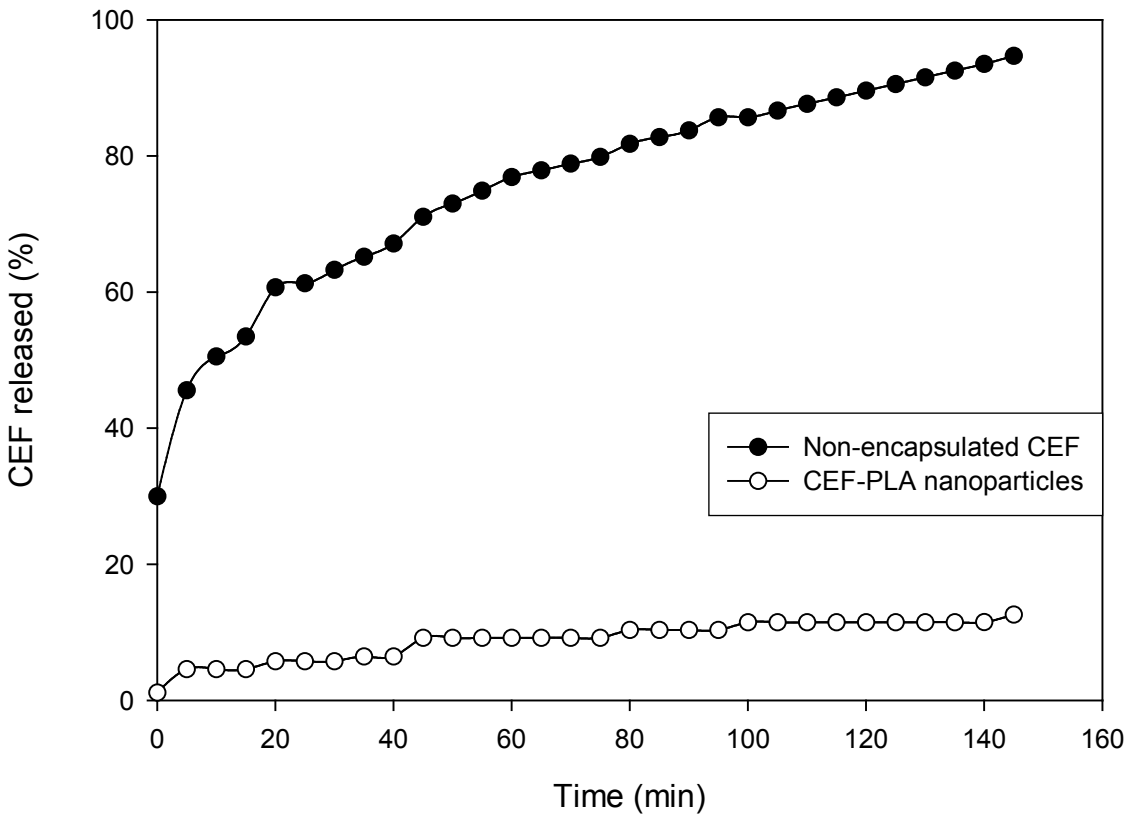


Figure 3: In vitro CEF release from PLA nanoparticles.

The results demonstrated a very slower drug release from the encapsulated system in comparison to the free drug in solution. Comparatively, at the end of the assay the nanoparticles released only approximately 15% of CEF whereas about 90% was released from the free drug.

Several mathematical models were applied to the CEF dissolution profile obtained. These results associated with the values obtained for the respective coefficients indicate that CEF was released by a predominant Fickian diffusion mechanism. Thus, the values found for coeficients *n* and *b*, for Peppas and Weibull models, respectively, were 0.3775 and 0.5105. These coefficients values are in agreement for those established for Fickian drug diffusion from spheres in the Peppas model (< 0.45) (Siepmann & Peppas, 2001) and for the Weibull model (< 0.75). On the other hand, they contribute to reinforce the linear correlation between the *n* exponent of the power law derived from the initial 60% of the release curves and the exponent of time *b* of the Weibull function (Papadopoulou et al., 2006).

DISCUSSION

Microencapsulation by the multiple emulsion/solvent evaporation technique consists in a two-steps emulsification process that generates a transitory emulsion (W_1/O) prior to the formation of the double emulsion (W_1/O/W_2). The principle of encapsulation is based on the induction of separation of polymer phase, due to the partial diffusion of the solvent into the voluminous aqueous external phase W_2, but mainly due to its further evaporation. In that way, the polymer builds a coacervate enclosing the internal drug containing aqueous phase (W_1). Micro- nanoparticles solidify as the residual solvent is removed (Schnieders et al., 2006). Although this technique seems to be simple, there is a number of parameters that can affect some of the nanoparticles properties, like their size, morphology and drug release (Chen et al., 2002).

Variation of internal phase (phosphate buffer pH 7.0) did not alter significantly the size of the particles obtained. This demonstrates that it is possible to increase the volume of this phase in order to increase the amount of encapsulated drug. Fuminori et al., (2007) observed that the particle size increased with increasing the volume ratio of W_1 phase against oil phase, W_1/O (v/v). However, the encapsulation efficiency is not affected by this parameter, but it is influenced by drug concentration in W_1 phase.

Concerning the influence of tenside amount, as its concentration in the external phase was increased there was a significant decreasing in the particles diameter. Similar effect was observed when the rotate evaporator was used for solvent evaporation and when a longer homogenisation time was applied in the multiple emulsion preparation. On the other hand, unfortunately, nanoparticles prepared at a longer sonication time showed contamination with titanium traces from the equipment rod, as could be visually assessed in the form of greyish spots. The stirring method used for the particles preparation showed to be an important parameter for the process. The use of ultra-homogenisation at 16,000 rpm (Ultra-Turrax mod. T25, Germany) for 3 min during the preparation of the primary emulsion (W_1/O), followed by 10 min for the multiple emulsion (W_1/O/W_2) resulted nanoparticles with mean size a bigger than those observed for the particles obtained by sonication (Figure 1). The same occurred with the data relative to tenside concentration. It was demonstrated that an increase in the stabiliser concentration has the effect to diminish the particles size (Mao et al., 2007). Apparently, there is a critical viscosity for the continuous phase, under which a homogeneous emulsification is not possible and the particles size increases. The addition of an efficient emulsifier, such as PVA, is required, because it builds a protective layer around the oily droplets, preventing the coalescence of the proto-nanoparticles during the step of solvent removal and avoiding the formation of particles agglomerates.

Emulsification involves a complex interaction between the mechanical force applied, which is dependent upon the reactor design, and viscosity of the emulsified millieu. During the emulsification step the most important parameter is the stirring condition during the formation of the primary emulsion W_1/O, since it will determine the size of the droplets built and, consequently, the size of the particles obtained. Studies have shown that the mean particles diameter decrease with the increase of both rate and duration of stirring (Moinard-Checot et al., 2006). In our work, both the stirring by a higher energetic homogenisation using sonication and a longer time of operation resulted in a decreasing in the particles diameter.

The choice of the organic solvent to dissolve the polymer is also important. Chlorinated solvents, like chloroform and methylene chloride, are widely employed (Quintanar-Guerrero et al., 1998) because they are immiscible and easily emulsified in water, show good dissolving properties for several molecules and low boiling point, which facilitates their easy removal from the system by evaporation. Besides, the interfacial tension between organic solvent and water phase influences the particle size and drug loading efficiency in PLGA microparticles (Fuminori et al., 2009).

During the evaporation step, the solvent in the form of droplets diffuse through the continuous phase and evaporates at the emulsion surface, due to the increase of temperature and decrease of pressure (Moinard-Checot et al., 2006). Chen et al., (2002) studied the mechanism involved in the structural change of microparticles during the solvent removal process. Actually, there are three stages through which the particles pass until their complete solidification. The first step comprises the first 60 min, including the preparation of the system and the beginning of the evaporation process, at which, in terms of morphology, only the multiple emulsion is observed. In the time period from 60 to about 90 min, one can visualize the formation of an agglomerated semi-solid system. Only after 90 min it is possible to observe the solidification of the microparticles into individualized units.

By analysing the evaporation stages, it can be suggested that as faster and more efficient the solvent removal is than faster is the solidification process. When the microencapsulation of hydrophilic drugs is the objective, this is decisive for the encapsulation efficiency, because the drug diffusion from the core of the droplets into the external phase can occur due to the concentration gradient during the first two steps of the evaporation process (Sing-Muk et al., 2010).

The use of multiple-emulsion/solvent evaporation method for the preparation of D,L-PLA nanoparticles did not prevent the loss of CEF to the external aqueous phase, resulting in an encapsulation efficiency lower than expected (Table II). Similar results are found in the literature, e.g., the encapsulation of dexametasone, gentamicin, betametasone and hydrogen peroxide gave entrapment rates of 3.4%, 13.3%, 15% and 10% respectively (Eroglu et al., 2001; Prior et al., 2000; Chaw et al., 2003; Sing-Muk et al., 2010). Concerning the drug concentration, generally the encapsulation efficiency is directly proportional to the increasing of drug concentration in the solution of the most internal phase (W_1). The results here obtained are contrary to this rule, but other authors have described such fact (Prior et al., 2000; Perugini et al., 2001).

Concerning the nanoparticles morphology (Figure 1), it can be difficult to observe the particles, especially when the emulsifier is PVA, as in the present study. This compound generally adsorbs on the particles surface at the external aqueous phase and builds a halo around them, distorting their contour (Moinard-Checot et al., 2006).

Thermal analytical studies of polymeric drug delivery systems are important, since the processes used to their preparation are able to modify the organization of the polymer chains (Dubernet, 1995). Thermal analysis data (Table III and Figure 2) showed a *Tg* for D,L-PLA around 46°C, of 52°C for inert nanoparticles and around 50°C for CEF-containing (5.5%) nanoparticles. From Figure 2, one can see that the physical mixture presents two endothermic peaks around 50 ºC and 138 ºC, corresponding, respectively, to the *Tg* of the inert nanoparticles and to the melting point of the drug. For the lactic acid polymers, the *Tg* represents a measure of the polymer chain flexibility and indicates how the hydrolysis of the ester bonds will occur (Ford & Timmins, 1989).

The thermogram of the drug alone shows an endotherm corresponding to the CEF melting at 135°C. However, such a peak is not visible in the thermogram of the nanoparticles containing drug (5.5%). In this way, it can be suggested that the drug is dispersed throughout the system (Ford & Timmins, 1989). It is well known that through the determination of the *Tg* it is possible to evaluate in what level the drug is dispersed within the carrier system and that the disappearance of the peak referred to the crystalline melting of the drug is indicative that it is uniformly dispersed throughout the polymer matrix at a molecular level (Hariharan & Price, 2002).

The *in vitro* release assays (Figure 3) showed that the CEF release occurred in a sustained fashion when compared with the free drug, which was similarly described in other experiments involving micro- or nanoencapsulated antibiotics (Yang & Chung, 2001; Chaw et al., 2003; Liu et al., 2004).

Drug release from matrices made of poly(ortho-esters) is initially mediated by diffusion processes through the matrix whereas in later steps, additionally, matrix degradation plays an important role (Crotts & Park, 1995).

Additionally, the agreement of the release data with the mathematical models referred reinforces the matricial nature of the structure of the nanoparticles produced, that was also assessed by thermal analysis (Huang & Brazel, 2001; Jantzen & Robinson, 2002; Manca & Rovaglio, 2003; Ranade & Hollinger, 2003; Yang et al., 2001; Yang & Washington, 2005).

Although CEF presents high water solubility and presumable chemical instability, it was possible by the first time to prepare PLA-nanoparticles containing this drug by a crude multiple-emulsification solvent evaporation method, i. e. without additional improvements. Despite the low encapsulation efficiency, comparable, however, with other results from the literature for substances with similar characteristics and experimental conditions, the results represent a promising first attempt to nanoencapsulate this drug. The nanoparticles obtained were of regular and near spherical shape with narrow size distribution, their diameters ranging, for example, from 100 to 240 nm when sonication was used in the emulsification step. Thermal analyses showed that the drug could be homogeneously mixed with the polymer, originating homogeneous multiparticulate matrix systems. Although the Weibull model showed the best dissolution curve fit at all, other mathematical kinetics approaches, such as of Higuchi, Baker & Lonsdale, and Korsmeyer & Peppas provided almost the same agreement, indicating that the CEF release from the nanoparticles predominantly occurs by a Fick diffusion mechanism.

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RESUMO

Desenvolvimento e Caracterização de Nanopartículas de D,L-PLA contendo Cefoxitina

As nanopartículas de D, L-PLA (PLA) contendo cefoxitina (CEF) foram preparadas pelo método de emulsão múltipla / evaporação do solvente. As partículas foram avaliadas em relação à morfologia, à eficiência de encapsulação, às interações polímero-fármaco, bem como à cinética de liberação do fármaco *in vitro* . As nanopartículas são esféricas e isoladas, com um diâmetro médio de cerca de 600 nm. O comportamento térmico (DSC) das nanopartículas contendo CEF sugeriu que o fármaco está disperso em um nível molecular dentro do sistema. A eficiência de encapsulação do fármaco no sistema quando a concentração de CEF é 30 mg / mL foi de 5,5%, determinada após a extração de fármaco, através de um método de HPLC validado. Esta baixa eficiência de encapsulação é compreensível, uma vez que a CEF é altamente hidrofílica. Os ensaios *in vitro* mostraram um perfil de liberação do fármaco a partir das nanopartículas fortemente sustentado e com uma cinética de difusão Fickiana pura.

Palavras-chave: Nanopartículas. Liberação controlada. D,L-PLA. Cefoxitina. Liberação In vitro.

REFERENCES

Benita S. Microencapsulation: Methods and industrial applications. In: Kissel T et al., ed. Microencapsulation techniques for parenteral depot systems and their application in the pharmaceutical industry. Nova York: CRC Press Taylor & Francis Group; 2006. p. 99-118.

Brazel CS, Peppas NA. Modeling of drug release from swellable polymers. Eur J Pharm Biopharm. 2000; 49(1):47-58.

Cevher E, Orhan Z, Mülazimoglu L, Sensoy D, Alper M, Yildiz A, Özsoy Y. Characterization of biodegradable chitosan microspheres containing vancomycin and treatment of experimental osteomyelitis caused by methicillin-resistant Staphylococcus aureus with prepared microspheres. Int J Pharm. 2006; 317(2):127–35.

Chaw CS, Yang YY, Lim IJ, Pahn TT. Water-soluble betamethasone-loaded poly(lactide-co-glycolide)hollow microparticles as a sustained release dosage form. J Microencapsul. 2003; 20(3):349–59.

Chen JL, Chiang CH, Yeh MK. The mechanism of PLA microparticle formation by water-in-oil-water sovent evaporation method. J Microencapsul. 2002; 19(3):333–46.

Costa P, Lobo JMS. Modeling and comparison of dissolution profiles. Eur J Pharm Sci. 2001; 13(1):123-33.

Couvreur P, Fatal E, Andremont A. Liposomes and nanoparticles in the treatment of intracellular bacterial infections. Pharm Res. 1991; 8(9):1079–86.

Crotts G, Park TG. Preparation of porous biodegradable polymeric hollow microspheres. J Control Release 1995; 35(2-3):91–105.

Cynamon MH, Klemens SP. Chemotherapeutic agents for mycobacterial infections. In: Friedman L N., ed. Tuberculosis - current concepts and treatment. Boca Raton: CRC; 1994. p. 301-32.

Dubernet C. Thermoanalysis of microspheres. Thermochim Acta 1995; 248:259–69.

Durán N, Alvarenga MA, Da Silva EC, Melo PS, Marcato PD. Microencapsulation of antibiotic rifampicin in poly(3-hydroxybutyrate-co-3-hydroxyvalerate). Arch Pharm Res. 2008; 31(11):1509-16.

Eroglu H, Kas HS, Oner L, Türkoglu ÖF, Akalan NS, Argon MF, Ozer N. The in-vitro and in-vivo characterization of PLGA: L-PLA microspheres containing dexamethasone sodium phosphate. J Microencapsul. 2001; 18(5):603–12.

Farmacopéia Portuguesa. 8.ed. Lisboa: INFARMED; 2002.

Ford JL, Timmins P. Pharmaceutical thermal analysis. Chichester: John Wiley & Sons; 1989.

Freitas S, Merkle HP, Gander B. Microencapsulation by solvent extraction/evaporation: reviewing the state of the art of microsphere preparation process technology. J Control Release 2005; 102(2):313–32.

Fuminori I, Fujimori H, Honnami H, Kawakami H, Kanamura K, Makino K. Study of types and mixture ratio of organic solvent used to dissolve polymers for preparation of drug-containing PLGA microspheres. Eur Poly J. 2009 45(3): 658-67.

Fuminori I, Hiroyuki F, Kimiko M. Incorporation of water-soluble drugs in PLGA microspheres. Colloids Surf B Biointerfaces 2007; 54(2):173–78.

Giunchedi P, Genta I, Conti B, Muzzarelli RAA, Conte U. Preparation and characterization of ampicillian loaded methylpyrrolidinone chitosan and chitosan microspheres. Biomaterials 1998; 19(1-3):157–61.

Hariharan M, Price JC. Solvent, emulsifier and drug concentration factors in poly-(D,L-Lactic acid) microspheres contaning hexamethylmelamine. J Microencapsul. 2002; 19(1):95–109.

Huang YY, Chung TW. Microencapsulation of gentamicin in biodegradable PLA and/or PLA/PEG copolymer. J Microencapsul. 2001; 18(4):457–65.

Huang X, Brazel CS. On the importance and mechanisms of burst release in matrix-controlled drug delivery systems. J Control Release 2001; 73(2-3):121-36.

Jantzen GM, Robinson JR. Sustained- and controlled-release drug-delivery systems. In: Banker GS, Rhodes CT, eds. Modern Pharmaceutics. 4th ed. New York: Marcel Dekker; 2002. p. 501-28.

Jyothi NV, Prasanna M, Prabha S, Seetha Ramaiah P, Srawan G, Sakarkar SN. Microencapsulation Techniques, Factors Influencing Encapsulation Efficiency. J Microencapsul. 2010; 27(3):187-97.

Liu DZ, Chen WP, Lee CP, Wu SL, Wang YC, Chung TW. Effects of alginate coated on PLGA microspheres for delivery tetracycline hydrochloride to periodontal pockets. J Microencapsul. 2004; 21(6):643–52.

Mainardes RM, Evangelista RC. Praziquantel-loaded PLGA nanoparticles: preparation and characterization. J Microencapsul. 2005; 22(1):13–24.

Manca D, Rovaglio M. Modeling the controlled release of microencapsulated drugs: theory and experimental validation. Chem Eng Sci. 2003; 58:1337-51.

Mao S, Xu J, Cai C, Germershaus O, Schaper A, Kissel T. Effect of WOW process parameters on morphology and burst release of FITC-dextran loaded PLGA microspheres. Int J Pharm. 2007; 334(1-2):137–48.

Moinard-Checot D, Chevalier Y, Briançon S, Fessi H, Guinebretiere S. Nanoparticles for durg delivery: Review of the formulation and process difficulties illustrated by emulsion-diffusion process. J Nanosci Nanotechnol. 2006; 6(9/10):2664–81.

Nihant N, Schungens C, Grandfils C, Jerome R, Teyssie P. Polylactide microparticles prepared by double emulsion/evaporation technique. I. Effect of primary emulsion. Pharm Res. 1994; 11(10):1479–84.

Orhan Z, Cevher E, Mülazimoglu L, Gürcan D, Alper M, Araman A, Özsoy Y. The preparation of ciprofloxacin hydrochloride-loaded chitosan and pectin Microspheres their evaluation in an animal osteomyelitis model. J Bone Joint Surg. 2006; 88(2):270–75.

Panyam J, Labhasetwar V. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. Adv Drug Deliv Rev. 2003; 55(3):329–47.

Papadopoulou V, Kosmidis K, Vlachou M, Macheras P. On the use of Weibull function for the discernment of drug release mechanisms. Int J Pharm. 2006; 309(1-2):44-50.

Perugini P, Genta I, Conti B, Modena T, Pavanetto F. Long-term release of clodronate from biodegradable microspheres. AAPS PharmSciTech. 2001; 2(3):1–9.

Pinto Reis C, Neufeld RJ, Ribeiro AJ, Veiga F. Biomedical applications and current status of peptide and protein nanoparticulate delivery systems. Nanomedicine 2006a; 2(2):53–65.

Pinto Reis C, Neufeld RJ, Ribeiro AJ, Veiga F. Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles. Nanomedicine 2006; 2(1):8–21.

Prior S, Gamazo C, Irache JM, Merkle HP, Gander, B. Gentamicin encapsulation in PLA/PLGA microspheres in view of treating Brucella infections. Int J Pharm. 2000; 196(1):115–25.

Quintanar-Guerrero D, Allémand E, Fessi H, Doelker E. Preparation tecniques and mechanism of formation of biodegradable nanoparticles from preformed polymers. Drug Dev Ind Pharm. 1998; 24(12):1113–28.

Rajeev JA. 2000. The manufacturing techniques of various drug loaded biodegradable poly(lactide/glicolide) devices. Biomaterials 2000; 21(23):2475–90.

Ranade VV, Hollinger MA. Role of polymers in drug delivery. In: Ranade VV, Hollinger MA, eds. Drug Delivery Systems. 2nd ed. Boca Raton: CRC Press; 2003. p. 63-114.

Rang HP, Dale MM, Ritter JM. Farmacologia. 6.ed. Rio de Janeiro: Guanabara Koogan; 2007. 668 p.

Ravichandran R. Nanotechnology-Based drug delivery systems. Nanobiotechnology 2009; 5:17–33.

Robbers EJ, Speedie KM, Tyler EV. Farmacognosia e biotecnologia. São Paulo: Premier; 1997. p.246-317.

Schnieders J, Gbureck U, Thull R, and Kissel T. Controlled release of gentamicin from calcium phosphate—poly(lactic acid-co-glycolic acid) composite bone cement. Biomaterials 2006; 27(23):4239-49.

Semete B, Booysen L, Lemmer Y, Kalombo L, Katata L, Verschoor J, Swai HS. In vivo evaluation of the biodistribution and safety of PLGA nanoparticles as drug delivery systems. Nanomedicine: Forthcoming 2010; Corrected Proof. DOI: 10.1016/j.nano2010.02

Siepmann J, Peppas NA. Modelling of drug release from delivery systems based on hydroxypropyl methylcellulose (HPMC). Adv Drug Deliv Rev. 2001; 48(2-3):139-57.

Sing-Muk Ng, Jeong-Yeon C, Hyung-Soo H, Jeung-Soo H, Jeong O L. Novel microencapsulation of potential drugs with low molecular weight and high hydrophilicity: Hydrogen peroxide as a candidate compound. Int J Pharm. 2010; 384(1-2): 120-7.

Sonnengaard JM. On the misinterpretation of the correlation coefficient in pharmaceutical sciences. Int J Pharm. 2006; 321(1-2):12-17.

The United States Pharmacopeia Convention. 22th. ed. Rockville: United State Pharmacopeial Convention; 1990. p. 303-04.

The United States Pharmacopeia Convention. 28th. ed. Rockville: United State Pharmacopeial Convention; 2005.

Trabulsi LR, Toledo MRF. Microbiologia. 3 ed. São Paulo: Editora Atheneu; 2002.

Uchegbu IF, Schatzlein AG. Polymers in Drug Delivery. In: Bouissou C, van der Walle C. Poly(lactic-co-glycolic acid) Microspheres. New York: CRC Press: Taylor & Francis Group; 2006.

Vieira BD, Carmona-Ribeiro AM. Cationic nanoparticles for delivery of amphotericin B: preparation, characterization and activity in vitro. J Nanobiotechnol. 2008; 6(6):1-13.

Wu PC, Tsai YH, Liao CC, Chang JS, Huang YB. The characterization and biodistribution of cefoxitin-loaded liposomes. Int J Pharm. 2004; 271(1-2): 31–39.

Yang YY, Chung TS, Ng .N.P. Morphology, drug distribution, and in vitro release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. Biomaterials 2001; 22 (3):231-41.

Yang S, Washington C. Drug release from microparticulate systems. In: Benita S, ed. Microencapsulation Methods and Industrial Applications. 2nd ed. London: Taylor & Francis; 2005. p. 183-206.

Yeh MK, Chen JL, Chiang CH. In vivo and in vitro characteristics for insulin-loaded PLA microparticles prepared by w/o/w solvent evaporation method with electrolytes in the continuous phase. J Microencapsul. 2004; 21(7):719–28.

Zambaux MF, Bonneaux F, Gref R, Maincent P, Dellacherie E, Alonso MJ, Labrude P, Vigneron C. Influence of experimental parameters on the caracteristics of poly (lactic acid) nanoparticles prepared by a double emulsion method. J Control Release 1998; 50(1-3):31–40.