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International Journal of Pharmaceutics 208 (2000) 71–80

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Colloidal carriers for benzathine penicillin G: Nanoemulsions and nanocapsules

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Received 23 October 1999; received in revised form 7 August 2000; accepted 8 August 2000

Abstract

The main purpose of this work is to formulate benzathine penicillin G nanoemulsion and nanocapsules, to evaluate their physicochemical and stabilising characteristics, and to determine their antimicrobial activity and penicillin in vitro release kinetics. Nanoemulsions were produced by the spontaneous emulsification approach and nanocapsules of poly (D,L-lactic acid-co-glycolic acid) polymer (PLGA) were prepared by the method of interfacial deposition of a pre-formed polymer. A 207 ± 8 nm mean diameter nanoemulsion formulation maintained stability for more than 5 months at 4°C. Stable nanocapsules with 224 ± 58 nm mean diameter were obtained, which remained stabilised over 120 days at 4°C. The penicillin encapsulation ratio in the nanocapsules was 85%. The in vitro release profiles indicated that penicillin released from the nanoemulsion was similar to the one observed from nanocapsules. However it can be clearly deduced from the in vitro kinetic analysis that the antibiotic cannot be protected in colloidal delivery systems. Nevertheless, stable formulations obtained in this investigation supply a potential dosage form to encapsulate more easily soluble drugs.

Keywords: Nanoemulsions; Nanocapsules; Benzathine penicillin G; PLGA poly (D,L-lactic acid-co-glycolic acid) polymer; In vitro release kinetic

1. Introduction

The main purpose of nanotechnology is the design of miniaturised drug carrier systems to achieve adequate stability, improved absorption, controlled release, quantitative transfer and, therefore, the expected pharmacodynamic activity (Speiser, 1991). The extreme low size range of particles (around 50–300 nm) guarantees an ameliorated tissue tolerance, uptake and transfer, and no foreign body reactions. The major goal of the nanotherapy is to guide drug molecules specifically and directly to diseased tissues. The aim of site-specific delivery is to minimise the host of

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0378-5173/00/$ - see front matter
PII: S0378-5173(00)00546-9

undesired effects encountered with conventional therapy. Research on controlled delivery systems attained huge progress regarding what it must accomplish to develop such dosage forms. However, the two issues concerning nanotechnology are low physicochemical stability and rapid uptake of nanoparticles by the reticuloendothelial system (RES) in the liver, spleen and bone marrow associated with the plasma proteins opsonisation (Gref et al., 1995). If the involved parameters are correctly defined, viable and practical particulate parenteral forms can be developed for more controlled systemic delivery or for target to site at cellular or sub-cellular levels with an optimised release of the drug (Rosoff, 1989).

There has been a renewed interest in emulsions as a vehicle for delivering drugs to the human body, especially into the blood stream through parenteral administration. For this purpose, nanoemulsions, which are emulsions of droplets under 1 μm size, have ordinarily been used through intravenous injection (Benita and Levy, 1993). Nanoemulsions possess many appealing biological and pharmaceutical properties such as biodegradability, biocompatibility, physical stability and ease of production. Therefore, they have been utilised as carriers for lipophilic drugs, for the stabilisation of compounds susceptible to hydrolysis, for the prevention of drug uptake by infusion sets, and for reduction of irritation or drug toxicity. Nanoemulsions also have usefulness as sustained-release systems by means of depot formation after subcutaneous injection, and as site-specific drug delivery systems through binding of ligands in various cell-surface receptors to the particle surface (Pankerd and Stella, 1990; Lundberg, 1994; Mbela and Verschuren, 1997).

Nanocapsules are colloidal polymeric particles useful as efficient carriers for both drugs (particularly antibiotics) and vaccines, especially because of their biodegradability and biocompatibility. Lysossomotropic nanoparticles can be utilised for the treatment of various intracellular infections, such as rheumatism, schistosomiasis, leyschimaniosis, bacterial and viral infections (Bender et al., 1996; Londenberg and Kreuter, 1996) and for cancer chemotherapy (Morin et al., 1994; Manil et al., 1995; Reska et al., 1997).

Benzathine penicillin G (PenG) is a semi-synthetic compound derived from penicillin through the inclusion of a benzyl ring in the β-lactamic group. This chemical structural modification provides better stability against acids and decreases the solubility in water. The solubility of the penicillin in the blood and biological fluids is rather limited, hence it is used as a depot formulation in treatments that require low and constant blood levels for a long period of time (Graham, 1995). Intramuscular PenG has been accepted as the standard drug for treatment of patients with streptococcal pharyngitis since it was first introduced in 1952. A preparation containing 600 000 UI PenG has been considered suitable for children weighing less than 25 kg (Lamas et al., 1992; Bass, 1996).

The present work is concerned with the preparation of injectable nanoemulsion and nanocapsules of poly (lactic-co-glycolic acid) (PLGA) containing PenG intended for the prophylactic treatment of rheumatic fever. The physicochemical properties and antimicrobial characterisation of those colloidal carriers are also investigated. Nanocapsules systems were introduced in order to be compared with nanoemulsion in terms of loaded drug stability, and the influence of the polymeric barrier on the in vitro penicillin release profile kinetics.

2. Materials and methods

2.1. Materials

PenG, sunflower and soybean oils, benzyl-benzoate and dextran 70 were obtained from Sigma Chemicals (USA). Huls (France) supplied medium-chain triglycerides (Miglyol® 812). Penicillin G benzathine salt sodium (European standard) was kindly offered by the Institute Pasteur (France); the poly (D,L-lactic-acid-co-glycolic acid) polymer (PLGA) 50/50 was purchased from Birmingham Polymers (USA); soya phosphatidylcholine (Epikuron 200) was obtained from Lucas Meyer (Germany); poloxamer (Synperonic F-68) was generously supplied by ICI (France); monobasic potassium phosphate, sodium hydrox-

ide and all other reagents with analytical grade were purchased from Merck (USA); high performance liquid chromatography (HPLC) grade acetonitrile was obtained from Aldrich (USA).

2.2. Methods

2.2.1. Formulation of nanoemulsions

The nanoemulsions were obtained by the spontaneous emulsification process (Tabosa do Egito et al., 1994). The soya phospholipid was initially dissolved in 20 ml of methanol at 40°C. The soybean oil or Miglyol 812® was added to the phospholipid organic solution and then the PenG previously dissolved in 5.0 ml of methanol was added to this solution so as to constitute the organic phase. The aqueous phase consisted of 50 ml phosphate buffer solution at pH 7.4 with poloxamer dissolved in it. The nanoemulsion was formed through the slow injection of the organic phase into the aqueous phase, under magnetic stirring at 150 rpm during 30 min at 25°C. The solvent was then evaporated under reduced pressure at 40°C, and the volume of nanoemulsion was concentrated to the initial volume of the aqueous phase. The amount of hydrophilic and lipophilic surfactants was fixed to 1% with a 1:1 weight ratio.

The influence of the nature of the oily phase (soybean oil and Miglyol® 812) and solvents (acetone, ethanol and methanol) on the stability of the nanoemulsions was evaluated.

2.2.2. Formulation of nanocapsules

Several batches of penicillin-loaded PLGA nanocapsules were prepared according to the interfacial deposition of a pre-formed copolymer method (Fessi et al., 1989). The organic phase was initially prepared as follows. The polymer and the Soya phospholipid were each separately dissolved in 25 ml of acetone. Acetonic phospholipid solution was obtained by increasing the temperature to 40°C. After the dissolution, both organic solutions were mixed and maintained at 40°C under magnetic stirring at 150 rpm. An aqueous phase was prepared in two steps: the first one consisted of dissolving the poloxamer in 50 ml of a phosphate buffer solution at pH 7.4. In the second step, 5.0 ml of a methanolic solution containing penicillin was added to the poloxamer solution. Finally, the organic solution was poured into the aqueous phase under magnetic stirring at 150 rpm. The acetone, which rapidly diffused towards the aqueous phase, was then removed under reduced pressure at 40°C. The colloidal suspension was concentrated to a 10 ml final volume by removing water under the same conditions.

Formulation parameters such as the nature and the volume of the organic and aqueous phase, the nature and the concentration of surfactants and polymer have relevant implications on the particle size distributions (Jullienne et al., 1992). Thus, all these parameters were taken into account in the nanocapsule formulations (Table 1).

2.2.3. Physicochemical characterisation of the formulations

The physicochemical analysis of nanoemulsions and nanocapsules was carried out immediately after preparation. Parameters such as the macroscopic aspect, morphological examination, particle mean diameter, pH changes, penicillin content and encapsulation ratio were analysed.

Aiming to evaluate the long-life of the penicillin incorporated into nanocapsules and nanoemulsions stored at 4°C, the penicillin content was determined by both a chromatographic and a microbiological method.

The penicillin content was assayed by HPLC (Waters, USA). A NovaPak 4 μm, 150 × 3.9 mm column (Waters, USA) was utilised and the mobile phase consisted of acetonitrile and 0.1 M ammonium acetate (1:4) at a flow rate of 1 ml/min. The detection was performed at a wavelength of 254 nm. The calibration curve was prepared with methanolic solutions of penicillin G sodium standard at concentrations ranging from 10 to 250 μg/ml. A nanoemulsion sample was diluted with methanol (1:10, v/v) under agitation at 15 min. The extraction of PenG from nanocapsules samples was performed with acetone (1:10, v/v) under ultrasonic agitation at 15 min. For both preparations a sample aliquot was diluted with methanol to a theoretical concentration of 50 μg/ml, then the solutions were filtered and 10 μl was injected.

Table 1
The nature and concentration of constituents in nanocapsules formulations

Constituents	Formulations										
	NC1	NC2	NC3	NC4	NC5	NC6	NC7	NC8	NC9	NC10	NC11
Organic phase											
PLGA (mg)	300	300	125	125	125	125	125	125	120	125	125
Epikuron 200 (mg)	600	600	250	250	250	250	250	250	250	250	125
Ethanol (ml)	–	2	–	–	–	–	–	–	–	–	–
Acetone (ml)	12.5	25	18	18	18	18	18	18	18.0	18	18
Benzyl benzoate (ml)	–	–	–	–	–	–	–	–	0.5	–	–
Sunflower oil (mg)	500	250	250	250	250	250	250	250	–	250	250
Aqueous phase											
Benzathine											
Penicillin G (mg)	3	3	6	–	12	15	18	23.9	6	6	6
Deionized water (ml)	25	–	–	–	–	–	–	–	–	–	–
pH 7.4 phosphate											
Buffer solution (ml)	–	50	50	50	50	50	50	50	50	50	50
Methanol (ml)	–	–	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Poloxamer (mg)	–	250	250	250	250	250	250	250	500	500	250

The microbiological assay of the penicillin encapsulated into nanocapsules was carried out according to the FDA plate diffusion method using *Staphylococcus aureus ATCC 6538P* (Grove and Randall, 1955). The standard penicillin G sodium solution was prepared at concentrations ranging from 0.64 to 1.56 μg/ml. Nanocapsules suspension were dissolved with acetone (1:10, v/v) and diluted with a pH 7.4 phosphate buffer solution to reach a 1 μg/ml concentration.

The antimicrobial activity of the penicillin encapsulated into nanocapsules was measured in comparison with a standard penicillin solution against *Streptococcus pyogenes* ATCC 12346.

The penicillin encapsulation ratio of nanocapsules was determined by the ultra-centrifugation technique (Seijo et al., 1990; Fresta et al., 1996). The nanocapsules suspension was submitted to an ultra-centrifugation process at 19 000 × *g* for 2 h. The penicillin content was measured by HPLC in the supernatant, and then related to the total concentration of the nanocapsules preparation.

2.2.4. Evaluation of formulations stability

The formulations were submitted to both accelerated and long-term stability tests. The nanocapsules and nanoemulsions were subjected to centrifugation (3500 rpm/min for 1 h), mechanical stress (150 strokes/min at 37°C for 48 h) and freeze–thaw cycles (16 h at −18°C and 8 h at 25°C; Santos Magalhães et al., 1991).

Long-term stability of the formulations was examined at different temperatures: nanocapsules (4 ± 1°C and 25 ± 1°C) and nanoemulsions (4 ± 1°C, 25 ± 1°C and 50 ± 1°C). Physicochemical properties of the preparations were evaluated at regular time intervals of 7, 15, 30, 45, 60 days or until instability.

2.2.5. In vitro release profile kinetic of penicillin G

In vitro release profile of PenG from the preparations was determined using the bulk-equilibrium reverse dialysis bag technique (Levy and Benita, 1989). Seven dialysis bags each containing 1 ml of pH 7.4 buffer solution were previously immersed into 100 ml of an identical sink solution. After system equilibrium, an aliquot of 10 ml of the nanocapsules suspension or nanoemulsion was then placed directly into the sink solution. A bag and an aliquot of 1 ml of the sink solution were collected at different time intervals, and the penicillin content was determined. The kinetic experiments were performed at 37 ± 1°C under constant magnetic stirring of 50 rpm.

3. Results and discussion

3.1. Nanoemulsions preparation

Results showed that the solvent nature has a marginal influence on the particle diameter, for both soybean and Miglyol® nanoemulsions (Table 2). The NE1 formulation (acetone) and NE2 (ethanol) were produced with soybean and they presented particles with 199 ± 67 and 175 ± 59 nm mean diameter, respectively. Formulations elaborated with Miglyol® 812, namely NE3 (acetone) and NE4 (ethanol), showed a difference in the droplet diameter, from 180 ± 52 to 136 ± 32 nm, respectively. Regarding the oily phase nature, it was observed that the Miglyol® 812 supplied more stable nanoemulsions than soybean. The resistance to the accelerated stability testing was increased for Miglyol® nanoemulsions. Yu et al. (1993) also reported similar results in a comparative study on the influence of the oily phase nature on the nanoemulsion particle size. They suggested that the soybean, which has a higher viscosity than the ethyloleate might turn the dispersion hard during the emulsification.

Results reported by Yu and collaborators (1993) suggested ethanol is more adapted to manufacturing stable nanoemulsions. However, methanol was chosen because it is the best solvent for the PenG, despite the potential toxic effect of eventual residuals.

The most stable nanoemulsion formulation (NE5) consisted of 250 mg of Miglyol®, 250 mg of soya phospholipid, 25 mg of PenG, 25 ml of methanol, 250 mg of poloxamer and 50 ml of pH 6.4 phosphate buffer solution.

3.2. Nanocapsules preparation

In order to maintain a suitable pH in the final nanocapsules formulation, the aqueous phase consisted of a pH 7.4 phosphate buffer solution. A very low pH value (equal to 2.0) was observed in nanocapsules suspension when deionised water was used as the aqueous phase. This can be attributed to the low initial pH value of the organic polymer solution. Besides stabilising penicillin, the final pH 7.4 prevents the polymer hydrolysis, which rapidly occurs in the acidic environment (Dunn and Ottenbrite, 1991).

The influence of the type of oil and concentration on the nanocapsules stability was analysed. Concerning oil nature it was verified that sunflower oil allowed more stable nanocapsules than benzyl-benzoate (Table 2). Formulations with penicillin content above 6 mg exhibited precipitation 48 h after preparation. Results suggested that the low penicillin encapsulation ratio is due to its weak solubility in the vegetable oil, polymer or water. In fact, it was shown that the association of penicillin in PLGA films was 10% and the penicillin entrapment into PLGA nanocapsules was barely 0.9% (Verecchia, 1993).

3.3. Physicochemical characterisation of formulations

The physicochemical characteristics of selected nanocapsules and nanoemulsion formulations after the preparation and also after accelerated stability tests were presented in Table 3.

Microscopic photography of the nanocapsules suspension showed a spherical shape of the dispersed particles (Fig. 1a). Particle size evaluations of nanocapsules confirmed an unimodal distribution with a mean diameter of 180 ± 52 nm.

Nanocapsules penicillin contents were 99.8 and 100.0%, determined by HPLC and microbiological assay, respectively. Such results guarantee that integrity of penicillin was maintained during the manufacture of the dosage form. According to the HPLC assay an initial concentration of 101% was determined in the nanoemulsion.

The encapsulation rate of penicillin G into nanocapsules was 85%, which is higher than the values previously reported (Calvo et al., 1996).

3.4. Evaluation of formulations stability

The stable nanocapsules suspension (NC11) and nanoemulsion (NE5) were submitted to accel-

Table 2
The influence of the oily phase nature and concentration on the stability of nanocapsules and nanoemulsion formulations stored at 25°C

Physicochemical characterisation	Nanocapsules		Nanoemulsions	
	Sunflower oil (NC10)	Benzyl-benzoate (NC9)	Soybean oil (NE2)	Miglyol 812 (NE4)
Macroscopic aspect	Fluid milky white bluish opalescent	Fluid milky white bluish opalescent	Fluid milky white bluish opalescent	Fluid white bluish opalescent
pH	7.63	7.62	6.62	6.40
Diameter mean size (nm)	180 ± 52	310 ± 93	175 ± 59	136 ± 32
Accelerated stability testing				
Centrifugation	Redispersible creaming	Fine precipitate	Redispersible creaming	Stable
Freeze–thaw cycles	Stable at 6th cycle	Stable at 3rd cycle	Stable at 3rd cycle	Stable at 3rd cycle
Mechanical stress	Stable	Stable	Light creaming	Stable

Table 3
Physicochemical characteristics and accelerated stability of the nanocapsules (NC11) and nanoemulsion (NE5), formulations stored at 25°C

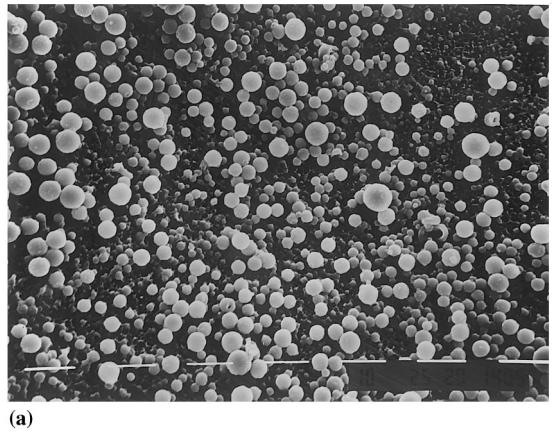
Physicochemical assay	Characteristics	
	Nanocapsules (NC11)	Nanoemulsion (NE5)
Macroscopic aspect	Fluid milky white bluish opalescent	Fluid white bluish opalescent
Microscopic aspect	Spherical particles	–
pH	7.63	6.70
Diameter mean size (nm)	180 ± 52	207 ± 8
PenG content (%)		
HPLC	99.8	101.32
Microbiological assay	100.0	–
PenG encapsulation content (%)[a]	8.50	–
Accelerated stability tests		
Centrifugation	Light creaming	Stable
Freeze–thaw cycles	Stable at 6th cycle	Stable at 3rd cycle
Mechanical stress	Stable	Stable

[a] Microbiological assay.

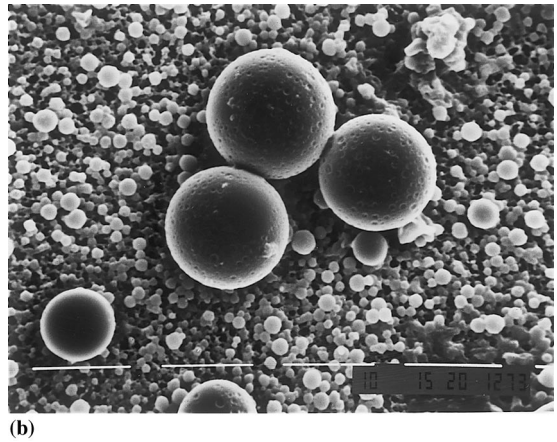
erated stability testing. Nanocapsules presented a light hand-shaking dispersible creaming after centrifugation, and nanoemulsion remained stabilised without remarkable physicochemical changes. No substantial variation in the particle size was observed. Both formulations also maintained the same macroscopic aspect when submitted to the mechanical stress and hold stability after six freeze–thaw cycles.

The stability of the nanocapsules suspension stored at 25 ± 1°C was maintained for 4 months, however, the penicillin was completely degraded in this period. After this time, the nanocapsules suspension presented instability signs such as creaming and oily exudate. No variations on the particle mean diameters were observed by the photocorrelation analysis during a 3 month of storage. However, the SEM analysis detected a second particle population of 2 μm (Fig. 1b). These contradictory results could be explained by the fact that the nanosizer is more accurate in the analysis of homogeneous nanoparticulated systems.

There was practically no change in the particle mean diameters at the storage temperature of 4 ± 1°C, varying from 207 ± 08 to 216 ± 74 nm after 4 months of nanoemulsion production. The particle size evolutions can be used to predict the stability of the preparation; small particle size provides a better emulsion stability. Furthermore, the invariance of particle sizes with time would be strong evidence that emulsion stability will be maintained (Garti et al., 1982).



(a)



(b)

Fig. 1. Scanning electron micrograph of PenG nanocapsules (NC11): (a) after the preparation, and (b) after 3 months of preparation.

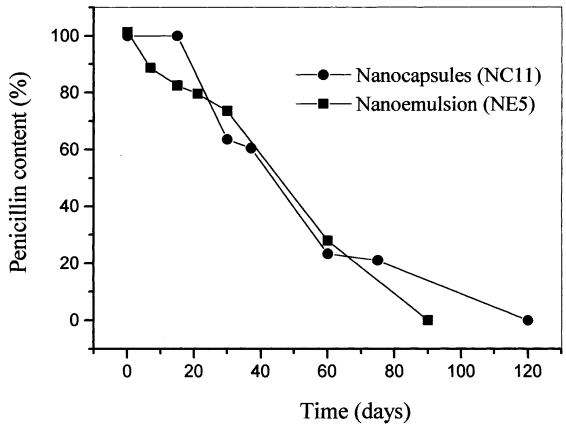


Fig. 2. The comparative analysis of penicillin content into colloidal carriers stored at 4°C.

The nanoemulsion stability at 50°C was maintained about 7 days. The nanoemulsion instability was observed by the formation of a transparent and fluid micellar solution. An analogous behaviour was observed at room temperature (approx. 25°C) after 2 months. This instability sign was probably induced by the hydrophilic surfactant at a concentration of 6×10^{-4} mol/l, much above the critical micellar concentration (5.3×10^{-6} mol/l). Solubility changes in the surfactant under the effect of the temperature affect the intermolecular interactions between aqueous and organic phases, provoking the loss of the stability, confirmed by phase separation after some days (Myers, 1991).

At all storage temperatures a gradual decrease in pH was found for nanoemulsions. This might indicate a lipidic degradation that induces the fatty acid formation. Such fatty acids could probably have interfered in the electrical conductivity and reduced the pH of the nanoemulsion, despite the use of the phosphate buffer solution as aqueous phase (Benita and Levy, 1993).

The comparative analysis of formulations revealed that the nanoemulsion and nanocapsules suspension present similar penicillin stability. After 60 days preparation, penicillin contents of 23.3 and 28% were observed respectively for nanocapsules and nanoemulsion, stored at 4°C (Fig. 2).

The results showed that the penicillin encapsulated exhibit 100% growth inhibition against *Strep. pyogenes* ATCC in comparison with a standard penicillin solution.

3.5. A comparative in vitro release analysis of penicillin from colloidal carriers

The release of PenG from nanoemulsions and nanocapsules yielded similar profiles (Fig. 3). A fast release was observed for both colloidal carriers. A similar profile was observed in previous works concerning different drugs (Santos Magalhães et al., 1995; Calvo et al., 1996). The release of PenG from nanocapsules was roughly twice that of nanoemulsions in the interval of 5–60 min. In this interval, the kinetic was assumed to be an apparent zero-order rate, and the rate constant values were derived from the minimum-squared fitting yielding 0.008 min^{-1} (nanoemulsion) and 0.017 min^{-1} (nanocapsules). The adjusted curves of PenG released (%) versus time (min) were $13.581 + 1.713\ t$ (min), $r^2 = 0.955$, and $26.626 + 0.800\ t$ (min), $r^2 = 0.979$, for nanocapsules and nanoemulsion, respectively. The whole quantity of drug was practically released within 120 min and 180 min, for nanoemulsion and nanocapsules, respectively.

The slight slower release of PenG from nanoemulsion as compared to that from nanocapsules could be attributed to the difference of the oily phase nature (Miglyol®) in relation to the sunflower oil used in the nanocapsules. Assuming

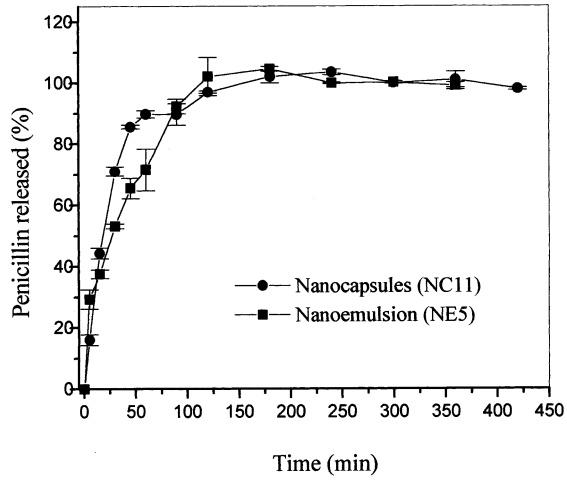


Fig. 3. Release profile kinetics of PenG from colloidal carriers.

that the kinetic process is governed by the oil–water partition coefficient (Benita and Levy, 1993), the difference of the ionic strength and dielectric constant of these oils could explain such different rates of PenG released.

4. Conclusions

Nanocapsules obtained from PLGA copolymer containing PenG were well-spherical-shaped and maintained their stability over 120 days when stored at 4°C. They presented an encapsulation ratio of about 85% according to microbiological assay for penicillin content. Stable preparations allowed the entrapment of PenG up to 0.6 mg (6300 UI)/ml and exhibited an in vitro antimicrobial activity against *Strep. pyogenes*.

A comparative analysis of penicillin-loaded formulations showed a higher stability of encapsulated penicillin in nanocapsules (120 days) against only 12 h for a reconstituted penicillin commercial suspension. However, the penicillin concentration in nanocapsules was about 100 times less than the therapeutically recommended child dose. In spite of such a drawback, these stable formulations supply a potential administration dosage form to encapsulate more easily soluble drugs.

The need of synthetic oil (Miglyol® 812) and the methanol as an appropriate organic solvent was verified to achieve a stable nanoemulsion formulation. Lipophilic (soya phosphatidylcholine) and hydrophilic (poloxamer) surfactants in molar ration (1:1) promote the stabilisation of the system. The nanoemulsion remained stable after accelerated stability testing and it presented physical stability for more than 5 months at 4°C storage. The penicillin content in the nanoemulsion (101%) decreased with time when stored at 4°C until 90 days after preparation. Both nanocapsules and nanoemulsions were not able to prevent penicillin degradation even stored at 4°C. About 20% reduction in drug content is observed after 20 days at 4°C.

In vitro kinetic pictured similar penicillin release profiles from both nanoemulsion and nanocapsules. However the release of penicillin from nanocapsules was faster than from nanoemulsion. It can be clearly deduced from the in vitro kinetic analysis that the antibiotic cannot be protected in colloidal delivery systems. In order to prevent PenG degradation and nanocapsules instability, the lyophilization could be used as an alternative issue to the storage of the nanocapsules.

Experiments are presently being carried out in our laboratory aiming to increase the penicillin entrapment by using microencapsulation techniques.

Acknowledgements

This work received partial support from the Brazilian-French CAPES-COFECUB agreement (UFPE-Université PARIS-Sud XI, grant no. 160/94). The first author is also grateful for the partial financial support from the Brazilian National Council for Scientific and Technological Development (CNPq), under grant no. 304946/85-1. The authors also thank an anonymous referee for interesting suggestions to improve the discussion and presentation of this paper.

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