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Primaquine-loaded poly(lactide) nanoparticles: physicochemical study and acute tolerance in mice

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Abstract

This paper describes the preparation of primaquine loaded-poly (D,L-lactide) nanoparticles. A simple and reproducible method of preparation was developed in chemically soft conditions. The morphological analysis of the colloidal suspension obtained showed a unimodal size distribution, ranging from 150 to 200 nm with a narrow dispersion. The binding of primaquine was highly dependent on pH and drug concentration. The stability of nanoparticles is related to the nature and quantity of hydrophilic and lipophilic surfactants. Intravenously injected primaquine-loaded nanoparticles were well tolerated by healthy and *Leishmania donovani*-infected mice. The 50% lethal dose of primaquine-loaded nanoparticles was significantly reduced when compared to that of free primaquine.

Keywords: Poly (D,L-lactide); Nanoparticles; Stability; Primaquine; Toxicity

1. Introduction

The potential of colloidal drug carriers in the targeted and controlled delivery of anti-parasitic compounds has received much interest. Most of the studies on these carriers concerned liposomes (Alving, 1986, Croft, 1986, Lopez-Berestein, 1987). Fewer studies have been carried out on biocompatible and biodegradable polymeric carriers than on liposomes but these carriers are an alternative in an attempt to overcome the problem of stability of phopholipid vesicles during storage and in biological fluids. These systems have been assumed to be a good strategy for drug delivering in the treatment of liver diseases (Couvreur and Vauthier, 1994). Among polymeric drug delivery devices, nanoparticles and nanocapsules represent a promising approach to a parenteral carrier system, capable of a passive delivering of drugs to lysosomes of phagocytes of the mononuclear

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phagocyte system (MPS), after intravenous administration (Grislain et al., 1983, Krause et al., 1985, Bazile et al., 1992, Rodrigues Jr. et al., 1994). The interest in lactide polymers and copolymers as bioerodible drug delivery systems has been widely described (Lewis, 1990, Pitt, 1990). Poly (lactide) (PLA) is biocompatible and slowly hydrolyzed to lactic acid (Makino et al., 1985). This polymer has a long history of use as safe bioerodible suture materials and as a matrix for controlled delivery systems (Brannon-Peppas, 1995).

Primaquine was chosen as a drug model in this study. This 8-aminoquinoline has been used against malaria infections in man and its potentiality against *Leishmania sp.*, obligate parasites of the MPS, has previously been reported (Neal, 1987). Although reported as an active drug against visceral leishmaniasis, the clinical use of primaquine is not suitable due to its numerous adverse effects (methaemoglobinaemia and haemolysis) at therapeutic doses (WHO Drug Information, 1988).

The incorporation of primaquine in drug carriers might be an interesting tool to reduce its toxicity. Previous studies have evaluated other carriers of primaquine: liposomes (Alving et al., 1980, Pirson et al., 1980), protein-conjugates (Hofsteeng et al., 1986), microparticles (Stjärnkvist, 1993) and nanoparticles: gelatin, albumin, polyalcrylamide (Labhasetwar et al., 1990), polyisohexylcyanoacrylate (Gaspar et al., 1991) and polydiethylmethylidene malonate (Mbela et al., 1992). Regarding the polymeric carriers, despite the interesting physicochemical results reported, the lack of data on biocompatibility has led us to consider the possible advantages of using PLA as matrix. So, we focused on a poly(D,L-lactide) nanoparticle system since such polyesters are well tolerated and also known to be biodegradable to metabolites of the Krebs cycle, after intravenous administration (Bazile et al., 1992).

This paper describes a simple and reproducible method of preparation of primaquine loaded-PLA nanoparticles and discusses evaluation of the physico-chemical characteristics and preliminary acute tolerance in healthy and *Leishmania donovani*-infected mice.

2. Materials and methods

2.1. Materials

Primaquine diphosphate was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The phospholipid mixture (Epikuron 170®) was supplied by Lucas Meyer (Hamburger, Germany). Poloxamer (Symperonic PE/F-68®) was from ICI (Clamart, France). Polymer poly(d,l-lactide) (PLA, mol. wt. 88000) was from Boehringer Ingelheim (Germany). Solvents for HPLC and other analytical procedures were obtained from Prolabo (Paris, France). All other chemicals used were of pharmaceutical grade.

2.2. Production of the free base form of primaquine

Primaquine base (PQ) was previously obtained by alkalinization of a PQ diphosphate solution with ammonium hydroxide to pH 12.0, and extracted twice with chloroform. The organic phase was washed twice with water and twice with a saturated solution of sodium chloride. Before evaporation, the chloroform was dried with anhydrous sodium sulphate. PQ obtained in this way was characterized by infrared spectroscopy (Perkin Elmer 16 PC, ST. Quentin en Yvelines, France) and ^{1}H-NMR (Brucker 80 MHz, CT, U.S.A.).

2.3. Preparation of PLA nanoparticles

PLA nanoparticles were prepared by modification of the method reported by Fessi et al. (1987). 7,5 mg of PQ base, 150 mg of PLA and 150 mg of phospholipids were dissolved in acetone (40 ml) and the solution was mixed, under magnetic stirring, to an aqueous phase (80 ml) containing 110 mg of Poloxamer. Before mixing, the pH was adjusted to 9.0 with sodium hydroxide 0.2 M. After 10 min of agitation, the acetone and water were evaporated under vacuum until a volume of 15 ml was reached. The pH of the suspensions was adjusted to 8.0 with a solution of potassium dihydrogen phosphate 0.2 M. Unloaded nanoparticles were prepared according to the same formulation omitting PQ.

2.4. Separation of free PQ from PQ-loaded nanoparticles

The aqueous phase containing free PQ was separated by ultrafiltration-centrifugation technique on Millipore 'Ultra-free MC units' (Bedford, U.S.A.). Ultrafiltrate was then analysed by HPLC.

2.5. PQ entrapment efficiency

Total PQ was assayed after dissolving the nanoparticles in acetonitrile. Free and bound-PQ was assessed by HPLC as described by Clark et al. (1984). Briefly: HPLC system consisted of a Waters system (St. Quentin en Yvelines, France): a F6000A pump, a TM 717 Autosampler, a 484 UV detector (wavelength = 254 nm). The column was a μBondapack C_{18} and the mobile phase was methanol:phosphate buffer pH4.5 (40/60) and the flow rate was 1.0 ml/min. The amount of bound-PQ was calculated as the difference between the total drug content in the suspension and the free PQ content in the ultrafiltrate.

2.6. Physico-chemical characterization

Photon correlation spectroscopy: particle size distribution, average size and polydispersity index were measured by laser light scattering using a monochromatic laser ray diffusion counter (Nanosizer N4, Coultronics, Margency, France).

Transmission electronic microscopy: morphological examination of nanoparticles was performed using a transmission electron microscope (TEM) following negative staining with 1% sodium phosphotungstate solution (pH 7.4) on carbon-coated copper grids.

Zeta Potential: Zeta potential was determined by laser Doppler velocimetry (Zetasizer 4, Malvern, England). All preparations were diluted with a 20 mM phosphate buffer, pH 7.0, solution in order to maintain a constant ionic strength.

Stability : Short-term stability was assessed by measuring particle size, drug loss and the pH of the suspension stored at 4°C, protected from the light during 3 months.

2.7. In vitro release

In order to investigate the release of PQ from nanoparticles, PQ-loaded PLA nanoparticles (1 ml) were diluted to different volumes of phosphate-buffered saline pH 7.4 (PBS) at 37°C (10–1000 ml). Aliquots (0.4 ml) were obtained just after dilution and filtered by centrifugal ultrafiltration process (1000 g, 5 min) using 'Ultrafree MC filter units'. The ultrafiltrate obtained was finally assayed for free PQ by HPLC.

2.8. Acute tolerance

The acute lethal toxicity of intravenously administered free PQ diphosphate and PQ-loaded PLA nanoparticles was compared in healthy mice. Female CD1 mice (Charles Rivers, France), weighing 20–22 g, were divided into groups of 10 animals. They were injected via the tail vein with different doses of drug (10–30 mg/kg) either as the free form or the loaded PLA nanoparticles.

In order to evaluate the tolerance in *Leishmania*-infected animals, female BALB/c mice (Charles Rivers, France), weighing 18–20 g, were infected on day 0 via the tail vein with 0.2 ml of parasite suspension containing 1×10^7 amastigotes of *L. donovani* (MHOM/ET/67/L82; LV9) freshly isolated from hamster spleen (Rodrigues Jr. et al., 1994). On day 7 after infection, the mice were randomly divided into groups of ten and they later received three doses of 10 mg PQ base/kg over a 5 day period (days 7, 9 and 11) and correspondent polymeric doses in the tail vein with unloaded or PQ-loaded nanoparticles. Three days after the last injection (day 14), the weight of each mouse was determined and compared to the control group which had received injections of PBS alone.

3. Results and discussion

3.1. Preparation of the nanoparticles

After addition of the organic solution, the aqueous phase immediately turned milky as result of the formation of nanoparticles. The colloidal

suspension was monodisperse between 150 and 200 nm. Fig. 1 shows the appearance of the particles on TEM examination and confirms the particle size and homogeneity of size distribution (Fig. 1a). In both preparations, we observed bilayers of phospholipids around the nanoparticles which were more frequent in the unloaded preparations (Fig. 1b).

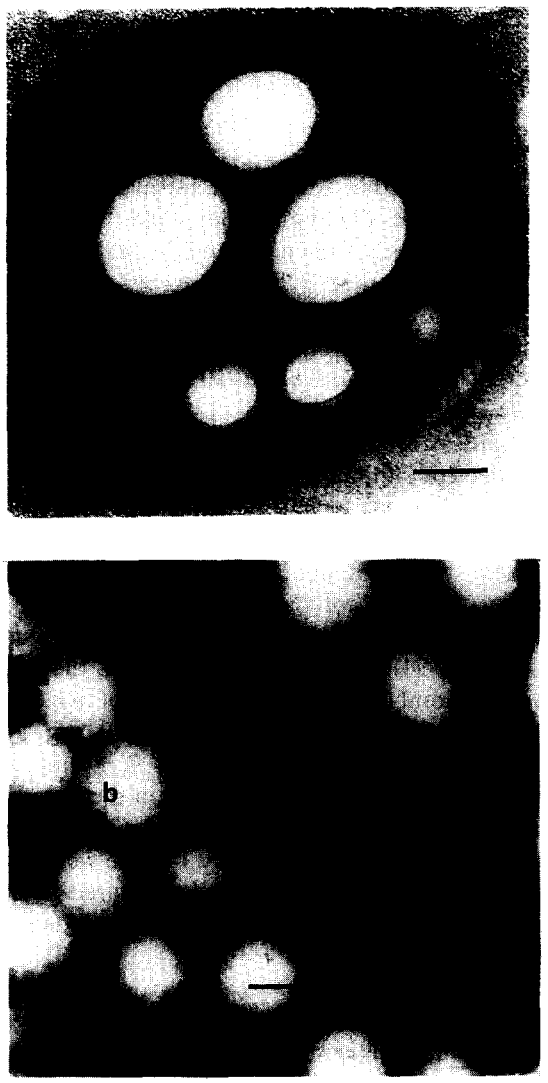

Fig. 1. Transmission electron microscopy following negative staining with 1% sodium phosphotungstate solution (pH 7.4) on carbon-coated copper grids. (a) primaquine-loaded PLA nanoparticles; (b) unloaded nanoparticles. Scale bar = 0.1 μm.

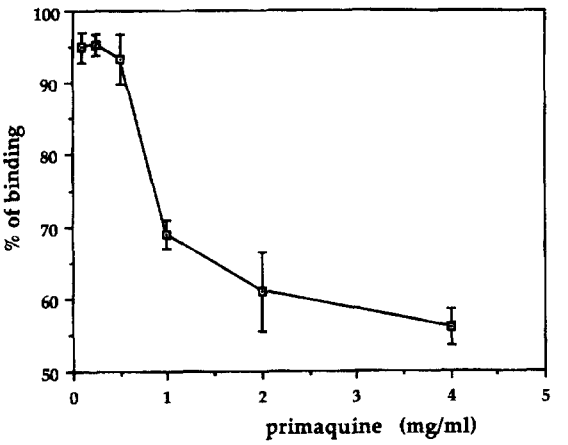

Fig. 2. Influence of the drug concentration on primaquine binding to PLA nanoparticles.

The incorporation of PQ into the nanoparticles was highly dependent on the lipophilic character of the drug and the pH of the medium. The use of PQ base was essential to obtain high levels of bound drug. We tried to incorporate PQ diphosphate to the aqueous phase before mixing with the organic phase, but the drug solubility in the aqueous phase had to be minimized in order to entrap the drug within the nanoparticles. By using the PQ base, we reduced the water solubility and increased the affinity for the polymer, which are also important factors when using other methods such as the solvent evaporation (Bodmeier and McGinity, 1988). Optimal drug loading was obtained by varying the concentration of PQ between 0.1 and 4.0 mg/ml. Above 0.5 mg/ml, significant amounts of free drug were found in the aqueous phase (Fig. 2). The efficiency of PQ incorporation ranged from 85 to 94% at 0.5 mg/ml.

In order to determine the optimal pH, boric-borate buffers were used. The pH of the aqueous phase had a considerable influence on the binding of PQ (Fig. 3). pH values above 9.0 yielded the highest percentage of drug binding. All unbuffered preparations showed a decrease in pH with the appearance of aggregates. Boric-borate buffers were useful for studying the influence of pH and for increasing the stability of prepara-

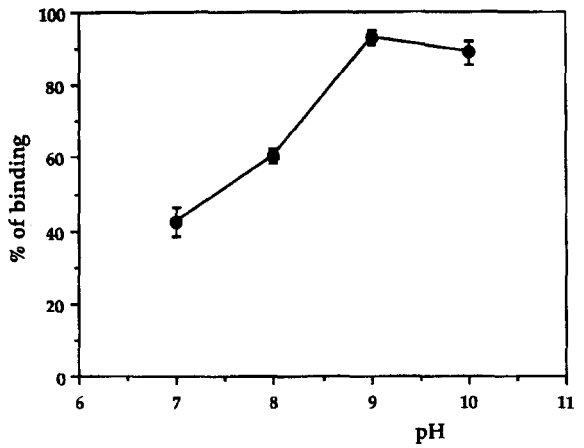

Fig. 3. Influence of pH on primaquine binding to PLA nanoparticles.

tions, but the inconvenience of their use by parenteral route prevented the in vivo studies (Deadorff, 1980). Therefore, we opted for preparing the nanoparticles at pH 9.0 and adjusting the pH to 8.0 with a solution of potassium dihydrogen phosphate. No significant release of PQ was observed after adjusting the pH to 8.0.

The nature and amount of surfactants are important factors for the size distribution and long-term stability of the preparations (Table 1). In the absence of phospholipids a good average size distribution was initially observed but these formulations were unstable due to aggregation of the particles. In contrast, in the absence of poloxamer, the initial size of the particles was greater than 1 μm. Maximum stability was achieved with a combination of both surfactants, at final concentrations of 0.75 and 1.0 g% for poloxamer and phospholipids, respectively. The zeta potential of unloaded nanoparticles was negative (Table 2).

Table 2
Influence of surfactants and primaquine concentrations on the Zeta potential of PLA nanoparticles

Preparation	Zeta potential
Unloaded nanoparticles	- 34.8
PQ-loaded nanop. 0.5 mg/ml	- 14.6
PQ-loaded nanop. 1.0 mg/ml	- 5.5
PQ-loaded nanop. 2.0 mg/ml	+ 1.6

Charge carried by the phospholipid mixture may be responsible for the better association of PQ with the surface of nanoparticles. This association is accompanied by an increase in the zeta potential of the preparations. In fact, preparations containing high concentrations of primaquine are very unstable, probably due to the absence of a repulsive effect at the surface of the particles.

3.2. Stability

The stability of nanoparticle suspension was evaluated over 3 months at 4°C protected from light. The physico-chemical characteristics were maintained during storage and no release of drug was observed (Table 3). The pH of the buffered preparations did not change.

3.3. Drug release

The 'ultrafiltration method' has been successfully applied to evaluate the release of indomethacin from nanoparticles (Magenheim et al., 1993). This method is based on direct dilution of the nanoparticle dispersion in the release medium avoiding the effects of membrane diffusion or dialysis sacs which have been criticized as

Table 1
Influence of surfactants concentrations on the average size and long-term stability of primaquine-loaded PLA nanoparticles

	% of surfactants in relation to the final volume				
Poloxamer	0.75	1.5	0.75	0.75	0
Phospholipids	0	0	0.75	1.0	0.75
Average size (nm)	154	163	169	165	
After preparation	± 39	± 40	± 46	± 42	> 1μm
After 1 month store	> 1μm	> 1μm	175 ± 81	153 ± 62	—

Table 3
Stability of primaquine loaded PLA nanoparticles (0.5 mg/ml)stored at 4°C, protected from light

Time (month)	Average size (nm)	% of PQ bound
0	169 ± 39	89.2
1	153 ± 62	83.9
2	158 ± 39	87.0
3	161 ± 46	89.2

not respecting perfect sink conditions (Washington, 1989). By diluting the colloidal carrier in the release medium, perfect sink conditions are maintained and the method is sensitive enough to study rapid release of drug from the carrier. The release of PQ as a function of the dilution factor is illustrated in Table 4. An increase in the release of PQ was observed when the dilution factor increased from 1/10 to 1/500. When compared to that of the 1/500 dilution, the release of PQ in 1/1000 dilution was not significantly different. The rapid release of PQ from the nanoparticles could be attributed to the fraction of PQ adsorbed to the surface of nanoparticles. Following dilution of the nanoparticle suspension with the release medium, the PQ rapidly partitioned in favour of the release medium, accounting for the immediate release rate phase. A similar effect was observed by Magenheim et al., 1993 who proposed that the exponential delayed release rate phase results probably from the sink solution penetration into nanoparticles, dissolution of the drug and diffusion out of the drug solution from the nanoparticles into the bulk release medium. This 'burst effect' is followed by a period without any significant release of PQ from the nanoparticles. The

Table 4
Effect of dilution on release of primaquine from PLA nanoparticles in phosphate buffer saline, pH7.4, at 37°C immediately after dilution

Dilutions	% drug released
1/10	30.6 ± 1.2
1/20	43.3 ± 2.3
1/100	58.3 ± 2.0
1/500	69.6 ± 2.9
1/1000	70.9 ± 3.5

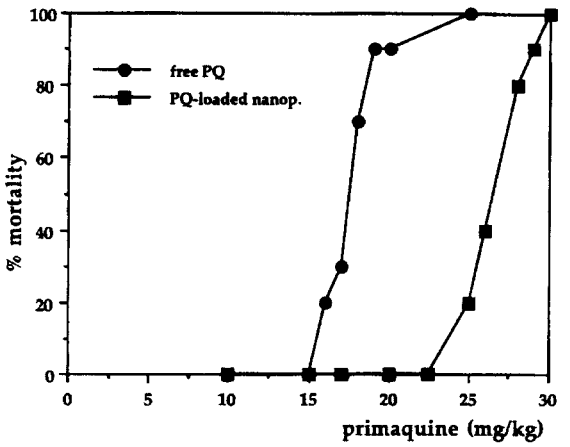

Fig. 4. Comparative acute lethal toxicity of free primaquine and primaquine-loaded PLA nanoparticles after intravenous administration in healthy CD1 female mice.

remaining PQ was not released over the time-course of our experiments (6 h, data not shown). It is reasonable to suppose that its release would be dependent on the bulk hydrolysis of the polymeric matrix in vivo.

3.4. Acute tolerance

The results of the acute lethal toxicity are summarized in Fig. 4. For free PQ, the LD_{50} and LD_{90} were 17.5 and 19.0 mg of PQ base/kg, respectively. For bound-PQ, no lethal effect was observed at these doses. The LD_{50} and DL_{90} for PQ-loaded nanoparticles were 26.9 and 29.2 mg PQ/kg, corresponding to 536 and 584 mg PLA/kg, respectively. Unloaded nanoparticles did not show lethal toxicity when mice were injected with a single-dose of 1,000 mgPLA/kg. The rapid clearance of the carrier from the circulation by the MPS could explain the reduction of the lethal toxicity, since it contributes to the accumulation of bound drug in phagocyte-rich tissues, such as liver and spleen, while the amount of the drug in other tissues is drastically reduced. On the basis of an equi-acute lethal toxicity of PQ, it is apparent that, under the conditions of our study, PQ-loaded nanoparticles was almost two-fold less toxic than free PQ. This small difference could be

explained by a significant release of PQ from the nanoparticles to the bloodstream after intravenous administration, since in vitro studies have shown that approximately 70% of bound-PQ is released just after high dilutions in sink conditions. In fact, the ratio of LD_{50} for free PQ and LD_{50} for PQ-loaded nanoparticles was calculated to be 0.65, quite similar to 0.70 which corresponds to the ratio of free PQ/total PQ after 1/500 and 1/1000 dilutions observed in release studies (Table 4).

Non-lethal toxicity was observed for free PQ in the form of weight loss of *L. donovani*-infected BALB/c mice (about 15% compared to the control group) after receiving a total dose of 30 mg/kg divided into three doses. No weight loss was observed for mice treated with PQ-loaded nanoparticles which received the same total dose. Gaspar et al. (1991) described a reduction of the acute toxicity of PQ bound to polyisohexylcyanoacrylate nanoparticles in healthy NMRI mice. The same author reported a high level of toxicity in *Leishmania*-infected BALB/c mice (Gaspar, 1991). Our nanoparticle formulation was shown to be well tolerated by this mouse strain, since no signs of acute toxicity were observed after three injections over a 5-day period (total injected dose of 30 mg/kg).

Taken together, these results have led us to test this system for experimental chemotherapy of visceral leishmaniasis. As reported elsewhere (Rodrigues Jr. et al., 1994), the effectiveness of PQ-loaded PLA nanoparticles was 3.3 times higher than free drug in the suppression of amastigotes of *L. donovani* in the liver of BALB/c mice. The 50% effective doses (ED_{50}) were 6.6 and 21.8 mg PQ/kg body weight for PQ-loaded nanoparticles and free PQ, respectively. So, the therapeutic index based on the ratio LD_{50}/ED_{50} of PQ-loaded nanoparticles, calculated to be 4.1, was 5 times higher than that of free primaquine (TI = 0.8).

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