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Atovaquone-loaded nanocapsules: influence of the nature of the polymer on their in vitro characteristics

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Abstract

Nanocapsules with atovaquone concentration of 1000 μg/ml were prepared according to the interfacial deposition technique using different polymers: poly-ε-caprolactone (PECL), poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLAGA). The following characteristics of nanoparticles were determined: percentage of encapsulation of atovaquone, percentage of encapsulation of benzyl benzoate (BB), nanoparticle size, nanoparticle wall thickness, suspension pH, and in vitro stability. The different formulations showed similar characteristics: maximal percentage of encapsulation (100%), particle size of approximately 230 nm, neutral pH and wall thickness of approximately 20 nm. The type of polymer used was the main factor influencing stability, in decreasing order: PECL > PLA > PLAGA. No release of atovaquone or benzylbenzoate was noted with PECL nanoparticles over 4 months. Release of atovaquone (25.9%) was found with PLA nanoparticles at 4 months. Release of both atovaquone (18.9%) and benzylbenzoate (54.2%) was noted with PLAGA nanoparticles from the third month, indicating a disruption of the nanoparticle membrane.

Keywords: Atovaquone; Nanocapsules; PLA; PLAGA; PECL

1. Introduction

Leishmania-HIV co-infection is frequent in the mediterranean area (Gangneux, 1999; Desjeux et al., 2001). Pentavalent antimonials and amphotericin B are the first lines of treatment despite irregular effectiveness (Gangneux, 1999). These treatments are effective in immunocompetent patients, but patients rendered T cell deficient by iatrogenic immunosuppression or advanced HIV

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infection fail to respond or promptly relapse (Davidson et al., 1994). New drugs or new formulations of drugs are used in the absence of response to classical treatments.

The potential of colloidal drug carriers in targeted and controlled delivery of anti-leishmanial compounds has received much interest. Leishmania are obligate intracellular parasites in mammals. They live exclusively in the cells of the mononuclear phagocyte system (MPS) which also clears drug particles from the circulation. Drug targeting has led to both a reduction in toxicity and an increase in effectiveness of antileishmanial drugs (Croft et al., 1989; Deniau et al., 1993). Liposomal amphotericin B is currently the most effective therapy in drug-resistant visceral leishmaniasis (Davidson et al., 1991; Gangneux, 1999). However, immunocompromized patients relapse and must receive permanent liposomal amphotericin B prophylaxis.

Several drugs (primaquine, inosine analogs, atovaquone), known to have a moderate activity against experimental leishmaniasis have shown an increased effectiveness through vectorization (Morishige et al., 1995; Rodrigues et al., 1995; Cauchetier et al., 2000). The use of colloidal systems increases the effectiveness of these drugs as it has been shown with primaquine (Rodrigues et al., 1995). Although the mechanism of action of these drugs is different, these vectorized drugs could represent an alternative to classical therapy.

Encapsulation of ATV into liposomes has already been realized and confirmed an increase in drug activity (Cauchetier et al., 1999, 2000). Hundred percent effectiveness may possibly be obtained by increasing doses. However, this effectiveness could not be attained due to the low percentage of encapsulation of ATV into liposomes (40%). The use of nanocapsules is attractive because of their oil-based central cavities which allow a high encapsulation level for lipophilic substances like ATV.

The aim of the study was to prepare and characterize ATV-nanocapsules made from four different biodegradable polymers according to the interfacial polymer deposition technique, and to evaluate their stability in vitro.

2. Materials and methods

2.1. Materials

ATV was obtained from Wellcome Foundation Ltd (Dartford, UK). Soybean lecithine (S75, 70% of phosphatidyl choline) was purchased from Lipoïd (Ludwigshafen, Germany). Poloxamer 188 (Symperonic PE/F-68) was purchased from ICI (Clamart, France). Polymers poly(D,L-lactide) (PLA, mol. wt. 200 000), poly-ε-caprolactone (PECL, mol. wt. 65 000 and 100 000) and poly(lactic-co-glycolic acid) (PLAGA, mol. wt. 40 000) were supplied by Boehringer Ingelheim (Ingelheim, Germany). Benzyl benzoate (BB, density at 20 °C: $d_{BB} = 1.118$) was purchased by Cooper (Melun, France). All organic solvants used were reagents of high performance liquid chromatography (HPLC) grade. Other reagents used were of analytical grade.

2.2. Preparation of nanoparticles

Nanocapsules with an ATV concentration of 1000 μg/ml were prepared using the process described by Fessi et al. (Fessi et al., 1989). Nanocapsules were prepared with each polymer (PECL65, PECL100, PLA and PLAGA): 100 mg of the selected polymer and 125 mg of lecithin were dissolved in 25 ml of acetone. ATV (10 mg) was then dissolved in 0.5 ml of BB and added to the acetone solution. This organic solution was poured, under moderate magnetic stirring, into 50 ml of distilled water containing 100 mg of Symperonic. The resulting mixed phase immediately turned opalescent as a result of the formation of nanocapsules, due to the diffusion of the acetone towards the aqueous phase. Acetone and water were removed under partial vacuum (60 °C, 0.8 bar) to obtain a final volume of 10 ml. Nanocapsules were kept in sealed glass bottles, in a dark room, at 4 °C throughout the study.

Free nanocapsules were prepared as previously described but omitting ATV.

2.3. Determination of ATV loading

Total drug concentration in the suspension containing the nanocapsules (C_1) was determined by dissolving the polymer membrane in acetonitrile and dosing for ATV. Atovaquone concentrations were determined by HPLC after dilution of the sample in the mobile phase (1/200) (48:36:16:0.001 v/v of acetonitrile:methanol:water:orthophosphoric acid 85%). A 250×4.6 mm i.d. 5 μm hypersil C-18 column was used. The detection wavelength was set to 258 nm. Typical injection volumes were 100 μl and the mobile phase flow rate was 1.5 ml/min. The retention time of ATV was 5.0 min.

Encapsulated drug levels were determined by removing unencapsulated drug by gel chromatography. Nanocapsules were passed through a sepharose CL 4 B gel column (Pharmacia Biotech, Orsay, France). A 1.5×20 cm column was used. The gel was initially equilibrated with the previously described symperonic solution with a 1 ml/min flow rate and pre-saturated with free nanocapsules of the same polymer composition. The nanocapsules fraction was then dissolved in acetonitrile and ATV concentration was determined (C_2).

The percentage of encapsulation (%PE) was determined according to:

$$\%\mathrm{PE} = C_1/C_2 \times 100$$

where C_1 is the total ATV content in the nanocapsules suspensions, and C_2 is the content of ATV encapsulated in nanocapsules.

2.4. Determination of BB content

The content of BB was determined as previously described for ATV. The retention time of BB was 2.7 min.

2.5. Particule size analysis

Particule size was determined with a dynamic light scattering spectrophotometer (Coulter® model N4MD, Coultronics, Margency, France) with nanoparticles filtered through the sepharose gel column (see above).

2.6. Determination of the pH of nanocapsules

PH of nanocapsules suspensions (after passage on the Sepharose column) was measured with a glass electrode and a digital pH meter (Bioblock Scientific 99622, Prolabo, Paris, France) at room temperature.

2.7. Determination of polymeric loss ($PL_{polymer}$)

The nanocapsules as well as the solvent used to clean the glass ware were filtered over a preweighed sintered glasses (porosity 16–40 μm)(P_1). The sintered glasses were then incubated at 100 °C for 24 h until their weight stabilized (P_2). Polymer content was then determined by the difference in weight $\mathrm{Pf} = P_2 - P_1$. Polymeric loss was expressed as the following percentage:

$$\mathrm{PL}_{\mathrm{polymer}} = \frac{(P_\mathrm{i} - P_\mathrm{f})}{P_\mathrm{i}} \times 100$$

where P_i was the initial polymer weight and P_f the filtered polymer weight.

2.8. Determination of nanocapsule wall thickness

Theorical values for wall thickness of nanocapsules were determined based on the following hypothesis: the polymer was the unique component of the wall of the nanocapsules.

determination of the fractional mass of the organic phase $\mathrm{fm}_{\mathrm{OP}}$:

$$\mathrm{fm}_{\mathrm{OP}} = f_{\mathrm{ATV}} + f_{\mathrm{PL}} + f_{\mathrm{BB}}$$

where f_{ATV} is the fractional mass of ATV:

$$f_{\mathrm{ATV}} = \frac{\%\mathrm{PE}_{\mathrm{ATV}} \times \text{quantity introduced}}{Q_\mathrm{T}} = \frac{Q_{\mathrm{ATV}}}{Q_\mathrm{T}},$$

f_{PL} is the fractional mass of PL:

$$f_{\mathrm{PL}} = \frac{\text{quantity introduced}}{Q_\mathrm{T}} = \frac{Q_{\mathrm{PL}}}{Q_\mathrm{T}},$$

f_{BB} is the fractional mass of BB:

$$f_{BB} = \frac{\%PE_{BB} \times (\text{volume introduced} \times d_{BB})}{Q_T} = \frac{Q_{BB}}{Q_T},$$

$$Q_T = \Sigma(Q_{ATV} + Q_{PL} + Q_{BB} + Q_{polymer}),$$

$$Q_{polymer} = (\text{quantity of polymer introduced} \quad (\text{quantity of polymer introduced} \times PL_{polymer}).$$

determination of the fractional volume of the organic phase fv_{OP}

$$fv_{OP} = \frac{fm_{OP}}{d_{OP}}$$

where d_{OP} was determined with a picnometer at 20 °C at 1.0866.
determination of the volume of the organic phase (V_{OP})

$$V_{OP} = \frac{\text{volume introduced of BB} \times \%PE_{BB} \times d_{BB}}{d_{OP}}$$

determination of the volume of a nanocapsule (V_p)

$$V_P = \frac{4}{3}\ \Pi R_p^3$$

where R_p is the mean radius of the nanoparticle.
determination of the number of nanoparticles per ml (N)

$$N = \frac{V_{OP}}{(V_P \times fv_{OP})}$$

determination of the radius of the oil sphere R_{OP}

$$R_{OP} = \left(\frac{3/4 \times V_{OP}}{\Pi}\right)^{-3}$$

determination of the wall thickness of the nanoparticle T

$$T = R_P - R_{OP}$$

2.9. Stability study

Nanocapsules, prepared according to the technique described in Section 2.2, were used to study the effect of storage (at 4 °C over 4 months). Particle size, pH, ATV and BB loading were investigated using the same technique as described above.

2.10. Statistical analysis

Results were expressed as mean ± S.D. Experiments were realized in triplicate.

3. Results and discussion

3.1. Characteristics of nanocapsules

The interfacial deposition method allows preparation of biodegradable nanocapsules with high drug loading capacity in an effective and reproducible manner. The maximum percentage (100%) of encapsulation of ATV was obtained with an ATV concentration of 1000 μg/ml, independently of the polymer used (Table 1). This result was comforted by Marchal-Heussler et al. (1999), Losa et al. (1993). Marchal-Heussler et al. (1999) have demonstrated that the type of polymer had no influence on encapsulation of indium oxine in PLAGA and PECL nanocapsules. Losa et al. (1993) reported that the nature of polymer had no significant effect on metipranolol-loading capacity of PECL and isobutylcyanoacrylate nanocapsules. Nevertheless, Marchais et al. (1998) showed that the efficiency of phenylbutazone loading on PECL was significantly lower in comparison with PLA and its copolymers.

Table 1
Physicochemical characterization of ATV-loaded nanocapsules: influence of the polymer on the percentage of encapsulation of ATV and BB, nanoparticle size, pH of the solution and the evaluation of the membrane thickness of the nanoparticle

	PLA	PLAGA	PECL65	PECL100
PE ATV (%)	103.3±4.6	97.5±3.4	97.9±2.8	97.5±5.7
PE BB (%)	87.7±9.6*	79.1±8.1	81.3±1.1	80.8±1.0
Nanoparticle size (nm)	236.6±13.2	228.8±9.8	228.0±16.1	241.7±32.5
PH	7.34±0.01	7.03±0.44	6.96±0.50	6.93±0.47
Polymeric loss (%)	6.5	6.4	6.5	6.7
Evaluation of the membrane thickness (nm)	21.8	20.9	20.9	22.2

Results as expressed as mean±S.D; *, $P < 0.05$: PE BB (%) into PLA nanoparticles vs. PLAGA ($P = 0.0117$), PECL 65 ($P = 0.0276$) and PECL 100 nanoparticles ($P = 0.0258$).

The maximum percentage (100%) of encapsulation of ATV (ATV concentration of 1000 μg/ml) (Table 1) was confirmed by Dalençon et al. (1997) in ATV-loading on PLA nanocapsules. The incorporation of drug into nanocapsules is highly dependent on the lipophilic character of the drugs, pH of the medium and thus on the oil core affinity (Ammoury et al., 1989; Rodrigues et al., 1995; Némati et al., 1996). Encapsulation in nanocapsules was found to be close to 100% for different lipophilic drugs such as phenylbutazone (Marchais et al., 1998), primaquine (Rodrigues et al., 1995), clofibride (Santos Magalhaes et al., 1995), or indomethacin (Ammoury et al., 1989; Fessi et al., 1989). The percentage of encapsulation is correlated to the solubility of drugs in oil (Guterres et al., 1995; Ferranti et al., 1999). The better solubility of ATV in BB, compared with other oils (vegetables oils: soybean, olive, ...), incitated to choose BB as the core oil despite its toxic nature (ATV solubility in BB = 2.4 mg/ml; Dalençon et al., 1997). Since ATV is poorly soluble in water (solubility $< 2.10^{-4}$ mg/ml, pH ≤ 7; Burroughs Wellcome, 1992), it was not surprising to find a high percentage of ATV encapsulation. This is based on the partition solubility characteristics of ATV between the oil core and the neutral aqueous phase. Since the solubility limits of ATV in BB were not attained, the solubility of ATV in BB was not a limiting factor in encapsulation percentage in our study. We obtained 85% BB encapsulation in our study (Table 1). This coud be due to BB solubility in the acetone suspension containing nanocapsules before evaporation. During the evaporation process, acetone and some water were removed, leading to a decrease of BB solubility in the final suspension of nanoparticles. This hypothesis was conforted by apparition of oil drops in the rotavapor balloon. BB is miscible in acetone and not miscible in water. During nanocapsule formation, a fraction of BB may be dissolved in the mixture of actone in symperonic solution. BB solubility in this acetone solution (0.33%, 75 ml) was calculated to be approximately 1.5 μg/ml. This corresponded to 112.5 μg of BB or 20% of initial quantity, correlated with the 85% BB loss found experimentally. Since ATV is poorly soluble in the acetonic suspension, the fraction supposed to precipitate during evaporation was weak.

Nanocaspule size, film membrane thickness, pH and percentage of BB encapsulation were also not modified with the polymer used (Table 1).

This one-step manufacturing process obtained instantaneous formation of nanometric nanocapsules (ca. 230 nm). This result was confirmed by Dalençon et al. (1997) who obtained a nanoparticle size of 205 nm with ATV-loaded PLA nanocapsules. This manufacturing process leads to the formation of nanoparticles close to 200 nm in size independently on the polymer used (Vauthier-Holtzscherer et al., 1991; Marchais et al., 1998).

Nanocapsule size is independent on the encapsulated drug, since similar results were obtained with indomethacin (Ammoury et al., 1989; Fessi et al., 1989), diclofenac (Guterres et al., 1995) or primidone (Ferranti et al., 1999) dissolved in BB. Different authors reported that nanoparticle size was significantly affected by the type of oil used

(Losa et al., 1993; Guterres et al., 1995). The influence of the quantity of polymer in the organic phase was described by Ferranti (Ferranti et al., 1999). The nanoparticles size increased from 264 to 352 nm when the amount of the polymer increased from 150 to 380 mg. Dalençon et al. (1997), Fessi et al. (1989), using similar amount of polymer and types of oil, obtained comparable nanoparticle sizes. The mean nanocapsule size was independent of both the phospholipid concentration and the ratioes of PL to poloxamer (Ammoury et al., 1989). The presence of both emulsifiers (poloxamer and PL) was needed for wall coating formation and for suspension stability. The choice of 125 mg of PL allowed us to obtain stable nanoparticles with no solvated bilayers of PL. Excess of PL in the formulation formed highly solvated bilayers of phospholipids around the nanocapsules which could be controlled by variation of initial PL concentration as shown by Ammoury et al. (1989), Fessi et al. (1989). These authors used 250 mg of PL for the same quantitative organic solution, compared with 125 mg of PL in our study.

The membrane thickness of ATV-nanocapsules was calculated in our study to be 20 nm (Table 1), which is twice the value found by Fessi and others authors using transmission electron microscopy (TEM) photography (Ammoury et al., 1989; Fessi et al., 1989; Santos Magalhaes et al., 1995; Dalençon et al., 1997). Membrane thickness is related to the quantity of polymer used and to the oil core (Fessi et al., 1989; Santos Magalhaes et al., 1995). Thus, membrane thickness is related to BB encapsulation percentage. Since the quantity of oil used did not affect the mean particle size but the number of nanocapsules formed (Losa et al., 1993), membrane thickness, for a same concentration of polymer, was inversely proportionnal to the content of oil volume. The difference observed in the membrane thickness in our study compared with the others can not be explained since the methodologic approach was different and can not be compared. Calculation of membrane thickness by TEM photography is not accurate. We determined membrane thickness by a mathematical approximation. The PL quantity was not determined but a 10% error on the PL quantity induce only an error of 0.1 nm on the film thickness.

The nanoparticle suspension had a neutral pH, which is an advantage for its use in animals (Table 1). We measured pH after separation of free ATV from the nanocapsules by gel chromatography. This technique leads to a dilution and neutralization of the nanocapsule suspension by the neutral symperonic solution. Nanocapsules are thus of interest for the delivery of lipophilic drug substances such as ATV, in comparison to ATV-loaded liposomes which needed alkaline conditions to obtain the higher encapsulation percentage (Cauchetier et al., 1999).

3.2. Stability study

Stability studies over a 4-month period indicated that the decrease of ATV and BB contents was different between the three types of polymers. PECL nanoparticles were more stable than PLA and PLAGA nanoparticles (Table 2). The percentage of ATV encapsulation with PLA decreased significantly by 25.9% ($P = 0.045$) over 4 months. With PLAGA, it decreased by 18.9% ($P = 0.0083$) over 3 months. Moreover, PLAGA nanoparticles showed a significant loss of BB (54%) in the aqueous medium ($P = 0.0122$; Fig. 2 and Table 2). The percentage of encapsulation of ATV with PECL 65 and PECL 100 did not decrease significantly over the 4-month period. Nanoparticle stability at 4 months depended on the type of polymer used for the preparation of nanoparticles in the following decreasing order of stability: PECL > PLA_{50} > PLAGA. This decreasing stability was also described by Lemoine et al. (1996). Crystallinity and hydrophobic nature of polymer are key factors in determining the rate of in vitro degradation. The most crystalline and hydrophobic polymers exhibit the slowest degradation rate (Coffin and McGinity, 1992; Lemoine et al., 1996). PECL is a semi-crystalline and more hydrophobic polymer compared with PLA or PLAGA, and PLA is more hydrophobic than PLAGA (Lemoine et al., 1996; Marchais et al., 1998).

The initial molecular weight of PECL did not influence the degradation profile of nanoparticles (Table 2) as previously shown by Lemoine et al.

Table 2
Stability studies: physicochemical characterization of ATV-loading nanocapsules after a storage of 3 months (PLAGA) or 4 months (PLA, PECL) at 4 °C

	PLA	PLAGA	PECL65	PECL100
PE ATV (%)	76.5±9.1*	79.1±3.8**	87.5±17.7	94.2±4.1
PE BB (%)	85.1±12.8	36.2±1.7*	86.5±13.6	85.1±0.7
Nanoparticle size (nm)	225.0±6.0	224.6±7.9	226.7±8.8	222.5±16.8
pH	6.75±0.90	6.59±0.28	6.98±0.13	6.96±0.44
Aspect of the suspension	White suspension	Oil sediment in the white suspension	White suspension	White suspension

Influence of the polymer on the percentage of encapsulation of ATV and BB, nanoparticle size, and pH of the solution; $P < 0.05$: PE ATV (%) into PLA nanoparticles at 4 months vs. at the beginning of the study, PE BB (%) into PLAGA nanoparticles at 3 months vs. at the beginning of the study; $P < 0.01$: PE ATV (%) into PLAGA nanoparticles at 3 months vs. at the beginning of the study.

(1996). The storage of nanocapsules in water at 4 °C may explain the stability of these nanocapsules. Under these storage conditions, there is a slow degradation process of PECL (Lemoine et al., 1996). The neutral conditions significantly limited ATV solubility in the aqueous phase and thus the release of ATV from nanocapsules. In sink conditions, a release of ATV from nanocapsules or/and a slow degradation of PECL nanocapsules would probably be described (Losa et al., 1993; Kedzierewicz et al., 1998; Marchais et al., 1998). The ATV content was slighty inclined to decrease (10.6 and 3.4% from PECL 65 and PECL 100 nanocapsules, respectively) over the 4-month period (Table 2). No release of BB from the core of the nanocapsules was noted. The pH of the suspension was not modified. Neither modification of nanoparticles size, nor of membrane thickness was noted (Table 2).

PLA nanocapsules showed no difference in terms of BB content or nanocapsule size. We noted a higher release of ATV compared with PECL nanoparticles. The explanation for this difference may be due to diffusional transport of ATV through the polymer membrane associated with an initial stage of PLA degradation. An indicator of polymer degradation is a drop in pH (Coffin and McGinity, 1992). pH tended to decrease during storage but not significantly (Table 2), perhaps indicating that PLA nanocapsules were in the initial stage of degradation. However, we were not able to confirm this by direct assaying of degradation products. There is no mathematical model in the literature concerning the release of drugs from nanocapsules. The mathematical model proposed by Ritger and Peppas for nanospheres would have predicted slow initial release followed by a phase of rapid release but this is not supported by our data (Ritger and Peppas, 1987). ATV release from nanoparticles was biphasic with an fast initial release, followed by a much slower second release phase (Fig. 1). The initial period called the burst effect, could be attributed either to the desorption of the drug located on the nanocapsule's surface, or to the degradation of the thin polymeric membrane surrounding the oily core containing the drug (Marchais et al., 1998).

The release of ATV (18.9%) and BB (54.2%) from PLAGA nanoparticles showed that the stability of PLAGA nanoparticles was less important than others polymers at 4 °C in water at 4 months. This result is supported by Lemoine's study (Lemoine et al., 1996). The BB and ATV

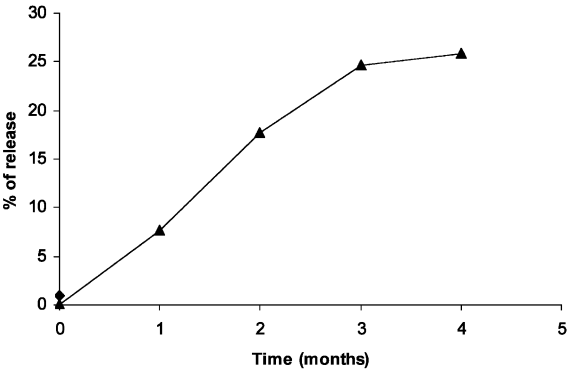

Fig. 1. ATV release profile from PLA nanoparticles with time.

contents decreased in a biphasic manner, slowly at first, then accelerating (Fig. 2). Leakage of the oil core was noted visually in glass bottles during the third month by the presence of sedimentation. This was attributed to the density of BB which is much more dense than water (Ammoury et al., 1989). The sedimentation of the oil and the formation of a cake, difficult to redisperse, led us to stop the stability study of PLAGA nanoparticles. The rapid leakage of BB up to the third month may indicate a disruption of nanocapsule polymeric coating. The release of ATV and BB as well as the tendency for a decrease in pH (Table 2) are in favor of degradation of the PLAGA nanocapsules. The release of ATV was slower than BB release (Fig. 2). Incomplete release of ATV from the nanoparticles may be attributed to the retention capacity of the polymer or phospholipids as described with ATV-loaded liposomes (Cauchetier et al., 1999), indomethacin-loaded nanocapsules (Ammoury et al., 1990) or primidone loaded-nanocapsules (Ferranti et al., 1999).

4. Conclusion

Colloidal nanoparticulate suspensions could be a suitable formulation for a lipophilic drug such as ATV. In order to assess the potential of ATV-loaded nanocapsules in vivo, it was necessary to prepare formulations with different types of polymers. In the present study, the highest percentage of encapsulation and the best stability was obtained with PECL, following by PLA and then by PLAGA. This study showed that PECL nanoparticles were stable over the 4-month period; the ATV release from PLA nanoparticles was biphasic with a diffusional transport of ATV through the polymer membrane associated with a first stage of polymer degradation; the ATV release from PLAGA nanoparticles is due to a disruption of the membrane. In order to assess the activity of ATV in an in vivo model, drugs would need to be administered three times a week and effectiveness evaluated 3 days after finally administration. The release of ATV from nanocapsules must be thus fast. The PLAGA formulation should be then administered intraveniously in vivo on a murine model of visceral leishmaniasis to determine the effectiveness of ATV on leishmania. As the percentage of encapsulation is higher than the one obtained in liposomes, we can possibly increase the effectiveness of ATV against *Leishmania.*

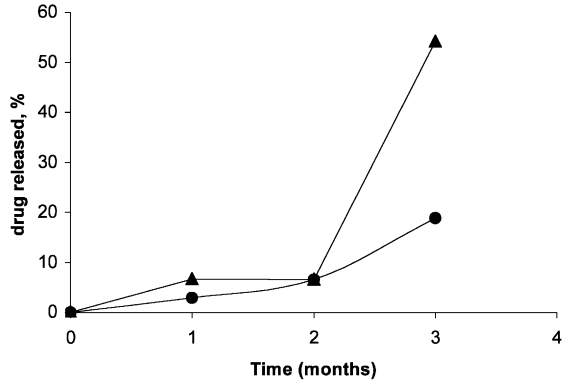

Fig. 2. ATV and BB release profiles from PLAGA nanoparticles with time (●, ATV released; ▲, BB released).

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