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In vitro evaluation of PLA nanoparticles containing a lipophilic drug in water-soluble or insoluble form

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Abstract

Cloricromene (AD6), an anti-ischemic drug, is rapidly metabolised into a stable and active metabolite (cloricromene acid, AD6-acid) poorly soluble in water and less lipophilic than cloricromene. The aim of this study was to evaluate which of the two forms has more possibility to be efficiently encapsulated in nanoparticles based on poly(D,L-lactide) and prepared using the nanoprecipitation method. Increasing the theoretical loading of AD6, an increase in drug actual loading and in the mean particle size occurred, while no formation of nanoparticles was observed when the highest theoretical loading (50 mg) was employed. Changing the pH of the aqueous phase the drug content dramatically increased. However, at a pH value of 11 a more rapid hydrolysis of AD6 occurred. When AD6-acid was embedded in the nanoparticles, suitable results concerning both drug content and encapsulation efficiency were achieved. A good control in the release of AD6 from the AD6-loaded nanoparticles was observed while the liberation of AD6-acid from the AD6-acid-loaded nanoparticles was faster than the dissolution of the AD6-acid free. These results confirm that the most easy encapsulable form in nanoparticles is AD6-acid probably owing to its poor water solubility. Further studies will be carried out in order to evaluate if the increase in the liberation of AD6-acid by nanoencapsulation may have outcomes in its bioavaibility in vivo.

Keywords: Poly(D,L-lactide); Nanoparticles; Cloricromene; In vitro release

1. Introduction

Leucocytes play an essential role in the pathogenesis of ischaemia and reperfusione injury. It is well known that inhibition of their adhesion and mediator release can reduce vascular and tissue damage. Numerous studies have shown that cloricromene, a semi-synthetic non-anticoagulant coumarin derivative, modifies several granulocyte as well as monocyte/macrophage functions and exerts a clear protective action in several experimental model of ischaemia and shock (Lidbury et al., 1993; Squadrito et al., 1993; Zatta and Bevilacqua, 1999). The antithrombotic and anti-ischaemic effects of AD6 are evident preminently in the peripheral ischemia for the difficult of the drug to pass the brain–blood barrier (BBB) (Squadrito et al., 1991). It has been found (Travagli et al., 1989) that cloricromene in the blood in vitro or in vivo is rapidly metabolised into a stable and active metabolite (cloricromene acid, AD6-acid) through the hydrolysis of an ester bound within the molecule (Fig. 1). Cloricromene (as chloride salt, AD6) is freely soluble in water even if it is very lipophilic ($\log P = 3.96$); on the contrary, its metabolite (AD6-acid) is less

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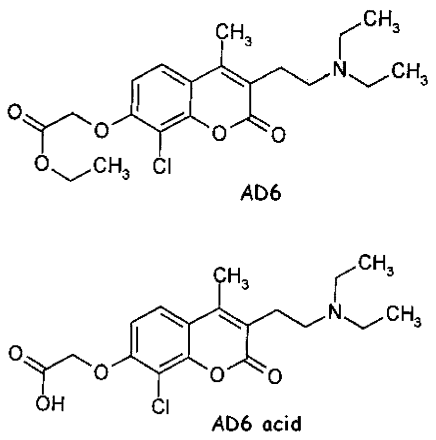

Fig. 1. Structure of cloricromene (AD6) and cloricromene acid (AD6-acid).

lipophilic ($\log P = 3.12$) but is poorly soluble in water and consequently is not used in the clinical practice.

Recently, polymeric nanoparticles have been proposed for brain targeting as interesting alternative to liposomes or to the traditional approaches to overcome brain drug delivery obstacles such as direct intracerebral drug injection or disruption of the BBB by infusion of hyperosmotic solution. Several articles have highlighted the ability of such nanoparticles, especially those with a prolonged half-life in blood (Troster et al., 1990; Moghimi, 1995), to improve the effect on central nervous system CNS) of many drugs which normally are not able to cross the BBB (Schröder and Sable, 1996; Alyautdin et al., 1997; Gulyaev et al., 1999). In the nanoparticle formulation particularly interest has been focused on the use of polyesters materials such as poly(D,L-lactide) (PLA) or poly(D,L-lactide-co-glycolide) (PLGA). Owing to their biocompatibility and biodegradability properties, nanoparticles of these polymers are investigated for a wide of applications using several preparation procedures. Some of the techniques commonly used include emulsification solvent diffusion method (Niwa et al., 1993), solvent evaporation procedure (Bodmeier and McGinity, 1988), salting-out procedure (Allemann et al., 1992) and nanoprecipitation procedure (Fessi et al., 1989). In order to produce small and low polydisperse nanoparticle population, the nanoprecipitation method results one of the most easy. Nevertheless, this technique suffers the drawback of a poor encapsulation efficacy of water-soluble drugs due to rapid migration and therefore loss of drug into aqueous phase. Various attemps have been performed in order to overcome this problem (Govender et al., 1999; Barichello et al., 1999). It was shown (Govender et al., 1999) that several formulation parameters (such as pH of water phase, addition of formulation excipient) can be exploited successfully for enhancing the incorporation of water-soluble drugs into PLGA nanoparticles prepared by the nanoprecipitation technique.

According to these premises, the aim of this work was to evaluate which of the two forms (AD6 or AD6-acid) has more possibility to be encapsulated efficiently in the nanoparticles formulated by the nanoprecipitation method, according to the parameters used in the preparation.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide) (Resomer® 203; mean MW 16,000) was supplied from Boehringer Ingelheim, (Ingelheim, Germany). Cloricromene (AD6, 8-monochloro-3-β-diethylaminoehyl-4-methyl-7-ethoxy-carbonylmethoxy coumarin) and cloricromene acid (AD6-acid, 8-monochloro-3-β-diethylaminoethyl-4-methyl-7-ethoxy-carbonyl coumarin) were donated by courtesy of Fidia Reserch Laboratory (Catania, Italy). Sodium cholate was suppled from Sigma Chemical (Milan, Italy). Acetonitrile (Sigma Chemical) was of HPLC grade. All other chemicals and solvents were of reagent grade and used as received.

2.2. Nanoparticle preparation

Nanoparticles were prepared according to the nanoprecipitation method (Fessi et al., 1989). The starting procedure was as follows. PLA polymer (100 mg) and different amounts of AD6 (10, 25, or 50 mg) were accurately weighed ad dissolved in acetonitrile (8 ml). The organic phase was added dropwise into the aqueous phase (25 ml) containing sodium cholate (12 mM). The aqueous phase was constituited by deionized water or deionized water adjusted at pH 10

or 11 with NaOH 0.1N. After stirring magnetically at room temperature for 10 min, the organic solvent was removed at 30 °C using a Rotavapor (Mod. B-480, Büchi Labortechnik, Flawil, Switzerland). The final volume of the suspension was adjusted to 10 ml with deionised water before nanoparticle recovery.

Nanoparticles loaded with AD6-acid were prepared dissolving the drug (10 mg) into 4 ml of methanol and the polymer into 4 ml of acetonitrile. The two organic phases were mixed and then added to the aqueous phase. Then, the procedure was the same as described above for the preparation of AD6-loaded nanoparticles.

Empty nanoparticles were prepared according both the procedures previously described, omitting obviously the drug. All samples were prepared in triplicate.

2.3. Nanoparticle recovery

The nanoparticles were purified from the suspension by gel-filtration chromatography. Sepharose CL4B (Sigma Chemical) gel was filled into a column, avoiding bubbles and cracks. The column had a length of 50 cm, an inner diameter of 2 cm and contained about 160 ml of gel. The nanoparticles were eluited using deionised water at a flow rate adjusted to 1 ml/min. Nanoparticles appeared within 30 min. The samples collected were freeze–dried during 24 h (Lyovac GT2; Leybold-Heraeus, Hanau, Germany) to obtain afine powder of nanoparticles. The yield of nanoparticles was calculated as the ratio of the amount of recovered nanoparticles to the total amount of polymer and drug added.

2.4. Scanning electron microscopy

A scanning electron microscope (SEM) (XL-40 Philips, The Netherlands) was used to evaluate both size and morphology of nanoparticles. Before the SEM analysis the samples were coated under argon atmosphere with a 10-nm gold palladium thickness (Emitech K550 Supper Coated, Emitech LTD, Ashford, Kent, UK). To determine the size distribution of the prepared particles, at least 500 particles for each preparation were sized from electron microphotographs by an image analysis (Image Proplus, Media Cybernetics, Silver Spring, MD, USA).

2.5. Determination of drug content in the nanoparticles

The amount of cloricromene encapsulated per unit weight of nanoparticles was determined dissolving a weight amount of nanoparticles (10 mg) in acetonitrile and than measuring the amount of the drug by an HPLC method previously described (Maltese and Bucolo, 2002). The HPLC system was equipped with a solvent delivery system (mod. 9012, Varian, Milan, Italy) connected with a pump (series 9012, Varian), a ChromSep HPLC column C_{18} reverse-phase (10 μm, 250 mm × 4.6 mm i.d.) (Varian Cromopack), a ChromSep guard column C_{18} (OmniSpher 10 μm) (Varian Superchrom), a UV-Vis detector (mod. 1575 Jasco, Tokyo, Japan) and interfaced to Star Chromatographic Workstation (Varian) software. The mobile phase was acetonitrile:phosphate buffer (monobasic ammonium phosphate 90 mM) 30:70 (v/v) adjusted to pH 3.5 with phosphoric acid and was delivered at flow rate of 1 ml/min. Before using, the mobile phase was filtered through a 0.2 μm hydrophilic polypropylene membrane filter (GH Polypro, Pall, MI, USA). The injected volume was 10 μl. The UV detector was set at 318 nm and the retention time of AD6-acid and AD6 was found to be 3.4 and 13.3 min, respectively. Actual loading and entrapment efficiency were calculated as following equations:

$$\text{Actual loading (\%, w/w)} = \frac{\text{mass of drug in nanoparticles}}{\text{mass of nanoparticles recovered}} \times 100$$

$$\text{Entrapment efficiency (\%)} = \frac{\text{mass of drug in nanoparticles}}{\text{mass of drug used in formulation}} \times 100$$

2.6. In vitro drug release study

The in vitro drug release studies were performed using the dialysis bag diffusion technique. Nanoparticles (20 mg) were suspended in 2 ml of phosphate buffer (20 mM, pH 7.4), placed in a dialysis bag (i.d. 18 mm; MWCO 12000, T3, Cellusep, Seguin, TX, USA) hermetically sealed and dropped into 50 ml of phosphate buffer (20 mM, pH 7.4) at 37 °C under magnetic stirring. At fixed time intervals an aliquot of receptor

media (50 μl) was withdrawn and replaced with fresh buffer. The drug content was determined by the HPLC method as previously described.

The diffusion rate across the dialysis membrane of the free drugs (AD6 and AD6-acid) was performed as follows. An amount exactly weighed of AD6 or AD6-acid was, respectively, solubilized or suspended in 2 ml of phosphate buffer (20 mM, pH 7.4), placed in the dialysis bag, hermetically sealed and dropped into 50 ml of phosphate buffer at 37 °C ± 0.2 under magnetic stirring. The drug content in the outer compartment was determined as described above for the drug-loaded nanoparticles.

3. Results and discussion

3.1. Effect of the theoretical drug loading

In order to establish the maximum amount of drug in its salt form (AD6) that could be incorporated into nanoparticles, the initial approach involved the increase of the theoretical drug loading. In Table 1 the variable parameter and the nanoparticle characteristics are reported. The aqueous phase was constituted by deionised water. As expected, an increase in the drug loading with an increase in the theoretical loading was observed. On the contrary, the drug entrapment efficiency decreased. The trend observed in the drug entrapment efficiency agrees to those observed for other water-soluble drugs, i.e. neurotensin analogue (Yamakawa et al., 1992), narfalein acetate (Niwa et al., 1994) and procaine hydrochloride (Govender et al., 1999). This occurrence indicates the rapid partitioning of the drug into the aqueous phase during nanoparticles formation owing to its water-soluble nature.

As far as nanoparticle yield, increasing the theoretical loading, a decrease in the nanoparticle recovery was observed. When the higher amount of drug was used (namely theoretical loading of 50 mg) no formation of nanoparticles occurred but only large particles or polymer aggregates were observed by SEM analysis (data not shown). Moreover, the mean particle size dramatically increased in the presence of the drug from 102 ± 12 nm (empty nanoparticles) to 320 ± 25 nm (AD6-loaded nanoparticles). No change in the particle morphology was observed since empty nanoparticles (Fig. 2A) as well as AD6-loaded nanoparticles (Fig. 2B) appeared spherical and discrete.

The increase in the particles size of drug-loaded nanoparticles respect of the empty ones is probably due to the entrapment of the drug during the nanoparticle formation as observed also by Govender et al. (1999). The dramatic decrease of the nanoparticle yield with the increase of the theoretical loading may be attributable to the rapid migration of the drug in the external aqueous phase during polymer nanoprecipitation. producing the precipitation of polymer fragments.

3.2. Effect of the pH aqueous phase on the AD6 loading

As show in Table 1, a low nanoparticle drug loading was achieved (the better value was of 0.6%, w/w). Therefore, other formulation approaches were investigated in order to improve AD6 entrapment, i.e. the change of the pH of the aqueous phase (Ueda and Kreuter, 1997; Leo et al., 1998; Govender et al., 1999; Vandervoort and Ludwig, 2001). Nanoparticles with a theoretical loading of 25% (recoverable with a satisfactory yield) were prepared using the conditions reported in the previous study. Only the pH value of

Table 1
Effect of AD6 theoretical loading on drug actual loading, entrapment efficiency, yield of nanoparticles and particle size

AD6 theoretical loading (mg)	AD6 actual loading (mg/100 mg nanoparticles)	Entrapment efficiency (%)	Yield of nanoparticles (%)	Mean particle size (nm)
Without drug	–	–	90.9 ± 1.3	102 ± 18
10	0.39 ± 0.02	3.9 ± 0.2	90.7 ± 1.2	320 ± 25
25	0.64 ± 0.03	2.6 ± 0.3	82.7 ± 2.1	340 ± 20
50	–	–	3.6 ± 0.4	–

Data are shown as mean ± S.E. obtained from three formulations.

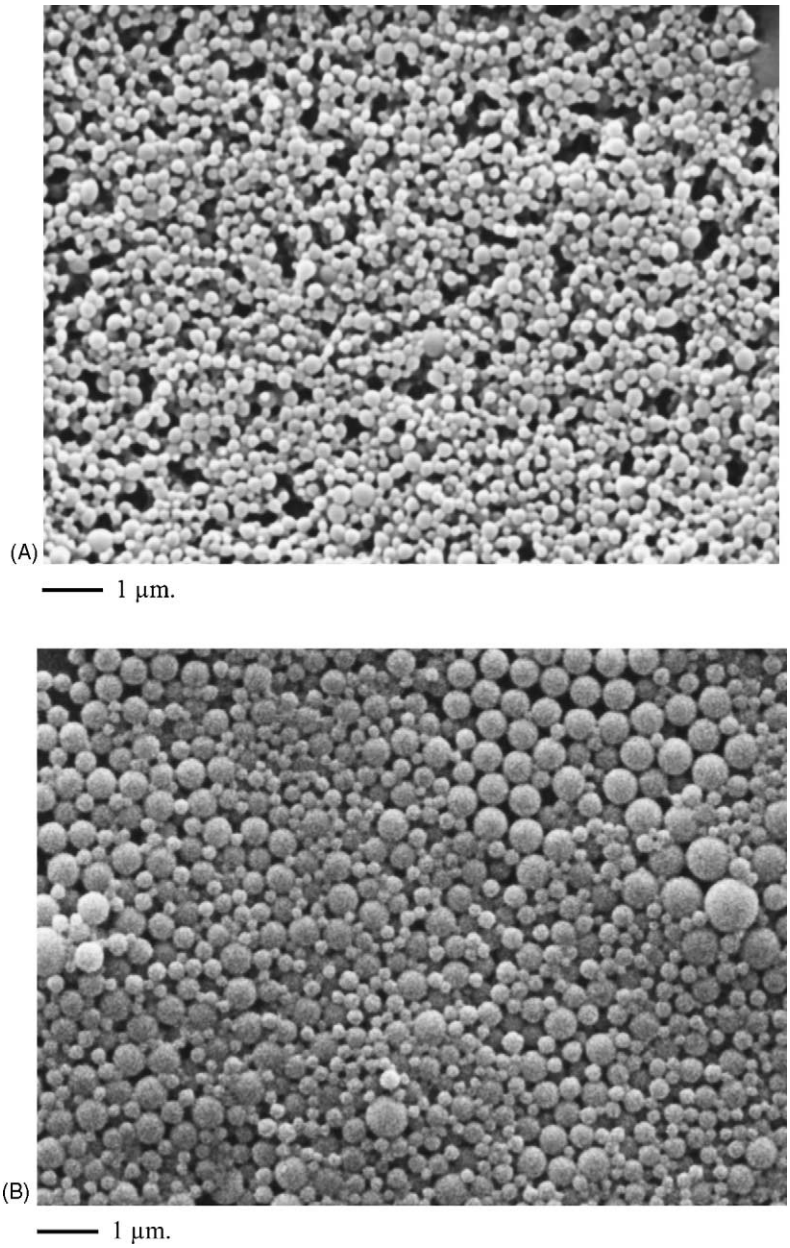

Fig. 2. Scanning electron micrographs of unloaded nanoparticles (A) and AD6-loaded nanoparticles (B).

the aqueous phase was adjusted to 10 or 11. The results in terms of drug content, loading efficiency and nanoparticle size and yield are listed in Table 2. It was observed that the change of the pH of the water phase did not modify the yield and the size of nanoparticles. As expected, an increase of the drug content and encapsulation efficiency was observed. Namely, employing a pH of the aqueous phase of 6.8 the drug loading resulted in only 0.6% (w/w) while employing pH 11 resulted in 2.3% (w/w). Nevertheless, at a pH value of 11 the hydrolysis of AD6 occurred rapidly and AD6-acid is formed. Therefore the two forms (AD6

Table 2
Effect of the pH of the aqueous phase on the drug (AD6) loading, entrapment efficiency, yield of nanoparticles, and particle mean diameter

Aqueous phase	Actual loading (mg/100 mg nanoparticles)	Entrapment efficiency (%)	Yield of nanoparticles (%)	Mean diameter (nm)
Deionised water	0.64 ± 0.03	2.6 ± 0.3	82.7 ± 2.1	340 ± 20
pH 10	0.83 ± 0.03	3.3 ± 0.3	85.8 ± 1.4	333 ± 25
pH 11	2.31 ± 0.7[a]	9.2 ± 0.8	84.2 ± 1.5	319 ± 15

Data are shown as mean ± S.E. obtained from three formulations.
[a] Corresponding of 1.48% of AD6 and 0.83% of AD-acid.

and AD6-acid) were loaded into the nanoparticles producing a drug delivery system with a not predictable bioavailability.

3.3. AD6-acid encapsulation

To prepare AD6-acid-loaded nanoparticles the procedure described previously was modified owing to the low solubility of the drug in the acetonitrile. Hence the starting theoretical loading was of 10% (w/w) instead of 25% (w/w) and a mixture 1:1 (v/v) of acetonitrile:methanol was used as organic phase. These modifications of the starting preparation procedure did not change the final yield of nanoparticles (81.7 ± 1.8%), their size and morphology. As shown in Fig. 3 we obtained small and quite spherical particles with a mean diameter ranged between 320 and 350 nm. Satisfactory both drug content (5.3 ± 1.1w/w) and encapsulation efficiency (53%) was achieved. These characteristics of the AD6-acid-loaded nanoparticles agree with the expectative. In fact, owing to the poor water-soluble nature of the drug, the migration of the drug molecule to the outer water phase should be slight.

3.4. Drug release studies

As concerning drug release experiments, the dialysis dynamic method was applied. Among the experimental method available for determining in vitro the release profiles from colloidal suspension, this method is the most suitable in order to separate rapidly

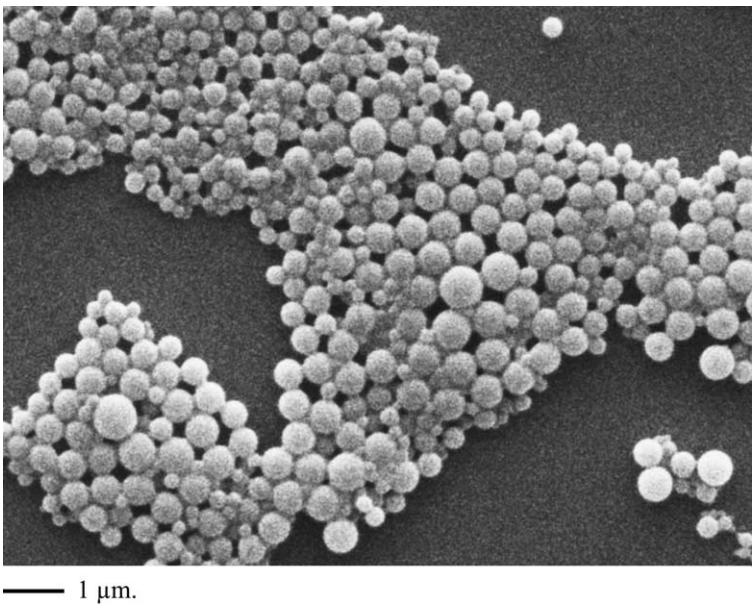

Fig. 3. Scanning electron micrographs of AD6-acid-loaded nanoparticles.

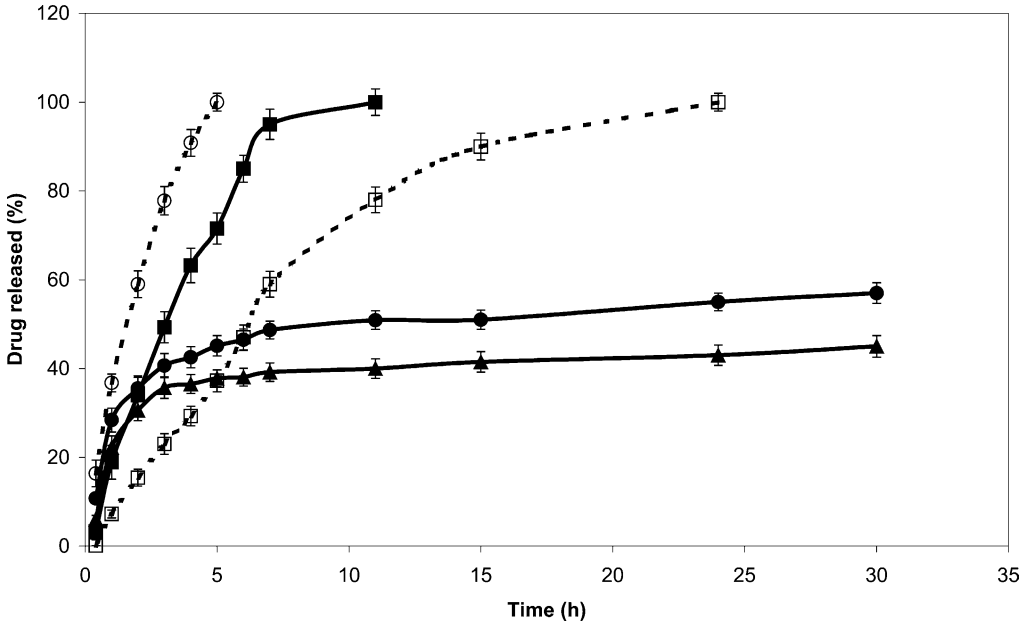

Fig. 4. Dissolution profiles of AD6-free (○) and AD6-acid free (□). Release profiles of AD6 from: AD6-loaded nanoparticles (deionised water) (▲) and AD6-loaded nanoparticles (pH aqueous phase of 10) (●). Release profile of AD6-acid from AD6-acid-loaded nanoparticles (■).

and completely nanoparticles from the release medium (Soppimath et al., 2001). However, dialysis is an indirect method in which the drug is determined in the outer compartment whereas it is release in the inner of the bag. The release rate of the drug and its appearance in the outer dissolution medium is governed by the partition coefficient of the drug between the polymer and the aqueous environment and by the diffusion of the drug across the membrane as well (Washington, 1989, 1990). For these considerations, the determination of the dialysis behaviour of the free drugs was also performed. In Fig. 4 are showed the release patterns of free drugs (AD6 and AD6-acid) and of the respective loaded nanoparticles.

The diffusion of AD6 as free drug was rapid and complete over 4 h. Concerning AD6-loaded nanoparticles the samples prepared in deionised water and at pH 10 were examined. These samples displayed a release pattern with two components: an immediate release ("burst effect") followed by a slower release profile. The burst effect was of about 35% for the sample prepared in deionised water and of 40% for nanoparticles prepared at pH 10. This first initial burst effect is probably due to the drug adsorbed onto the wall of the nanoparticles which would be immediately released during the initial stage. After this initial release phase, the drug release profile displayed a delayed release that may be attributed to diffusion of the drug entrapped within the core of the nanoparticles.

The diffusion profile of the AD6-acid as free drug (Fig. 4) indicates that the drug is able to diffuse across the membrane completely in 24 h. The 60% diffused after 7 h and the 90% after 15 h. The slow dissolution rate of the free drug may be attributable to the very poor solubility of the drug in the aqueous dissolution medium. However, the dissolution was complete and no phenomenon of absorption onto the dialysis membrane was observed. Interestingly, AD6-acid-loaded nanoparticles showed a drug release rate faster than the free drug. The 50% of the entrapped drug was released after 3 h and 95% after 7 h. This increase of dissolution rate of AD6-acid embedded in the nanoparticles may be outcome from the nanoprecipitation procedure during nanoparticle formulation (Müller et al., 2001). Moreover, the release pattern appeared monophasic. This finding may indicates that the most part of the drug was only adsorbed or close to the surface of the nanoparticles and not entrapped in the polymeric

core. Probably the use of a mixture of two solvents as acetonitrile for the polymer and methanol for the drug may cause the not contemporary precipitation of the drug and the polymer together. In other words, nanoparticles may take shape before the precipitation of the drug then, the drug precipitation occurs onto the nanoparticle surface.

In conclusion, AD6 or AD6-acid-loaded PLA nanoparticles were prepared by the nanoprecipitation method. The change of the pH of aqueous phase improves the entrapment of the drug in its water-soluble form (AD6) although at a pH value of 11 the hydrolysis of the AD6 occurs. Moreover, the results confirm that the most easy encapsulable drug form in polymeric nanoparticles is AD6-acid thanks to its poor water solubility. Our results show that the liberation of AD6-acid from the AD6-acid-loaded nanoparticles was faster than the AD6-acid free dissolution. Therefore, further studies will be carried out in order to evaluate if this unexpected result may have outcomes in the bioavailability of this drug as long as in its therapeutic use.

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