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Vitamin A palmitate and aciclovir biodegradable microspheres for intraocular sustained release

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Abstract

The aim of this study was to obtain a prolonged release of Vitamin A palmitate (RAP) and aciclovir from biodegradable microspheres for intraocular administration with an antiviral action and to be capable of preventing the inherent risks of intravitreal administration. The RAP effect on the microsphere characteristics was also studied. Poly(D,L-lactic-*co*-glycolic) acid microspheres were prepared by the solvent evaporation method. Different quantities of aciclovir (40–80 mg) and RAP (10–80 mg) were added to the internal phase of the emulsion. Microspheres were analysed by scanning electron microscopy, which revealed a spherical surface and a porous structure, and granulometric analysis that showed an adequate particle size for intraocular administration. The aciclovir loading efficiency increased when Vitamin A palmitate was added. Differential scanning calorimetry detected no differences in the polymer glass transition temperature and the aciclovir melting endotherm in all formulations. The release of aciclovir during the first days of the in vitro assay was improved with respect to microspheres without RAP. The microspheres showed a constant release of aciclovir and RAP for 49 days. Best results were obtained for microspheres prepared with 40 mg aciclovir, 80 mg RAP and 400 mg polymer. A dose of 4.74 mg of microspheres would be therapeutic for the herpes simplex and Epstein-Barr viruses' treatment in an animal model and would reduce the intravitreal adverse effects. The injectability of a suspension of microspheres in isotonic saline solution resulted appropriate for its injection through a 27 G needle.

Keywords: Vitamin A palmitate; Aciclovir; PLGA microspheres; Intraocular

1. Introduction

Intraocular drug administration is the most common route for the local treatment of the posterior segment eye diseases. This type of administration is recommended when less aggressive routes are discarded or for severe pathologies that affect the vitreous or the retina. These pathologies, such as macular edema, uveitis, endophthalmitis, proliferative vitreorethinopathy (PVR) or herpes viruses' infections, constitute the most frequent cause of blindness in the developed countries (Petrovich et al., 1992; Park et al., 1995; Yilmaz and Er, 2005; Avci et al., 2006). Some herpes viruses that infect humans are herpes simplex virus (HSV), types 1 and 2, varicella zoster and Epstein-Barr viruses. Infections are mainly due to a reactivation of the virus, which has remained latent since the primary infection. These infections are very common and important in immunosuppressed individuals and depending on the stage of immunosuppression the severity of clinical manifestations increase as a consequence of the viral reactivation (Meyers, 1985).

Intravitreal administration of antiviral agents is important to maintain therapeutic levels in the site of action and diminish or prevent the adverse effects associated to the systemic treatment. However, successive intraocular injections are poorly tolerated and have inherent risks, such as endophthalmitis, cataract, retinal detachment and vitreous haemorrhage (Herrero-Vanrell and Refojo, 2001). Moreover, the low therapeutic rate of some drugs used for the treatment of the posterior segment diseases can lead to toxic concentrations in the retina (Pulido et al., 1984). Some of these inconveniences can be overcome by the use of agents capable of preventing these effects such as substances with antiproliferative activity, or new dosage forms as controlled release systems, which allow a reduction in the number of injections. Vitamin A, as free alcohol and esters, has shown to be effective by injection for the treatment of proliferative vitreoretinopathy, tractional retinal detachment and other vitreoretinal diseases (Peyman and Schulman, 1986; Araiz et al.,

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1993; Giordano et al., 1993; Choi et al., 2001; Jeong et al., 2003).

The development of sustained release multiparticulate systems that can lead to a controlled release of the drug and are adapted to the intraocular route are also very useful because with a single injection it would be possible to achieve the same effect as with multiple doses, above all for drugs with a short half-life. Among these systems, microspheres are being developed to include a broad group of different active substances to be intravitreally administered. In fact drugs such as ganciclovir (Cochereau et al., 2000), retinoic acid (Giordano et al., 1993), adriamicyn (Moritera et al., 1992), fluorouracil (Moritera et al., 1991) and fluorescein (Khoobehi et al., 1991) have been included in microspheres. The advantage of these formulations is their simple administration to the patient as they can be administered by injection. The use of biodegradable polymers for microsphere preparation has the advantage of disappearing gradually from the site of action and reducing the inherent risks. PLGA has been broadly used in microspheres for ocular administration, owing to its biodegradability, biocompatibility and tolerance in humans (Ignatius and Clues, 1996). Aciclovir has proven to be effective in the treatment of intraocular herpes simplex and Epstein-Barr viruses' infections. In previous studies, aciclovir microspheres were prepared by the solvent evaporation method. Their use was mainly intended for the treatment of viral infections due to HSV. Nevertheless, the release of aciclovir from these microspheres was very slow. In order to improve the release of the drug, formulations of microspheres were developed containing different substances (Martínez-Sancho et al., 2003). For this reason, addition of substances such as Vitamin A palmitate that modify the release and, at the same time, are capable of preventing the adverse effects of intravitreal injection could represent a feasible alternative.

The purpose of this work was to obtain a formulation with an adequate long-term release of Vitamin A palmitate (RAP) and aciclovir for the administration of a single intraocular injection as well as to investigate the effect of RAP on the characteristics of biodegradable PLGA microspheres loaded with aciclovir. Different amounts of aciclovir and RAP (in the organic phase) were assayed, and the release rates of both active substances were evaluated. Microspheres were prepared by the solvent evaporation method from an O/W emulsion and their properties and characteristics were evaluated. Aciclovir release profiles of microspheres with and without RAP were compared, and the released RAP was quantified. Injectability of microparticles was performed, as an additional assay, to evaluate their suitability for intravitreal injection without dispersability modifier agents.

2. Materials and methods

2.1. Materials

Aciclovir (acicloguanosine, 9[2-(hydroxyethoxy)methyl]-guanine) was obtained from Reig Farma, S.A. (Spain). Poly(D,L-lactic-*co*-glicolic) acid (PLGA) 50:50, inherent viscosity 0.2 dl g^{-1} (Resomer®RG502), was supplied by Boehringer Ingelheim Chemicals Division (Germany). Polyvinyl alcohol (PVA) MW 72,000 Da was provided by Fluka Chemie AG (Germany) and Vitamin A palmitate (RAP) from Sigma Chemical Co. (Spain).

Table 1
Aciclovir and Vitamin A palmitate quantities added to microsphere formulations

Formulation (aciclovir:Vitamin A palmitate:polymer ratio)	Aciclovir (mg)	Vitamin A palmitate (mg)
1 (1:0.4:10)	40	10
2 (1:1:10)	40	40
3 (1:2:10)	40	80
4 (1.5:2:10)	60	80
5 (2:2:10)	80	80
RAP-free (1:0:10)	40	–

Methylene chloride (CH_2Cl_2), H_2KPO_4 and sodium hydroxide (OHNa) solution, analytical grade, were obtained from Merck (Spain). Distilled and deionised water (Millipore Corporation, USA) was used in preparation of solutions and buffers.

2.2. Microsphere preparation

Microparticle preparation was performed by the solvent evaporation technique from an o/w emulsion (Martínez-Sancho et al., 2003). Briefly, the inner phase was prepared by dissolving 400 mg of polymer in 1 ml CH_2Cl_2 with a vortex mixer (IKA Labortechnik, Germany). Different amounts of aciclovir (40, 60 and 80 mg) and Vitamin A palmitate (10, 40 and 80 mg) were incorporated into the organic phase of the emulsion as shown in Table 1. The aqueous phase consisted of a 0.1% PVA solution. The organic phase containing a suspension of the drug was slowly poured into the aqueous phase and the emulsion was continuously stirred for 3 h at room temperature until complete evaporation of the organic solvent. Then, the microspheres were vacuum-filtered through a 5 μm filter, washed three times with water and lyophilised (Flexy-Dry™, FTS Systems, USA). Microsphere batches were prepared in triplicate and kept in a desiccator until use. All processes were performed with minimum exposure of samples to light to protect them from degradation.

2.3. Loading efficiency

The aciclovir loading efficiency of microspheres was determined by dissolving 10 mg of microspheres in 1 ml of CH_2Cl_2 and extracting aciclovir three times with 9 ml of NaOH solution (10^{-4} M). After centrifugation (Eba 12R, Hettich, Germany) at 6000 × *g* for 5 min and before injection, the supernatant was filtered through a 0.45-μm syringe filter (Tracer, Spain). The drug was quantified by HPLC on the extracted aqueous solutions as described below. The total amount of aciclovir was calculated from the aliquots of each extract.

The Vitamin A palmitate loading efficiency of microspheres was calculated according to Giordano et al. (1993), 10 mg of microspheres was dissolved in 10 ml CH_2Cl_2 and the RAP content of the solution was determined with a spectrophotometer

(DU-6, Beckman, OH) at 330 nm. None of the microsphere components interfered at this wavelength.

2.4. Apparatus and chromatographic conditions

Chromatographic analyses were carried out using a Gilson HPLC instrument (USA), with a 305 solvent delivery pump, a 118 UV–vis detector and 712 system controller software. The injector was equipped with a 20 μl loop 7125 Rheodyne (USA). The separation was achieved by a reversed phase Hypersil ODS column (150 mm × 4.6 mm, 3 μm) directly connected to an ODS guard column, both purchased from Teknokroma (Spain).

The method for determination of aciclovir, described by Boulieu et al. (1997) was modified according to the following conditions: the mobile phase consisted of H_2KPO_4 0.02 mol l^{-1}, pH 3.5, which was premixed, vacuum-filtered through a 0.45 μm Nylon Millipore membrane (Millipore, USA) and degassed by ultrasonication for 15 min before use. The flow rate was 1.5 ml min^{-1}. The detection wavelength was set at 254 nm (detection sensitivity 0.01 aufs). After equilibration (20–30 min), aliquots of samples were injected. The aciclovir retention time was 8.5 min.

2.5. Microsphere characterisation

Scanning electron microscopy (SEM) was performed with a Jeol JSM-6400 (Japan). Samples were dried and gold sputter-coated under vacuum before examination at 20 kV.

Granulometric analysis was developed on a Galai Cis-1 computerised inspection system (Galai Production Ltd., Israel), with laser diffraction optics. The size range measurement was from 0.5 to 150 μm. Microsphere samples were suspended in distilled water and analysed while being gently stirred. Results were expressed as volume–density mean diameter.

2.6. Injectability

Aciclovir-loaded microspheres, approximately 10 mg, were suspended in 1 ml of isotonic saline solution. Assay was ascertained using a 2 ml syringe attached to a 27 G needle. The maximum force needed to inject the microsphere suspension was determined in an Instron 4501 instrument (Instron Corporation, USA).

2.7. Differential scanning calorimetry (DSC)

DSC measurements were carried out with a Mettler 820 DSC analyser (Mettler Toledo, Switzerland). Indium standards were used for system calibration. Samples of microspheres (5–10 mg) in sealed aluminium pans were heated from 25 to 300 °C at a heating rate 10 °C min^{-1} in nitrogen atmosphere (flow rate 40 ml min^{-1}). Scans were recorded only under heating conditions with an empty pan as reference. Data obtained from samples were processed by the system software, allowing the identification of glass transition temperatures (T_g) and crystalline melting points (T_m).

2.8. In vitro release assay

Samples of microspheres (10 mg) were weighed in tubes and filled with 3 ml of isotonic phosphate buffer saline (PBS) pH 7.4 (sink conditions). Tubes were placed in a water shaker bath (NE-5, Clifton, UK) at 37 °C with a constant agitation of 100 strokes min^{-1}. At certain time intervals, the dissolution medium was withdrawn with a syringe, filtered through a 0.45 μm filter and aciclovir concentration was determined by HPLC. The same volume of fresh medium was added to the tube to continue the release study. This release assay was performed in duplicate from each batch of microspheres.

Vitamin A palmitate is practically insoluble in water, so its in vitro release kinetic was carried out studying the remaining amount of RAP in the microspheres (Giordano et al., 1993). For this purpose, several samples of microspheres (10 mg) were suspended in isotonic PBS pH 7.4 (3 ml). At fixed time intervals, the samples were centrifuged, the supernatant was removed and the remaining microspheres were washed three times in distilled water (100 ml) and freeze dried. They were dissolved in 10 ml of CH_2Cl_2 and the RAP concentration was determined spectrophotometrically at 330 nm. Microsphere components did not interfere at this wavelength. This release assay was performed in triplicate from each batch.

2.9. Statistical analysis

The statistical significance of the different parameters (yield of production, loading efficiency and particle size) among different batches of microspheres was tested by an one-way analysis of variance (ANOVA) with the Pairwise Multiple Comparison Procedures (Student–Newman–Keuls Methods) for multiple comparison (Statgraphics Plus 4.0). Differences were considered to be significant at a level of $p < 0.05$. The Statgraphics Plus 4.0 (John Wiley and sons, New York) was also used to estimate the values of parameters of Peppas equation (1985).

3. Results and discussion

The effect of the addition of different amounts of aciclovir and Vitamin A palmitate (RAP) on the surface morphology, particle size, yield of production, loading efficiency, thermal behaviour and in vitro release of microspheres were evaluated.

Table 2 shows the results for the mean yield of production, aciclovir loading efficiency and particle size of microparticles from the microsphere batches.

The influence of incorporation of RAP on the microsphere yields of production was proved in certain cases. The mean yield values ranged from 56.92 ± 1.56% (formulation 5) to 79.57 ± 3.49% (formulation 2). No significant differences were found among formulations 3, 5 and non-loaded RAP microspheres. Formulations 3 and 5 were prepared with the same amount of RAP (80 mg) but different amounts of aciclovir (40 and 80 mg, respectively), so a clear influence of both factors (aciclovir and RAP) cannot be stated on the microsphere yield of production.

Table 2
Yield of production, aciclovir loading efficiency and particle size of microsphere formulations

Formulation (aciclovir:RAP ratio)	Yield of production (%) (mean ± S.D.)	Mean aciclovir loading efficiency ± S.D. (%) (mg/10 mg microspheres)	Mean particle size (μm)
1 (1:0.4)	65.41 ± 0.33	33.32 ± 2.82 (0.303)	29.81 ± 18.00
2 (1:1)	79.57 ± 3.49	36.94 ± 3.97 (0.336)	21.79 ± 12.54
3 (1:2)	56.98 ± 1.35	55.36 ± 1.34 (0.503)	22.45 ± 13.01
4 (1.5:2)	68.89 ± 1.01	39.29 ± 2.43 (0.512)	23.68 ± 15.38
5 (2:2)	56.92 ± 1.56	24.89 ± 1.74 (0.413)	24.85 ± 15.07
RAP-free	57.00 ± 6.85	60.42 ± 8.91 (0.571)	43.82 ± 17.88

Aciclovir loading efficiency values for RAP-loaded formulations were lower than those obtained for non-loaded RAP microspheres (60.42 ± 8.91%), except for formulation 3 (55.36 ± 1.34%). The loading efficiency increased when increasing the amount of RAP and decreased when increasing the aciclovir amount (Table 2). This behaviour would probably be due to the drug included in the inner phase as a suspension, facilitating the addition of RAP the partial incorporation of molecular dispersed aciclovir in the matrix. The higher RAP:aciclovir ratio corresponded to formulation 3. The mean percentage of loaded aciclovir ranged from 24.89 ± 1.74% (formulation 5) to 55.36 ± 1.34% (formulation 3). In the present study, high payloads could result advantageous to minimise the dose of microspheres to be administered because administration in the posterior segment of the eye, where there is a slowdown of the turnover and the biodegradation time of the polymer, has been described as slower than in other areas with a higher enzymatic activity (Herrero-Vanrell and Refojo, 2001).

The microparticle size is a primary determinant of drug release (Berkland et al., 2002; Siepmann et al., 2004) and an important limitation for intravitreal administration. The microsphere size distribution was not significantly influenced by the incorporation of RAP. A non-significant decrease was observed when RAP was added. The mean particle size of non-loaded RAP microspheres was 43.82 ± 17.88 μm, whereas microspheres containing RAP were from 21.79 ± 12.54 μm (formulation 2) to 29.81 ± 18.00 μm (formulation 1). These results suggest that all formulations have a suitable diameter for intraocular administration (Tice and Gilley, 1985).

Microspheres injectability depends on their own properties such as particle size, as well as the syringe, the needle size, the medium used for microparticle suspension and the use of a diluent and its properties. Vitamin A palmitate-loaded microspheres showed an appropriate injectability (≤12N over 10 s) through a 27 G needle when dispersed in saline solution without dispersability modifier agents. This assay showed that microspheres are suitable for clinical use by the intraocular route without surgical incision and minimal adverse effects on the ocular tissues.

Morphologically, SEM revealed a spherical shape with a smooth surface in microspheres without RAP, but with the presence of pores in microspheres containing RAP. Pores could be due to a change in the inner phase of the emulsion as a consequence of incorporation of RAP, which can modify the solvent evaporation rate making it faster and making pores appear. Microspheres were agglomerated tending to stick together owing to a remaining residue of RAP in their surface but redispersion characteristics were not affected (Fig. 1).

In previous studies, samples of polymer, aciclovir, physical mixtures and microspheres were analysed by DSC (Martínez-Sancho et al., 2003). The DSC profiles of PLGA and non-loaded microspheres showed a T_g at 44.83 °C. The trace for aciclovir showed a T_m at 255.33 °C. The thermal behaviour of microspheres was not influenced by addition of RAP (Fig. 2). These

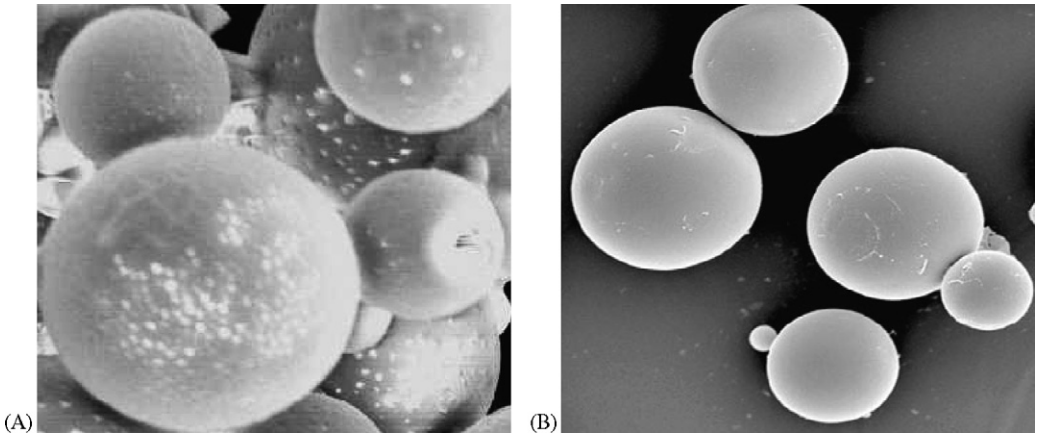

Fig. 1. SEM photographs of: (A) aciclovir-Vitamin A palmitate-loaded microspheres (formulation 3) and (B) aciclovir-loaded microspheres.

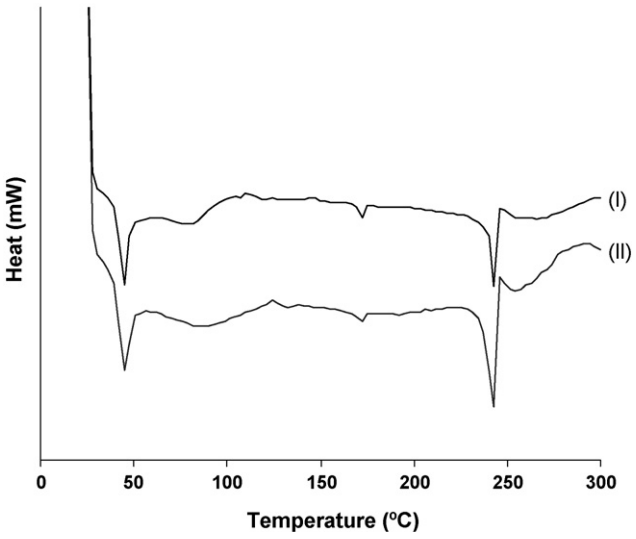

Fig. 2. DSC scan of: (I) aciclovir-loaded microspheres and (II) aciclovir-Vitamin A palmitate-loaded microspheres (formulation 3).

microspheres showed a T_g at 44.72 °C, a broad endotherm in the range 75–125 °C corresponding to a loss of residual water and a narrow T_m at 242.92 °C. The aciclovir T_m probably decreased because of an interaction among the formulation components. A small endothermic peak at 175.02 °C was also observed, attributed to the fusion of a morphous form of aciclovir, which was previously described when different substances were assayed to modify the aciclovir release rate from microspheres.

The aciclovir release profiles from RAP-loaded microspheres were compared to RAP non-loaded microspheres (Fig. 3). In the presence of buffered aqueous media, aciclovir was released from

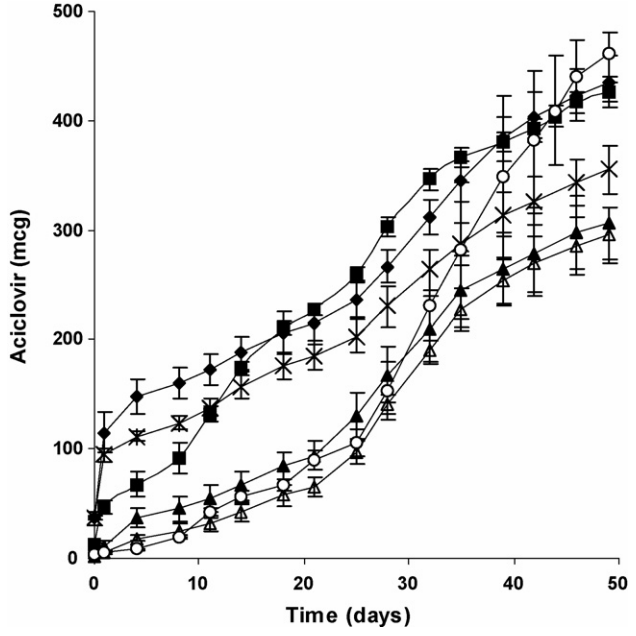

Fig. 3. Aciclovir cumulative release profiles in PBS from microspheres with and without Vitamin A palmitate ((△) formulation 1; (▲) formulation 2; (■) formulation 3; (♦) formulation 4; (✳) formulation 5; (◯) non-loaded Vitamin A palmitate microspheres).

50:50 PLGA microspheres for 49 days. The release kinetic of aciclovir showed a two-phase profile: a slow release (diffusion phase) followed by a faster release (bulk erosion).

The initial burst release of aciclovir was low for formulations 1 and 2, prepared with a lower amount of RAP (5.33 and 11.38 μg, respectively). This value resulted higher when increasing the amount of RAP; 94.57, 47.32 and 115.22 μg (formulations 3, 4 and 5, respectively). The increase in the burst release can be considered as a result of the presence of pores in the microspheres because the pore formation augmented when increasing the amount of RAP. The slow aciclovir release from microspheres without RAP can be explained as follows. Aciclovir is a hydrophobic drug, which can be partially crystallised inside the microspheres. As a result, the drug should dissolve and diffuse to the outer aqueous phase more slowly than as a molecular dispersion, thus the release rate results lower under the crystalline form than as a molecular dispersion. It can also be considered that the crystallised drug can be released by simple dissolution and diffusion (Guyot and Fawaz, 1998).

When RAP was added to the microspheres, the release profiles of the drug during the first days of the assay (1–8th day) were improved compared to RAP-free microspheres, with an important burst effect. For some authors, the addition of lipophilic substances, apart from Vitamin A, avoided the diffusion of drugs through the microspheres (Herrero-Vanrell et al., 2000; Puebla et al., 2005), but others have reported that fatty substances such as isopropyl myristate, significantly increase the release rate of drugs from PLA microspheres (Juni et al., 1985; Wang et al., 1996). In a previous study and in agreement with Sansdrap and Möes (1998), it was observed that isopropyl myristate did not improve the release rate of aciclovir with respect to additive-free microspheres. On the contrary, the release behaviour of Vitamin E and Labrafil® loaded microspheres were similar and both improved the release of aciclovir from days 14 to 32 (Martínez-Sancho et al., 2003).

In this study, the release was only significantly improved in one formulation, not being significantly improved from the 1st to the 20th day for the rest. There was a peak release on day 21 corresponding to the polymer degradation. The best results were obtained for formulation 3, with the highest aciclovir:RAP ratio (1:2). This proportion of drugs would allow a significant change in the matrix composition, increasing its liposolubility and improving the diffusion of aciclovir in the first 20 days. This mechanism cannot be applied from day 20 onwards because beginning on the day, the release of the drug from the microspheres was mainly due to polymer degradation, and not to diffusion. This behaviour would be applied to substances with similar characteristics to aciclovir.

To determine the model that will represent the best fit for this microsphere formulation, data were analysed using the exponential equation for general solute release behaviour from spheres proposed by Peppas (1985). The diffusion exponent value was found to be $n = 0.9$ showing that microspheres released aciclovir for 49 days with a zero-order kinetic and a release constant of 0.85 μg/day/mg microspheres.

Theoretically, the amount of microspheres to be administered in an animal model (rabbits) can be calculated applying the equa-

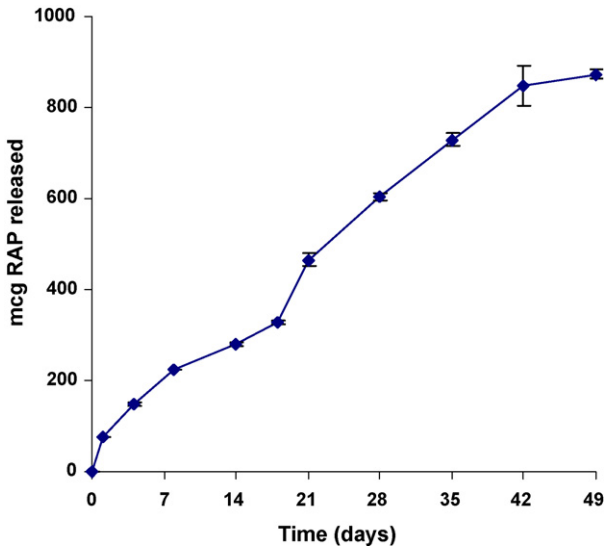

Fig. 4. In vitro release profiles of Vitamin A palmitate from 10 mg PLGA microspheres in PBS.

tion: $K_0 = c(0.693/t_{1/2})V_d$, where V_d is the vitreous distribution volume in rabbits (1.5 ml), $t_{1/2}$ the vitreous elimination half-life of the drug (2.98 h), and c is the in vitro sensitivity of herpes simplex and Epstein-Barr viruses to aciclovir (0.1 μg ml^{-1}) and of varicella zoster virus (1 μg ml^{-1}). It was estimated that 0.98 and 9.8 mg of microspheres, respectively, could deliver an adequate quantity of the drug in a single injection into the vitreous of an animal model, allowing therapeutic levels during at least 49 days.

The selected microspheres showed an adequate release of aciclovir but at the same time, RAP must be released at suitable rate in order to be used in the treatment of viral infections and to prevent inherent risks due to intravitreal injections. The RAP mean loading efficiency for formulation 3 resulted 60.07%. Fig. 4 shows the in vitro release profile of RAP from PLGA microspheres. The release of the drug was nearly constant and after 49 days the RAP release was practically completed. Microsphere formulation 3 showed a minimum daily release rate of RAP up to 0.9 μg/day/mg microspheres. Studies about PLGA microspheres prepared with retinoic acid (RA) (Giordano et al., 1993) had showed that 5 mg of microparticles were capable of preventing PVR and retinal detachment in rabbits. These microspheres, under the same conditions of our study, released approximately 0.46 μg/day/mg microspheres of RA for about 30 days, which corresponded to 0.88 μg RAP/day/mg microspheres. According to this release rate it would be necessary to administer an amount of 4.74 mg microspheres to obtain an antiproliferative effect and to provide protection against retinal detachment. At the same time, these microspheres would release enough of aciclovir to achieve an antiviral effect against herpes simplex and Epstein-Barr viruses.

4. Conclusions

Vitamin A palmitate showed an influence on the microsphere characteristics such as the particle size distribution, the aciclovir loading efficiency and the release pattern during the first days of the assay. The best results were obtained with microspheres prepared with 40 mg of aciclovir, 80 mg of Vitamin A palmitate and 400 mg of polymer. These microspheres provided an in vitro constant release rate within the therapeutic level for 49 days and resulted suitable for intraocular injection according to injectability data. A dose of 4.74 mg of microspheres would be therapeutic for the herpes simplex and Epstein-Barr viruses' treatment in an animal model and would reduce the adverse effects of intravitreal administration.

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