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ScienceDirect
International Journal of Pharmaceutics 338 (2007) 180–190
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

5-Fluorouracil plasma levels and biodegradation of subcutaneously injected drug-loaded microspheres prepared by spray-drying poly(D,L-lactide) and poly(D,L-lactide-co-glycolide) polymers

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Received 25 October 2006; received in revised form 31 January 2007; accepted 1 February 2007
Available online 9 February 2007

Abstract

Microspheres (MS) of 5-fluorouracil-loaded poly(D,L-lactide) (PLA), poly(D,L-lactide-co-glycolide) 75/25 (PLGA 75/25) and poly(D,L-lactide-co-glycolide) 50/50 (PLGA 50/50) prepared by the spray-drying technique were subcutaneously injected in the back of Wistar rats in order to evaluate the 5-fluorouracil (5-FU) release and the biodegradation characteristics. Determination of plasma 5-FU concentration by HPLC with analysis of data using a non-compartmental model showed drug in plasma between 9 and 14 days after administration of drug-loaded PLGA 50/50 or PLA and PLGA 75/25 microspheres, respectively, with a maximum drug concentration of 2.4 ± 0.2 μg/mL at 24 h (5-FU-loaded PLGA 50/50 MS), 2.5 ± 0.1 μg/mL at 48 h (5-FU-loaded PLGA 75/25 MS), and 2.3 ± 0.1 μg/mL at 24 h (5-FU-loaded PLA MS). Pharmacokinetically, a significant increase of AUC (up to 50 times) and MRT (up to 196 times) of 5-FU with regard to the administration of the drug in solution was observed. Scanning electron microscopy and histological studies indicated that a small fibrous capsule was observed around the microspheres in the site of injection. One month after the injection of PLGA 50/50 MS and 2 months after the injection of PLGA 75/25 and PLA MS, masses of polymers, instead of single microspheres, were observed. Close to them, macrophagic cells were present, and blood vessels were observed in the connective tissue. Total absence of fibrous capsule and injected microspheres was observed after 2 (for PLGA 50/50 MS) or 3 (PLGA 75/25 and PLA MS) months.

Keywords: 5-Fluorouracil; Poly(D,L-lactide); Poly(D,L-lactide-co-glycolide); Spray-dryer; Microspheres; Drug release; Biodegradation

1. Introduction

Among chemotherapeutic compounds, 5-fluorouracil is one of the most used for the treatment of colorectal cancer (Moehler et al., 2005; Segura et al., 2006; Goldberg et al., 2006; El-Khoueiry and Lenz, 2006), breast cancer (Yarde et al., 2004; Hutchins et al., 2005; Eniu et al., 2006; Rouëssé et al., 2006), pancreatic cancer (Günzburg et al., 2002; Li et al., 2004) or actinic keratosis (Jorizzo, 2004; Gupta et al., 2005). Since antitumour activity of a drug can be enhanced by improvements in the method of drug administration (Roullin et al., 2004; Larina et al., 2005), numerous studies have been carried out to obtain sustained drug delivery systems. In this type of systems, the drug is protected from degradation following administration, the delivery system can be administered close to the tumoural cells, the drug is released with a specific patron and the action of the drug on tumoural cells can be direct. Thus, lower amount of drug would be necessary to obtain a therapeutic effect, and the side effects due to a systemic administration could decrease. Different drug delivery systems have been prepared for 5-fluorouracil. Thus, biodegradable gelatin microspheres for drug targeting (Muvaffak et al., 2005), bovine serum albumin nanospheres containing 5-FU (Santhi et al., 2002), 5-FU-sustained release biodegradable microspheres of poly(methylidene malonate 2.1.2) for the treatment of brain cancer (Fournier et al., 2004), suspension of poly(D,L-lactide-co-glycolide) 5-FU-loaded microspheres for the treatment of malignant glioma (Menei et al., 2004), 5-FU-loaded

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doi:10.1016/j.ijpharm.2007.02.001

poly(2-hydroxyethyl methacrylate-co-acrylamide) hydrogels for *in vivo* drug administration (Blanco et al., 2000), matrix containing 5-FU spray-coated with Eudragit S100 (Zambito et al., 2005), or poly(butylcyanoacrylate) nanoparticles for topical delivery of 5-fluorouracil (Simeonova et al., 2003) are some of the drug delivery systems prepared for this antitumoural drug.

We have prepared small size microspheres with a significant load of 5-fluorouracil by spray-drying drug dispersions of PLA, PLGA 75/25 and PLGA 50/50 (Blanco et al., 2005). Unloaded microspheres were of small size (average diameter: 0.9 ± 0.4 μm for PLGA 50/50; 1.4 ± 0.8 μm for PLGA 75/25; 1.3 ± 0.7 μm for PLA), and their surface was smooth and slightly porous. Significant differences in size were not observed regarding 5-FU-loaded microspheres (average diameter: 1.1 ± 0.5 μm for 5-FU-PLGA 50/50; 1.7 ± 0.9 μm for 5-FU-PLGA 75/25; 1.5 ± 0.8 μm for 5-FU-PLA). The amount of 5-fluorouracil in drug-loaded microspheres was 47 ± 11 μg 5-FU/mg microspheres for PLGA 50/50, 67 ± 12 μg 5-FU/mg microspheres for PLGA 75/25, and 50 ± 9 μg 5-FU/mg microspheres for PLA. *In vitro* drug release studies were carried out previously (Blanco et al., 2005); total drug release took place *in vitro* in phosphate buffer (1 mM pH 7.4) between 28 h from PLGA 50/50 microspheres and 129 h from PLA microspheres, thus depending on the type of polymer of the microspheres (PLA > PLGA 75/25 > PLGA 50/50). The *in vitro* release characteristics of 5-FU from PLGA 50/50, PLGA 75/25 and PLA microspheres at pH and temperature similar to those of *in vivo* systems provide information on the utility of the systems for drug administration. However, it is necessary to know the behaviour of 5-FU-loaded microspheres when they are implanted in animals to know how the presence of physiological substances, such as enzymes, ions, or cells, influences the drug release and also whether the interaction between the 5-FU-loaded polymeric microspheres and the living tissues is harmless for the organism. The knowledge of pharmacokinetic parameter of 5-FU when it is subcutaneously administered to normal animals using the 5-FU-loaded microspheres would be the previous step to use a tumour model animal to evaluate the effect of 5-FU loaded microspheres on tumour remission.

Thus, in the present study, drug release and biodegradation of 5-fluorouracil-loaded poly(D,L-lactic acid) and poly(D,L-lactide-co-glycolide) microspheres prepared by the spray-dryer technique was studied after subcutaneous injection of the drug delivery systems in the back of rats, in order to assess the utility of these microspheres for 5-fluorouracil administration.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide) (PLA) (Sigma–Aldrich, Barcelona, Spain), Poly(D,L-lactide-co-glycolide) (PLGA) [lactide:glycolide 50:50 and lactide:glycolide 75:25] (Sigma–Aldrich), dichloromethane (Panreac, Barcelona, Spain), chloroform (Panreac), heparin (Laboratorios Leo, Madrid, Spain), ethyl acetate (Panreac), potassium monohydrogen phosphate (K_2HPO_4) (Panreac), potassium dihydrogen phosphate (KH_2PO_4) (Panreac), Tween-80 (Panreac), 5-fluorouracil (Sigma–Aldrich) were used as received. Milli-Q® water (Millipore, Madrid, Spain) was used.

2.2. Preparation of microspheres

Preparation of microspheres was carried out by the spray-drying process (Mini Spray-dryer B-190, Büchi, Switzerland) as it has been previously reported (Blanco et al., 2005). Briefly, to obtain microspheres without drug, poly(D,L-lactide) (PLA) ($M_n = 24100$), poly(D,L-lactide-co-glycolide) 50:50 (PLGA 50/50) ($M_n = 21700$) or poly(D,L-lactide-co-glycolide) 75:25 (PLGA 75/25) ($M_n = 36100$) was dissolved in dichloromethane (2 wt%). Microspheres with 5-fluorouracil were prepared from 1.8 wt% polymer dissolved in dichloromethane, and 0.2 wt% of 5-fluorouracil. The drug powder was dispersed in the polymer solution by stirring (1800 rpm, 10 min) and then ultrasonication for 60 s (Branson Sonifier® 450 Cell Disruptor) to obtain a homogeneous suspension. The polymeric solutions (100 mL) were maintained under constant stirring (900 rpm) and sprayed through the nozzle (0.5 mm diameter) of the spray-dryer. Microspheres were collected from the spray-dryer cyclone separator, and then they were placed in a vacuum oven (Bioblock Scientifics) for 24 h at 100 mBar of pressure and 37 °C. Microspheres were stored in a desiccator under vacuum condition.

The amount of 5-fluorouracil (5-FU) included in the polymeric microspheres was determined by dissolving 5-FU-loaded microspheres in chloroform, then extracting the drug in phosphate buffer (1 mM, pH 7.4), and quantifying 5-FU by UV/V spectroscopy (Blanco et al., 2005).

2.3. 5-Fluorouracil administration routes

Male Wistar rats, weighing 197 ± 10 g, were obtained from the Animalario of the Universidad Complutense de Madrid (Spain) which operates according to the requirements relating to animal experimentation regulations (DC 86/609/CEE; RD 223/1988; OM 13/X/1989, RD 1201/2005). Guidelines contained in NIH publication on the Principles of Laboratory Animal Care 85-23 revised in 1985 were followed throughout. The animals were kept on a 12:12 h light:dark cycle and were fed standard rat food and water *ad libitum*. Different groups of animals (six rats per group) were established. Before the drug release experiments were carried out, a group of animals (six rats) was administered with the vehicle used for microsphere administration, thus 1 mL of saline solution (0.9% NaCl) containing 0.06 wt% of Tween-80 was subcutaneously injected during one minute in the upper part of the back of rats. This solution was harmless, and histological changes in the tissue of the injection place were not observed. The animals administered with the drug were divided into three groups. Group 1: animals injected with 5-FU-loaded microspheres. The microspheres were dispersed in 1 mL of saline solution (0.9% NaCl) containing 0.06 wt% of Tween-80. The animals were anaesthetized with halothane [Burtons, Series 5 T.C.V.] and then the microsphere dispersion was subcutaneously injected during one minute in the upper part of the back of the rat using a sterile

syringe with a 1.2 mm × 40 mm nozzle (Microlance 3). In order to study drug release as well as biodegradation, a similar amount of drug-loaded microspheres was injected in the animals to obtain an adequate dose of 5-FU, and, due to the different 5-FU load of the microspheres (Blanco et al., 2005), that amount of microspheres resulted in two different 5-FU doses (35 mg of 5-FU per kg of body weight and 50 mg of 5-FU per kg of body weight). Group 1A (six rats): 140 mg of PLA microspheres, whose 5-fluorouracil content was 7 mg. Group 1B (six rats): 150 mg of PLGA 75/25 microspheres, whose 5-fluorouracil content was 10 mg. Group 1C (six rats): 150 mg of PLGA 50/50 microspheres, whose 5-fluorouracil content was 7 mg. Group 2: animals subcutaneously injected in the upper part of the back with 1 mL of an aqueous solution of 5-fluorouracil during 1 min. Group 2A (six rats): 7 mg of 5-FU/injection. Group 2B (six rats): 10 mg of 5-FU/injection Group 3: animals intraperitoneally injected with 1 mL of an aqueous solution of 5-fluorouracil during 1 min. Group 3A (six rats): 7 mg of 5-FU/injection. Group 3B (six rats): 10 mg of 5-FU/injection. Furthermore, a group of animals was subcutaneously injected with PLA (140 mg) (six rats), PLGA 75/25 (150 mg) (six rats) or PLGA 50/50 (150 mg) (six rats) microspheres without drug. The microspheres were dispersed in 1 mL of saline solution (0.9% NaCl) containing 0.06 wt% of Tween-80.

2.4. Determination of 5-fluorouracil in plasma

At predetermined times after the injection of 5-FU-loaded microspheres and the 5-FU solutions, animals were anaesthetized with halothane. Blood (1 mL) was collected by puncturing the jugular vein in heparinized (75 units = 15 μL) polypropylene tubes. The heparinized blood was centrifuged at 10,000 × *g* for 10 min in a Sigma 202 M centrifuge immediately after collection so as to obtain plasma. Plasma proteins were precipitated by addition of trichloroacetic acid 2 M (5 μL of TCA per 100 μL of plasma), and after centrifugation (10,000 × *g*, 5 min) plasma samples were then stored at −20 °C.

5-Fluorouracil was extracted from plasma samples by ethyl acetate according to the method proposed by Buckpitt and Boyd (Buckpitt and Boyd, 1980): 100 μL of phosphate buffer (0.5 M, pH 8) and 6 mL of ethyl acetate were added to 500 μL of plasma, after vigorous shaking for 5 min and centrifugation (4000 × *g*, 3 min), the organic phase was collected. This organic phase was evaporated with N_2 at 55 °C, and then the samples were reconstituted with 100 μL of KH_2PO_4 0.01 M, pH 4, and 5-fluorouracil concentration in the sample determined by HPLC (Spectra-Physics SP8800 HPLC pump, SP 100 UV absorbance detector and SP 4400 computing integrator). The stationary phase was Spherisorb ODS2, C_{18}, 5 μ (25 cm × 0.46 cm; Waters). The eluent was KH_2PO_4 0.01 M, pH 4. The flow rate was set at 1 mL/min and the detector wavelength was 266 nm. For calibration, 5-FU standards of 0.1–100 μg/mL in phosphate buffer (1 mM, pH 7.4), as well as drug-free plasma pooled with known amounts of 5-FU, to obtain a 5-FU concentration between 0.01 and 100 μg/mL, were used after undergoing the same procedure. In both cases, 5-FU standards were run for external standardization and linear curves with a correlation coefficient of 0.98 were generated from the area under the peak measurements. The 5-fluorouracil retention time was 10.3 ± 0.2 min.

From plots of plasma 5-FU concentration versus time, a decrease rate of 5-FU plasma concentration was calculated from the slope of the straight line from the time of maximum drug concentration up to the absence of drug in plasma, and the circulating blood volume of rats. For calculating the circulating blood volume of rats, the recommended mean value of 64 mL/kg of body weight (Diehl et al., 2001) was used.

2.5. Pharmacokinetics parameters

Non-compartmental methods can be used to determine certain pharmacokinetic parameters without deciding on a particular compartmental model. The basic calculations are based on the area under the plasma concentration versus times curve (AUC) (zero moment) and the first moment curve (AUMC). The AUC can be calculated by the trapezoidal rule. The AUMC is the area under the concentration times time versus time curve, and it can be also calculated by the trapezoidal rule. From the AUC and AUMC values, the mean residence time (MRT) can be calculated (MRT = AUMC/AUC) (Doménech et al., 1997). Data analysis of the pharmacokinetic parameters was performed by unpaired Student's *t*-test. A value of $p < 0.05$ was considered significant.

2.6. In vivo/in vitro correlation (IVIVC)

In order to establish level B IVIVC (Uppoor, 2001), the mean *in vitro* dissolution time (MDT) was compared to the mean residence time *in vivo* (MRT) (Blanco-Prieto et al., 2004). The MDT, a model-independent *in vitro* parameter that shows the mean time for the drug to release from the microspheres under *in vitro* release conditions, was calculated from the previously published *in vitro* 5-fluorouracil release data (Blanco et al., 2005) according to the equation:

$$\mathrm{MDT} = \frac{\mathrm{ABC}_{in\ vitro}}{M_\infty}$$

where $\mathrm{ABC}_{in\ vitro}$ is the area between the release curve and its asymptote, calculated by the trapezoidal rule from time zero to the last measure time point, and M_∞ is the total amount of released drug at this time point.

2.7. Biodegradation studies

Animals were sacrificed in CO_2 atmosphere 1, 2 and 3 months after the subcutaneous injection of the microspheres. An incision was made on the rat back to remove the tissue around the place of the injection.

The removed tissue was divided in two pieces and one of them was cut in slices, which were dried. These slices were fixed with an adhesive sheet on a rigid support and shadowed with gold. Thus, the morphology of the microspheres was studied by scanning electron microscopy (SEM).

Histological studies were also carried out. A piece of the removed tissue, fixed with formol (10% v/v), was immersed

in paraffin. Cuts (10 μm) were carried out with a paraffin microtome (Minot type). Samples were dyed using the alcian blue hemalum picro-indigo and the hematoxilin-eosin methods (Humason, 1979).

3. Results

3.1. 5-Fluorouracil level in plasma

Unloaded and 5-fluorouracil-loaded PLA, PLGA 75/25 and PLGA 50/50 microspheres have been prepared by spray-drying technology. In previously published studies microsphere size and morphology, and *in vitro* drug release (Blanco et al., 2005) as well as *in vitro* degradation (Blanco et al., 2006) were evaluated. The same batch of microspheres has been used for *in vivo* studies. Thus, the small size of the microspheres (Fig. 1) allowed their subcutaneous implantation in the upper part of the back of rats by injection using a conventional syringe. The total dose of 5-FU administered in this study was 35 mg/kg when 5-FU-loaded PLGA 50/50 and PLA microspheres were administered, and 50 mg/kg for the administration of the drug-loaded PLGA 75/25 microspheres. Blood samples were taken from rats subcutaneously injected with 5-FU-loaded microspheres 8 and 24 h after the injection and at 24-h intervals thereafter. The release of 5-FU from the different types of microspheres gave place to a similar profile of variation of the drug plasma concentration with time (Fig. 2). There were a quick release of 5-FU from the microspheres, and the 5-FU plasma concentration reached a maximum (Table 1) of 2.4 ± 0.5 μg/mL from PLGA 50/50 at 24 h after injection, 2.5 ± 0.8 μg/mL from PLGA 75/25 at 48 h, and 2.3 ± 0.9 μg/mL from PLA at 24 h. Although significant differences were not detected regarding the maximum 5-FU plasma concentration, the decrease rate of the drug plasma concentration and the total time that the drug was detected in plasma depended on the type of injected microsphere. A decrease rate of 5-FU plasma concentration can be calculated from the slope of the straight line from the time of maximum drug concentration up to the absence of drug in plasma, and the circulating blood volume of rats. The circulating blood volume of rats (Diehl et al., 2001) is 58–70 mL/kg of body weight, and the recommended mean value is 64 mL/kg of body weight. Thus, the decrease rate of 5-FU plasma concentration was 2.52 ± 0.39 μg/day and 1.57 ± 0.10 μg/day from PLGA 50/50 and PLA microspheres, respectively. When 5-FU-loaded PLGA 75/25 microspheres were injected, the decrease in plasma drug concentration (Fig. 2B) took place in two stages. The rate of the first stage, between second and seventh day after injection, was 7.85 ± 0.94 μg/day, and the decrease rate of the second stage, between seventh and sixteenth day, was 0.60 ± 0.31 μg/day. The subcutaneous administration of aqueous solutions of 5-FU at 35 and 50 mg/kg doses resulted in a maximum concentration of 18 ± 1 and 37 ± 2 μg/mL, respectively, 30 min after injection with the drug being detected in plasma for 5 h. Administration of aqueous solutions of the drug by intraperitoneal injection resulted in a maximum drug concentration of 16 ± 1 and 30 ± 4 μg/mL, for 35 and 50 mg/kg doses, at 15 min with the drug being detected in plasma for 1.33 h (Fig. 2). The AUC (area under the blood level-time curve) of 5-FU administered from drug-loaded microspheres was between 5 and 11 times greater than that obtained when the drug was administered in aqueous solution by subcutaneous injection (Table 1), and between 14 and 50 times when the drug solution was administered by intraperitoneal injection. The mean residence time (MRT) that can be considered an average time of residence of the drug in the organism, showed a larger value when 5-FU was subcutaneously administered by microspheres, and the largest value was obtained using PLA microspheres. A significant difference was observed with regard to the MRT values for subcutaneous injection of the drug in solution. When the drug was administered in solution, the route of administration had a significant effect, and the MRT of 5-FU increased between 3.5 and 4.3 times when the drug is administered by subcutaneous

Table 1
Pharmacokinetic parameters of 5-fluorouracil (5-FU) after subcutaneous injection of the drug-loaded microspheres, subcutaneous injection of a solution of the drug, and intraperitoneal injection of a solution of the drug

5-FU Administration route (5-FU dose)	C_{max} (μg/mL)	T_{max} (h)	AUC (μg h/mL)	AUMC (μg h^2/mL)	MRT (h)
Subcutaneous injection of 5-FU-loaded PLGA 50/50 microspheres (35 mg/kg)	$2.4 \pm 0.2^{a,b}$	24	$291 \pm 70^{a,b,c}$	$24040 \pm 7101^{a,b,c}$	81 ± 5^{c}
Subcutaneous injection of 5-FU-loaded PLGA 75/25 microspheres (50 mg/kg)	$2.5 \pm 0.1^{d,e}$	48	$372 \pm 100^{d,e}$	$31692 \pm 12758^{d,e,f}$	85 ± 13^{f}
Subcutaneous injection of 5-FU-loaded PLA microspheres (35 mg/kg)	$2.3 \pm 0.1^{a,b}$	24	$503 \pm 124^{a,b}$	$69990 \pm 21421^{a,b}$	137 ± 9
Subcutaneous injection of 5-FU solution (35 mg/kg)	18 ± 1	0.5	45 ± 8^{g}	135 ± 31^{g}	3.00 ± 0.21^{g}
Subcutaneous injection of 5-FU solution (50 mg/kg)	37 ± 1	0.5	72 ± 8^{h}	181 ± 22^{h}	2.51 ± 0.05^{h}
Intraperitoneal injection of 5-FU solution (35 mg/kg)	16 ± 1	0.25	10 ± 3	7 ± 2	0.70 ± 0.05
Intraperitoneal injection of 5-FU solution (50 mg/kg)	30 ± 4	0.25	26 ± 5	19 ± 4	0.71 ± 0.01

[a] Significant difference with regard to subcutaneous injection group (5-FU dose 35 mg/kg), $p < 0.05$.
[b] Significant difference with regard to intraperitoneal injection group (5-FU dose 35 mg/kg), $p < 0.05$.
[c] Significant difference with regard to PLA group, $p < 0.05$.
[d] Significant difference with regard to subcutaneous injection group (5-FU dose 50 mg/kg), $p < 0.05$.
[e] Significant difference with regard to intraperitoneal injection group (5-FU dose 50 mg/kg), $p < 0.05$.
[f] Significant difference with regard to PLA group, $p < 0.05$.
[g] Significant difference with regard to intraperitoneal injection group (5-FU dose 35 mg/kg), $p < 0.05$.
[h] Significant difference with regard to intraperitoneal injection group (5-FU dose 50 mg/kg), $p < 0.05$.

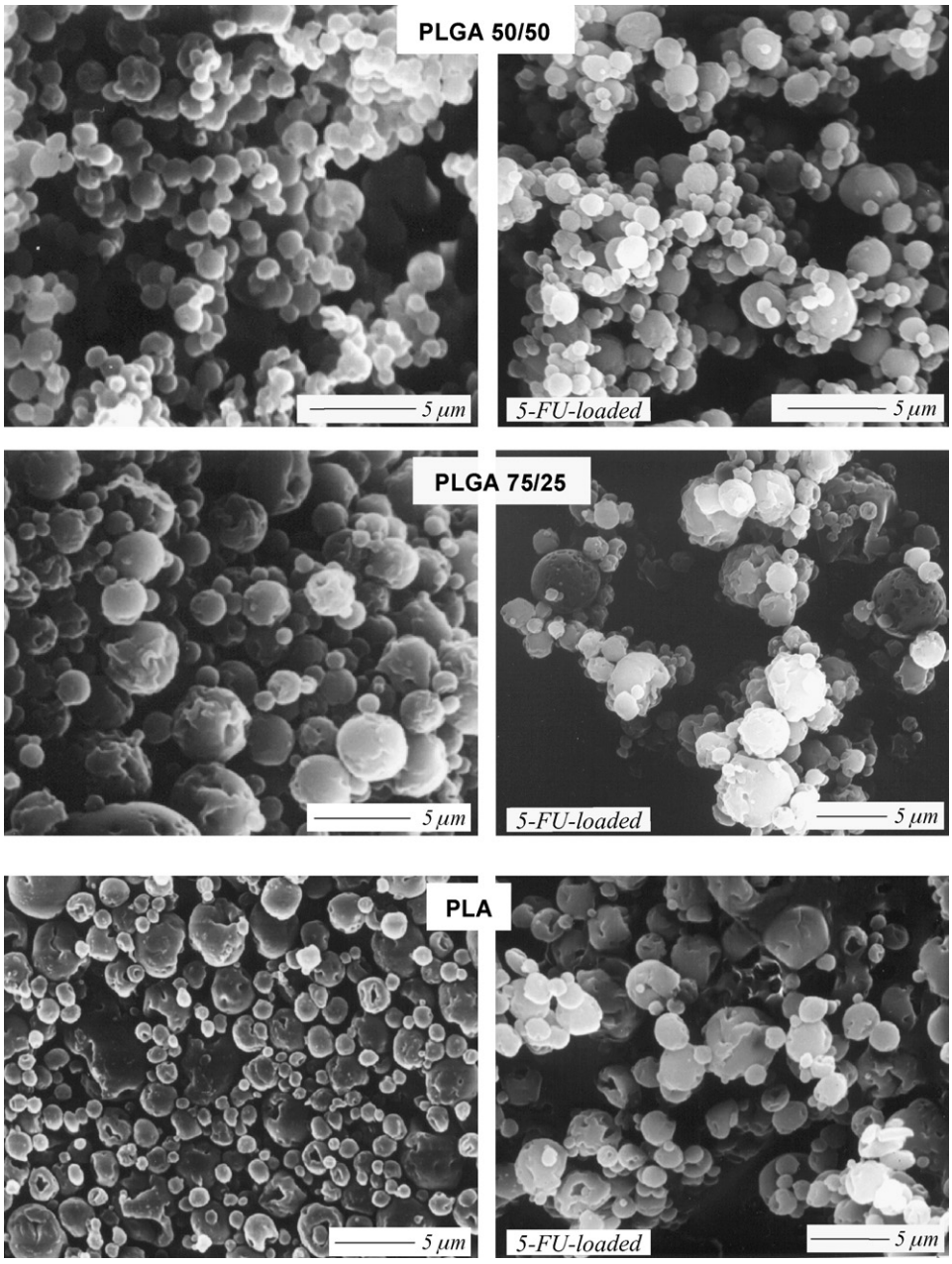

Fig. 1. Scanning electron (SEM) micrographs of unloaded and 5-FU-loaded PLGA 50/50, PLGA 75/25 and PLA microspheres.

injection regarding intraperitoneal injection. However, the effect of the form of 5-FU administration on the MRT value was larger than that of the administration route; thus, the MRT was up to 46 or 196 times higher when the drug was administered by drug-loaded microspheres with respect to the subcutaneous or the intraperitoneal injection of 5-FU solutions.

In order to establish *in vitro*/*in vivo* level B correlation, MDT values were calculated from *in vitro* 5-fluorouracil release from microspheres (Blanco et al., 2005): 106 ± 1 h for 5-FU loaded PLA microspheres; 76.6 ± 0.5 h for 5-FU-loaded PLGA 75/25 microspheres; 24.3 ± 0.1 h for 5-FU-loaded PLGA 50/50 microspheres. A linear relationship for *in vitro* MDT and *in vivo* MRT with a correlation of 0.822 was obtained (MRT = 58.43 + 0.623 MDT).

3.2. Biodegradation studies

To follow the morphological changes of subcutaneously administered PLA, PLGA 75/25 and PLGA 50/50 microspheres, animals were sacrificed 1, 2 and 3 months after the injection, and the tissue of the injection site was removed. Differences between animals injected with 5-FU-loaded microspheres and animals injected with unloaded microspheres were not observed in any case and at any time. In the animals sacrificed 1 month after the unloaded or 5-FU-loaded PLGA50/50 microsphere injection, at the injection site, under the skin, a capsule had been formed around the injected microspheres (Fig. 3). In the histological studies masses of polymers, instead of single microspheres, were observed. It is in accordance with *in vitro* degradation studies

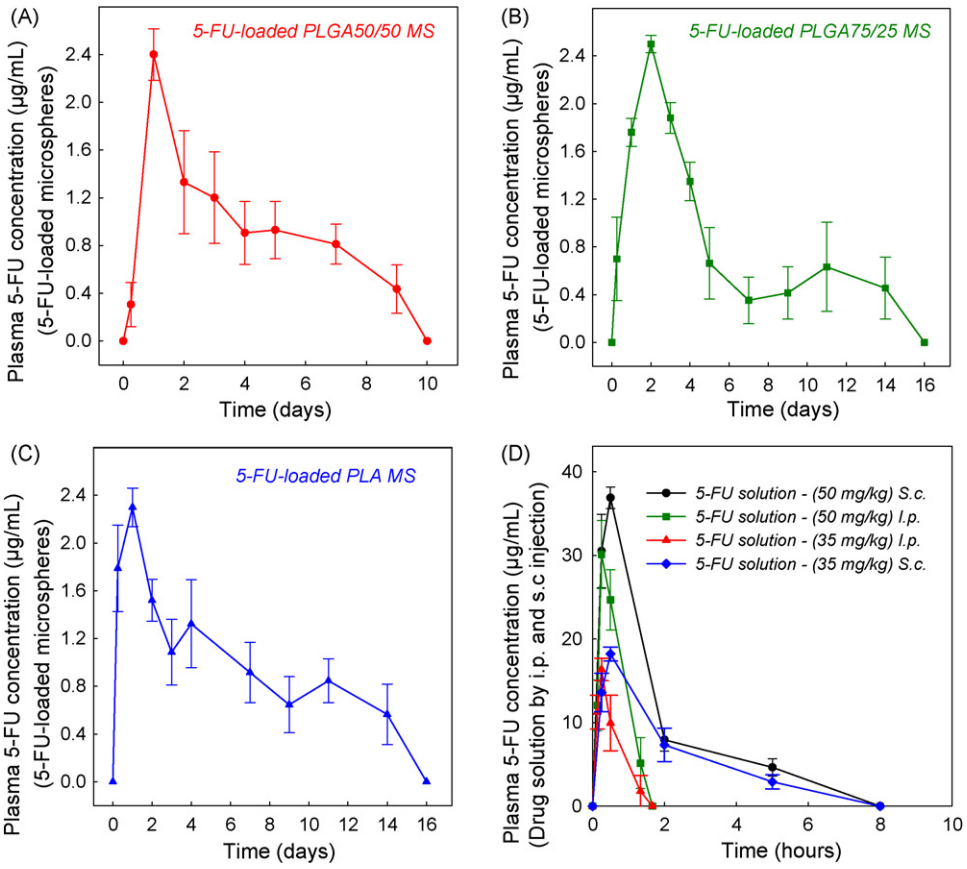

Fig. 2. Plasma concentration of 5-fluorouracil (5-FU) after subcutaneous injection of: 5-FU-loaded PLGA 50/50 microspheres (A); 5-FU-loaded PLGA 75/25 microspheres (B); 5-FU-loaded PLA microspheres (C). Administration of 5-FU solution: dose of 50 mg 5-FU/kg body weight by subcutaneous (●) and intraperitoneal (■) injection, and dose of 35 mg 5-FU/kg body weight by subcutaneous (◆) and intraperitoneal (▲) injection (D).

of these microspheres (Blanco et al., 2006), which indicate the fusion of microspheres to form large particles when mass loss of the polymeric system takes place. Close to these masses of polymers, macrophagic cells were present, and blood vessels were observed in the connective tissue. In the animals sacrificed 2 months after the PLGA 50/50 microsphere injection a fibrous capsule or injected microspheres were not detected. In the case of unloaded and 5-FU-loaded PLGA 75/25 microspheres the degradation sequence was similar but slower (Fig. 4). Thus, 2 months after the microsphere injection polymeric groupings surrounded by connective tissue were observed inside the capsule, and at the surface or interface of the microspheres with the tissue multinucleated foreign body giant cells were present. Three months after unloaded and 5-FU-loaded PLGA 75/25 microsphere injection, total absence of fibrous capsule and injected microspheres was observed. The degradation of unloaded and 5-FU-loaded PLA microspheres (Fig. 5) was equivalent to that above described for PLGA microspheres. Two months after the injection polymeric groupings surrounded by connective tissue were observed. Close to these groups of microspheres multinucleated foreign body giant cells, as well as blood vessels were detected. In this case, also 3 months after the microsphere injection neither fibrous capsule nor microspheres were detected.

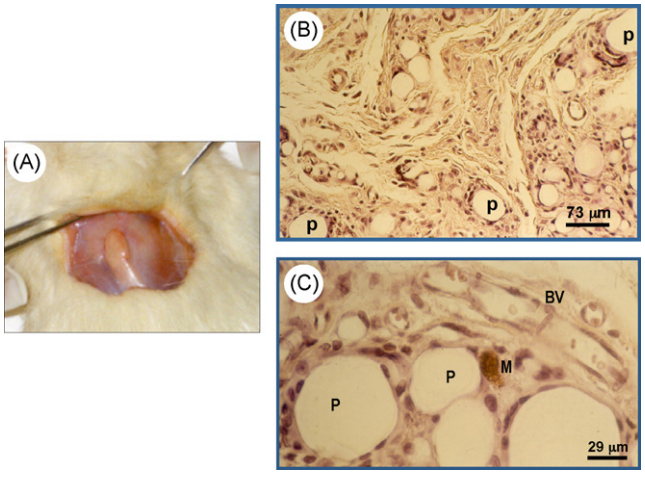

Fig. 3. 5-FU-loaded PLGA 50/50 microspheres after 1 month of the subcutaneous injection in the back of Wistar rats. Capsule around of injected microspheres at the injection site (A). Photomicrograph of polymeric groupings of microspheres and the surrounding tissue (B, C). P: polymeric groupings of microspheres; BV: blood vessel; M: macrophage.

4. Discussion

The design of anticancer drug delivery systems may offer important improvement in cancer therapy by limiting toxic side effects, increasing mean residence time of the drug in plasma and allowing higher specificity in the delivery of the drug to

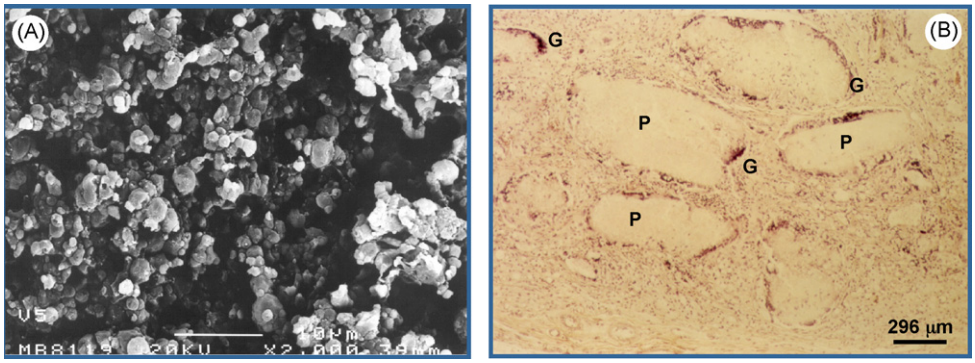

Fig. 4. 5-FU-loaded PLGA 75/25 microspheres after 2 months of subcutaneous injection in the back of Wistar rats. Scanning electron micrograph of a transversal cut of the removed tissue from the injection site (A). Photomicrograph of polymeric groupings of microspheres and the surrounding tissue (B). P: polymeric groupings of microspheres; G: foreign body giant cell.

the tumour target cells. Thus, the drug release as well as the biodegradation behaviour of 5-fluorouracil-loaded PLGA 50/50, PLGA 75/25 and PLA microspheres prepared by spray-drying technology after subcutaneous injection in rats has been studied in order to evaluate the utility of these systems for 5-FU administration.

The maximum tolerated dose of 5-FU in rats is 100 mg/kg of body weight (Spector et al., 1995), and doses of 0.5–80 mg/kg/h for 6 h (Collins, 1985) has been used when 5-FU was administered by perfusion. Furthermore, 20 mg/kg of 5-FU i.p. for 7–10 days has been used in rats for the treatment of colonrectal cancer (Weiber et al., 1994). The total dose of 5-FU administered in this study, 35 mg/kg when 5-FU-loaded PLGA 50/50 and PLA microspheres were administered, and 50 mg/kg for the administration of the drug-loaded PLGA 75/25 microspheres, was in accordance with the above mentioned doses. These two different doses were used as a result of injecting a similar amount of microspheres (140 or 150 mg) of different 5-FU load (47 ± 11 μg 5-FU/mg microspheres for PLGA 50/50, 67 ± 12 μg 5-FU/mg microspheres for PLGA 75/25, and 50 ± 9 μg 5-FU/mg microspheres for PLA) (Blanco et al., 2005).

The values of pharmacokinetic parameters of 5-FU (Table 1) showed the effect of the administration route and the different polymer formulations of the drug. Except when a drug is

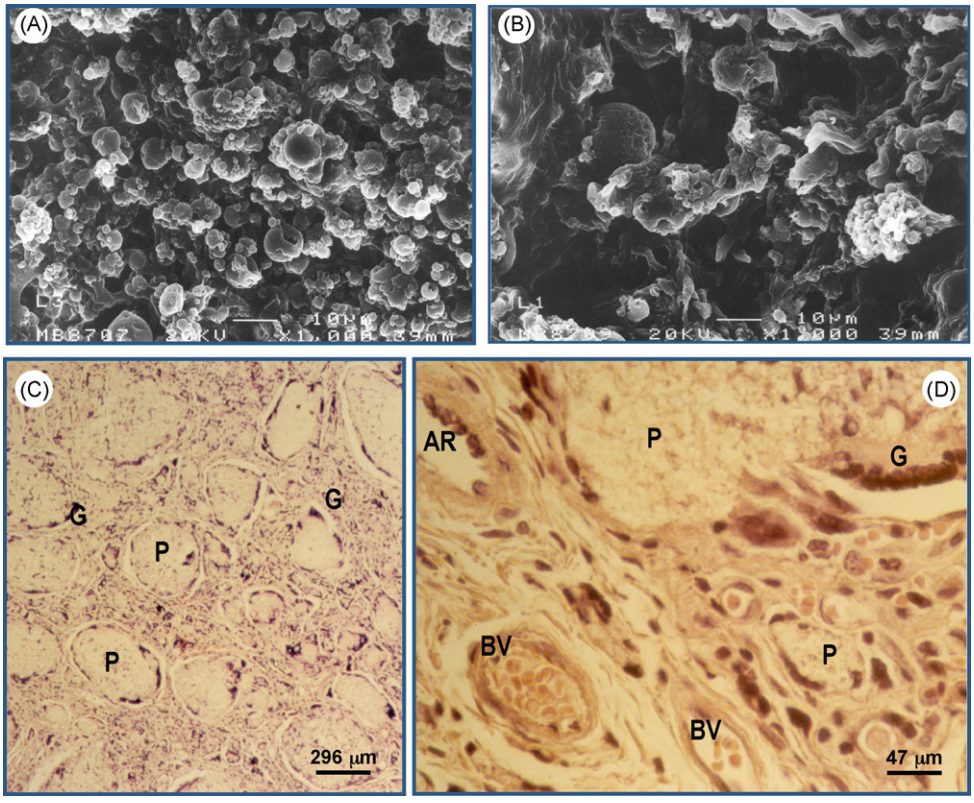

Fig. 5. 5-FU-loaded PLA microspheres after subcutaneous injection in the back of Wistar rats. Scanning electron micrograph of a transversal cut of the removed tissue from the injection site after 1 month (A) and 2 months (B). Photomicrograph of polymeric groupings of microspheres and the surrounding tissue after 2 months of injection (C, D). P: polymeric groupings of microspheres; BV: blood vessel; AR: arteriole; G: foreign body giant cell.

administered intravenously in the form of a solution, the drug has to be released from the dosage form and then be absorbed into the systemic circulation by passing through various membranes (Ritschel and Kearns, 2004). A drug given in different dosage forms or by different routes of administration will yield varying amounts of drug absorbed and, hence, differences in onset, intensity, and duration of the pharmacologic or clinical effect (Ritschel and Kearns, 2004). Thus, the subcutaneous administration of 5-FU solutions originates an increase of AUC and MRT with regard their intraperitoneal administration. Intraperitoneal injection is a parenteral via of drug administration, usually used in animal experimentation due to its very simple use and fast absorption of the drug into the blood, essentially by the portal vein (Benet and Sheiner, 1989) allowing a comparison between plasma levels of a drug and its pharmacokinetic parameters with regard to subcutaneous administration. Pharmacokinetic studies of 5-FU following intravenous administration to rats (Jarugula et al., 1997) at dose of 50 mg/kg showed a maximum drug plasma concentration of 70 μg/mL and a value of AUC of 25.1 ± 8.3 μg h/mL; whereas the AUC was quite similar to that obtained in our study when the drug was administered by intraperitoneal injection, the C_{max} was larger. The difference in maximum 5-FU plasma concentration must be due to the complete and immediate absorption of the drug that takes place when it is intravenously administered.

The polymer composition of 5-FU-loaded microspheres influenced their drug release characteristics, as it was observed *in vitro* studies (Blanco et al., 2005), and it determined not only the maximum plasma 5-FU concentration but the time that the drug was present in plasma and also its decrease rate in plasma. Significant differences in MRT and AUC, pharmacokinetic parameters reflecting the residence time and amount of free 5-FU, respectively, in systemic circulation, between the subcutaneous administration of the drug by 5-FU-loaded microspheres and 5-FU solution, as well as between different types of microspheres subcutaneously administered were observed. The higher MRT values of 5-FU suggested a longer prolonged action when 5-FU is formulated with PLA, PLGA 75/25 or PLGA 50/50. This effect of different PLA/PLGA microsphere formulations on pharmacokinetic parameter of a drug has been observed for vapreodite after the intramuscular administration of drug-loaded microspheres (Blanco-Prieto et al., 2004). A similar effect on AUC and MRT has been observed for buserelin after subcutaneous administration of different buserelin-loaded biodegradable implants (Schliecker et al., 2004). Thus, starting from AUC values, the relative bioavailability (Ritschel and Kearns, 2004) of 5-FU after the administration of the drug-loaded PLA, PLGA 75/25 and PLGA 50/50 microspheres significantly increases. Polymer drug delivery systems exerts a protection on the loaded drug against its degradation or metabolism; thus, the drug absorption from this type of systems after subcutaneous administration depends on the release of the drug from the polymer system, the permeability across the tissues barriers, and the dissolution of the drug under physiological conditions.

From *in vitro* 5-FU release studies (Blanco et al., 2005) and *in vitro* degradation PLA and PLGA microspheres (Blanco et al., 2006) can be deduced that 5-FU release from PLA and PLGA microspheres occurs mainly by dissolution and then diffusion of the drug, although a combination of diffusion and erosion processes also takes place. A level B IVIVC can be established not only when drug dissolution is the rate-limiting step for the *in vivo* ADME (Blanco-Prieto et al., 2004) but also when drug release occurs by a combination of diffusion and erosion (Schliecker et al., 2004). In this study with 5-FU-loaded PLA and PLGA microspheres, a linear relationship has been established between mean *in vitro* dissolution time (MDT) and *in vivo* mean residence time (MRT), with a correlation coefficient of $r = 0.822$. Although the r value cannot be considered optimum to predict MRT values of 5-FU from *in vitro* experiments, a clear relationship exits between both parameters and the largest AUC value of 5-FU when it was released from PLA microspheres corresponds with the longer release observed *in vitro* (Blanco et al., 2005), whereas the lower AUC of the drug is obtained when 5-FU is released from PLGA 50/50, that showed the quickest *in vitro* release.

5-Fluorouracil (5-FU) is one of the most commonly used drugs in the treatment of different malignant tumours but causes numerous secondary effects, and its plasma half-life is very short, about 15 min. When 5-FU is administered to patients with different types of solid tumours (Takimoto et al., 1999) using a dose of 1750 mg/m^2/day (about 45.5 mg/kg/day for a 70 kg body weight and 170 cm height man) for 3 days by intravenous infusion, 5-FU plasma concentration was between 23 and 80 μg/mL. With those 5-FU plasma concentrations toxicity was observed: stomatitis, diarrhoea, hand and foot syndrome, nausea/vomiting. Overall toxicities were mild to moderate with no grade 3 or 4 toxicities. Although real comparisons between human beings and rats cannot be considered, relative information can be obtained. Thus, the maximum 5-FU plasma concentrations obtained when solutions of the drug were administered to rats by subcutaneous or intraperitoneal injections using doses of 35 and 50 mg/kg were close to that obtained in the above mentioned study with patients. The administration of low dose of 5-FU during a protracted venous infusion (300 mg/m^2/day; about 7.8 mg/kg/day for a 70 kg body weight and 170 cm height man) to patients with metastatic tumour of liver (Findlay et al., 1996) caused drug plasma concentrations between 25 ng/mL and 25 μg/mL; plasma 5-FU levels may show greater interpatient variation when given as a protracted venous infusion. The 5-FU plasma levels when the drug was administered to rats using subcutaneously injected 5-FU-loaded PLA and PLGA microspheres were in the above mentioned concentration range.

Furthermore, the presence of these subcutaneously injected unloaded or 5-FU-loaded microspheres was not observed in the injection place between 2 months, for PLGA 50/50 microspheres, or 3 months, for PLGA 75/25 and PLA microspheres, after the injection. In all cases, lymphocytes or mast cells were not observed in the implants, and signs of rejection of the implanted microspheres did not exist. Accumulation of extravessel liquids in the implant area was not detected, which was indicative of that there was not an acute inflammatory response when the animals were sacrificed. The sequence of events following implantation of a biodegradable microsphere drug delivery system had been characterized as occurring in three

phases (Anderson and Shive, 1997). The acute and chronic inflammatory responses take place during the first phase, they are of short duration, 1 or 2 weeks, and the presence of leukocytes, monocytes and lymphocytes is detected. During the second phase, macrophages and foreign body giant cells at the material-tissue interface are observed, and fibrous capsule development is initiated at the onset of the second phase of the tissue response. The normal body reaction to a biocompatible material consists in walling it off in an avascular, collagenous bag, 50-200-μm-thick (Ratner, 2002), as it has been observed after the injection of these unloaded and 5-FU-loaded PLA and PLGA microspheres. However, this biological encapsulation prevents intimate contact between drug delivery systems and tissue, and the inability to accurately predict the thickness of the collagenous capsule around the device can make difficult drug release was accurately controlled (Ratner, 2002). Since the first time the animals were sacrificed was a month after the subcutaneous injection of the microspheres, time at which the presence of the collagenous capsule was observed, it is possible that its development around to microspheres took place when 5-FU was still being released.

When these *in vivo* degradation results are considered along with previously obtained *in vitro* degradation data (Blanco et al., 2006), an outline of the degradative process of the unloaded and 5-FU-loaded PLGA 50/50, PLGA 75/25 and PLA microspheres prepared by the spray-drying process after subcutaneous injection can be proposed. Thus, after the injection of the microspheres with the vehicle, a quick absorption of the vehicle by the surrounded tissue takes place. A fibrous capsule is formed around the microspheres, groups of microspheres are formed, and this makes the degradation of the polymer easier due to the reduced ability of acidic PLGA and PLA oligomers to escape from larger or less porous devices (Grizzi et al., 1995). Breaks of links and decrease of the molecular weight of the polymer have to take place, which will cause a mass loss of the particles, in a similar way to that described *in vitro* degradation (Blanco et al., 2006), in which a significant mass loss began to be observed in PLGA 50/50 and PLGA 75/25 microspheres when the number-average molecular weight of the polymers decrease up to 5400 and 6200, respectively, which took place at day 35 and 100 of incubation. A sharp decrease of glass transition temperature was observed coinciding with the start of mass loss, and this fact was accompanied by a physical change of the samples, fusion of microspheres to form large particles, which also fusion to form a unique mass of polymer; moment from that the degradation process was quicker. Decrease in average-number molecular weight of PLA microspheres was observed during 5 months of incubation *in vitro* in phosphate buffer; however no mass loss was determined. Thus, the quick microsphere degradation after subcutaneous injection might be related to the biological environment. Macrophagic activity can work in two ways. As a consequence of the small size of the microspheres, macrophagic cells can act forming phagolysosomes that contain microspheres (Anderson and Shive, 1997; Torché et al., 1999). These microspheres can be degraded by the hydrolytic action of the acidic conditions inside phagolysosomes. Macrophagic cells also contain degradative enzymes as carboxyesterases. Degradation studies of aliphatic polyesters in the presence of different enzymes had shown that enzymes as proteinase K (Reeve et al., 1994), lipase (Fukuzaki et al., 1989) or carboxylic esterase (Makino et al., 1985) accelerated the hydrolytic degradation of PLA, whereas enzymes as trypsin seemed to be inactive and only plays a second role by enhancing the degradation products of PLGA to be dispersed into the water (Cai et al., 2003). Furthermore, the toxic oxygen products of macrophagic cells (superoxide and hydroxyl radical) are highly reactive and cause extensive, non-specific chemical damage to contents of the phagolysosomes. Other possibility is the release of the lysosomal content to the medium. In this case, the environment close to the macrophages makes acid, which makes the degradation of the microspheres quicker. Comparison of *in vitro* and *in vivo* decrease in molecular weight of PLGA microspheres, prepared by very-low temperature casting using low-molecular weight polymers, has shown that the relative rate of degradation of microspheres *in vivo* is 1.7–2.6 times faster than *in vitro* (Tracy et al., 1999). This faster degradation *in vivo* may be due not only to the presence of macrophages or foreign body giant cells around the microspheres, but also to lipids or other biological compounds present *in vivo* acting as plasticizers favouring the uptake of water into the polymer (Menei et al., 1993).

The efficacy of different antineoplastic drug delivery systems has been evaluated thought tumour growth suppression experiments. The intratumoural administration of cisplatin and adriamycin to tumour bearing mice by implanting under the tumour mass gelatine hydrogels incorporating cisplatin and adriamycin by skin incision (Konishi et al., 2005) showed significant higher antitumour effect on the tumour growth suppression and on survival period than solutions of the drugs, and a synergistic effect was observed when both drugs were simultaneously released. Equivalent results were obtained when paclitaxel was released from chitosan hydrogels subcutaneously injected beneath the tumour subcutaneously induced in mice with lung cancer cells (Obara et al., 2005). In the same way, the intratumoural injection of paclitaxel entrapped in emulsifying wax nanoparticles to mice with a tumour (Koziara et al., 2006), which was induced with a drug resistance human colon adenocarcinoma cell line, caused a significant inhibition in tumour growth, and the enhancement of efficacy of these nanoparticles over Taxol was attributed to the ability of the nanoparticles to overcome multidrug resistance via enhanced delivery as well as an antiangiogenic effect. Therapeutic efficacy of 5-fluorouracil-loaded microspheres have been evaluated in C6-glioma-bearing rats; intratumoural injection of slow releasing type 5-FU-loaded PLGA microspheres after implantation of C6 malignant glioma cells in the brain significantly decreased the mortality of the rats with regard the group injected with 5-FU solution (Menei et al., 1996). Studies of the same research group with the same animal tumour model using 5-FU-loaded poly(methylidene malonate 2.1.2) microspheres (Fournier et al., 2003) have demonstrated the significant improved of the median survival of C6-glioma-bearing rats and also the decreased of the tumour burden. Although the efficacy of 5-FU-loaded PLGA 50/50, PLGA 75/25 and PLA microspheres prepared by the spray-dryer technology must be evaluated in an animal tumour model, the tumour remission studies carried out by different

research groups with anticancer drug-loaded delivery systems joint to the release characteristics of these 5-FU-loaded microspheres after subcutaneous injection in rats make think us that they may be useful in the remission of solid tumour.

In conclusion, these 5-FU-loaded PLGA 50/50, PLGA 75/25 and PLA microspheres prepared by the spray-dryer technology can be subcutaneously injected for sustained drug release. Microspheres are degraded and they are not detected at the injection site between 2 and 3 months. The mean residence time of 5-FU in plasma significantly increases regarding the injection of solutions of the drug, the drug is continuously detected in plasma between 9 and 14 days, and the 5-FU plasma concentrations obtained can be considered in the therapeutic range.

Acknowledgements

This work was founded by grant Proyecto de Investigación Científica y Desarrollo Tecnológico (ref. MAT2003-08179) from Ministerio de Ciencia y Tecnología (Spain) and grant UCM research groups (group 920613) from CAM-UCM (Spain).

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