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Sustained release of a water-soluble GP IIb/IIIa antagonist from copoly(DL-lactic/glycolic)acid microspheres

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Abstract

Sustained release of TAK-029 [4-(4-amidinobenzoylglycyl)-3-methoxycarbonyl-2-oxopiperazine-1-acetic acid], a glycoprotein (GP) IIb/IIIa antagonist, from injectable microspheres was achieved by a W/O/W emulsion solvent evaporation technique using copoly(DL-lactic/glycolic)acid (PLGA). Entrapment of the drug into microspheres increased with addition of sodium chloride into an external aqueous polyvinyl alcohol solution. Addition of L-arginine to an internal water phase dose-dependently reduced initial burst of the drug from the microspheres in vitro and in vivo, probably by forming hydrophobic diffusion barriers with rigid inner structure and increased glass transition temperature (T_g). Microspheres obtained using sodium chloride and L-arginine demonstrated sustained plasma levels of TAK-029 for 3 weeks after subcutaneous injection in rats, while causing a slight increase of its plasma levels in 2–3 weeks. © 1997 Elsevier Science B.V.

Keywords: GP IIb/IIIa antagonist; L-Arginine; Microspheres; PLGA; Sustained release

1. Introduction

Parenteral dosage forms for sustained release are useful to improve the efficacy of many drugs by reducing the frequency of injections and by decreasing plasma level fluctuations. Injectable microspheres prepared by using biodegradable polymers, polylactic acid (PLA) and copoly(DL-lactic/glycolic)acid (PLGA) have been widely used for controlled release of many drugs including peptides and proteins (Sanders et al., 1984; Kwong et al., 1986; Cohen et al., 1991). Among several microencapsulation techniques, a solvent evaporation technique is generally limited to the entrapment of lipophilic drugs (Fong et al., 1986;

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Bodmeier and McGinity, 1987; Jalil and Nixon, 1990), but on the other hand concerning watesoluble basic drugs, there are some papers describing successful preparation of injectable microspheres for sustained release of the drugs (Ogawa et al., 1988; Okada et al., 1988; Heya et al., 1991). Efficient entrapment of these basic drugs was probably due to the ionic interaction between the drugs and PLGA (Okada et al., 1994b). For acidic or neutral drugs with high water-solubility, however, it is difficult in solvent evaporation techniques using a W/O/W emulsion to achieve high entrapment and small initial burst of the drugs, because of drug leak to an external water phase during emulsifying process.

TAK-029 [4-(4-amidinobenzoylglycyl)-3-methoxycarbonyl-2-oxopiperazine-1-acetic acid] is a highly potent antagonist to fibrinogen receptor GP IIb/IIIa (Kawamura et al., 1996), which might be used as an anti-platelet drug for treatment of various cardiovascular and cerebrovascular thromboembolic disorders such as unstable angina, myocardial infarction, transient ischemic attack (TIA), stroke, and atheroscrelosis (Peerlinck et al., 1993; The EPIC Investigators, 1994). These antagonists, however, often cause prolongation of bleeding time at high doses, and a well-controlled release system is needed for clinical application of the drugs. The pharmacological effect—inhibition of ADP-induced platelet aggregation—is expected in plasma levels above 20 ng/ml, but higher plasma levels above 100 ng/ml may significandy cause prolongation of bleeding time. TAK-029 is of high water-solubility of more than 200 mg/ml, and behaves as a zwitterion at neutral pHs with an amidino residue and a carboxylic acid group. We considered that PLGA microspheres are one of the most desirable dosage forms for the drug to maintain the therapeutic range for more than 2 weeks following a single injection. The present paper describes the preparation of TAK-029-containing microspheres by a solvent evaporation technique using a W/O/W emulsion and the characterization of the microspheres in vitro and in vivo.

2. Materials and methods

2.1. Materials and animals

TAK-029 was synthesized in Production Research Laboratories of our company. Copoly(DL-lactic/glycolic)acid (lactic/glycolic = 75/25 mol%, weight average molecular weight = 9000) (PLGA) obtained from Wako Pure Chemicals (Osaka, Japan) was used for preparation of microspheres throughout the present study. L-Arginine and sodium chloride were also supplied by Wako (Osaka, Japan). Polyvinyl alcohol (Gosenol EG-40, molecular weight 79 000) was commercially obtained from Nihon Synthetic Chemical Ind. (Osaka, Japan). Other chemicals were of reagent grade. Male Sprague-Dawley rats (8 weeks of age) were purchased from Clea Japan (Tokyo, Japan).

2.2. Preparation of PLGA microspheres by a W/O/W emulsion solvent evaporation technique

Microspheres were prepared by a W/O/W emulsion solvent evaporation technique using PLGA as described previously (Ogawa et al., 1988). In brief, a weighed amount of TAK-029 (0.15 or 0.45 g) was dissolved in water (0.75 or 2.25 ml) with or without L-arginine (0.045–0.135 g), and a weighed amount of PLGA (4.35 or 4.05 g) was dissolved in 4 ml of dichloromethane. Both solutions were mixed and vigorously homogenized with a Polytron (Kinematica, Luzern, Switzerland) for 1 min. The W/O emulsion, after being cooled to 15°C, was poured through a thin nozzle into 800 ml of a 0.1% polyvinyl alcohol aqueous solution with or without sodium chloride (0.9–2.7%) under stirring with an Autohomomixer (Tokushu Kika Kogyo, Osaka, Japan), and the resulting mixture was stirred for a few minutes to make a W/O/W emulsion. To evaporate dichloromethane, the W/O/W emulsion was further stirred gently with a propeller mixer for 3 h at 800 rpm. After removing coarse particles larger than 125 μm by sieving, the resulting microspheres were collected by centrifugation for 5 min at 2000 rpm, rinsed with water three times and then freeze-dried.

2.3. Determination of TAK-029 by a high performance liquid chromatography (HPLC)

TAK-029 was determined by HPLC using a reversed phase column (μ-Bondasphere C_{18}; 3.9 mm i.d. × 150 mm) and a 1:9 mixture of methanol and 0.01 M ammonium formate (pH 4.5) as mobile phase. The flow rate was 1.0 ml/min and the eluate was monitored at 237 nm by ultraviolet detection

2.4. Extraction of TAK-029 from microspheres

Microspheres (20 mg) were dissolved in 10 ml of dichloromethane and TAK-029 was extracted into 15 ml of 0.01 M ammonium formate buffer (pH 4.5). TAK-029 in the aqueous layer was determined by the HPLC procedure described above.

2.5. In vitro release study

Microspheres (20 mg) were suspended in 10 ml of phosphate buffered saline (PBS) (pH 7.4) containing 0.02% Tween 80 (Kao Atlas, Tokyo, Japan) (PBS-T), and stirred continuously at 37°C with a horizontally reciprocating shaker (Taiyo Scientific, Tokyo, Japan). The PBS-T solution was periodically taken out and determined for released TAK-029 by the HPLC procedure after filtrating through a Millipore 0.45 μm-membrane.

2.6. Determination of plasma levels of TAK-029 by an enzyme-linked immunosorbent assay (ELISA)

Plasma specimens containing TAK-029 were mixed with one-fourth volume of 10% trichloro acetic acid (TCA) solution and kept in ice for 30 min. The mixture was centrifuged for 10 min at 12 000 rpm and the supernatant was diluted ten folds with PBS containing 0.2% bovine serum albumin (BSA). Reference plasma specimens were prepared by adding TAK-029 to normal plasma and treated in the same way. Sample solutions thus obtained were mixed with an equal volume of biotinylated TAK-029 solution (20 pg/ml), and 100 μl portions of the mixed solutions were applied on the microtiter plate coated with rabbit anti-TAK-029 antibody. The plate was incubated for 2 h at room temperature. After washing three times with PBS containing 0.01% Tween 20 (PBS-T20), 100 μl of horse-radish peroxidase (HRPO)-avidin D conjugate solution (Vector Labs, Burlingame, CA) was added to the plate, followed by incubation for 2 h at room temperature. After additional washing with PBS-T20, the plate was developed with 100 μl of 3,3′,5,5′-Tetramethyl Benzidine dihydrochloride (TMBLUE) (Intergen-CDP, Milford, MA) for 5 min at room temperature. After the reaction was stopped by addition of 100 μl of 1N-sulfuric acid, the absorbance at 450 nm was measured using a Titertek Multiskan (Flow Labs, McLean, VA).

2.7. Plasma levels of TAK-029 after subcutaneous injection of microspheres to rats

The microsphere-suspending vehicle consisted of 5% mannitol, 0.5% carboxymethyl cellulose and 0.2% Tween 80 in an aqueous solution. The PLGA microspheres containing TAK-029 were injected subcutaneously into the back of male rats at a dose of 20 mg/kg as TAK-029. Plasma was serially collected from the tail vein and its TAK-029 levels were determined in duplicate by the ELISA described above.

2.8. Characterization of microspheres

The particle size distribution of microspheres was determined using a Coulter Multisizer (Coulter Electronics, England). The shape, surface and cross-section of the microspheres were observed using a scanning electron microscope (Model S-2300, Hitachi, Tokyo, Japan).

2.9. Arginine content in microspheres

Arginine content in microspheres was determined using an amino acid analyzer (L8500A, Hitachi, Tokyo) after alkaline hydrolysis. In brief, 20 mg of microspheres were dissolved in the mixture of acetonitrile (2 ml) and distilled water (1 ml). Then, a 3 ml portion of ethanol containing KOH (0.5N KOH-EtOH) was added to the solu-

tion and incubated for 20 h at 25°C to hydrolyse the polymer. After evaporation to dryness under nitrogen gas, the residue was dissolved in 3 ml of 0.5M HCl to neutralize the sample, then diluted with 0.01 M HCl to adjust the arginine concentration to approximately 20 nmol/ml for amino acid analysis.

2.10. Determination of glass transition temperature (T_g)

Glass transition temperature (T_g) was determined by thermal analysis using a Perkin Elmer DSC7 (Differential Scanning Calorimeter). Samples were heated to 100°C at an ascending rate of 20°C/min and then cooled to −10°C at a descending rate of 200°C/min, followed by heating to 100°C at an ascending rate of 10°C/min again. The midpoint and onset point were determined using the microcomputer software supplied by Perkin Elmer. The midpoint was used as the T_g.

2.11. Remaining TAK-029 and PLGA in microspheres after subcutaneous injection to rats

The PLGA microspheres containing TAK-029 were injected subcutaneously into the back of male rats at a dose of 20 mg/kg as TAK-029. The remaining microspheres were taken out from the injection site and homogenized with a Polytron in 15 ml of 0.01 M ammonium formate buffer (pH 4.5) and then extracted with 15 ml of dichloromethane. TAK-029 in the aqueous layer was determined by the HPLC procedure described above.

Molecular weight of PLGA in dichloromethane was determined by a gel permeation chromatography (GPC) procedure described below.

2.12. Determination of the weight-average molecular weight of PLGA by a gel permeation chromatography

After the extraction of the remaining microspheres as described above, 5 ml of the dichloromethane layer was removed and evaporated to dryness under nitrogen gas, and the residue was dissolved in 1 ml of chloroform as a sample solution for GPC. The molecular weight of PLGA was determined under the following conditions: column, two series of Shodex KF 804L (8 mm i.d. × 300 mm, Showa Denko KK, Japan); column temperature, 50°C; mobile phase, chloroform; flow rate, 1 ml/min; detector, refractive index (Shodex RI SE-31). The GPC system was calibrated using polystyrene reference standards (Showa Denko KK). The weight-average molecular weight was calculated using the microcomputer software (Maxima 820) developed by Millipore Japan. The weight of remaining PLGA was calculated from peak area of the GPC chromatogram.

3. Results

3.1. Effect of sodium chloride on drug entrapment and in vitro initial burst

PLGA microspheres containing TAK-029 were prepared by a solvent evaporation technique through a W/O/W emulsion with different concentrations of sodium chloride in an external aqueous phase. Effect of sodium chloride on drug entrapment into microspheres and in vitro initial burst, the released drug percentage from microspheres during the first day, was studied at a loading amount of 10% drug. Addition of sodium chloride into the external aqueous phase remarkably increased the drug entrapment, and slightly reduced the in vitro initial burst, with increasing amounts of sodium chloride (Fig. 1).

3.2. Effect of L-arginine on drug entrapment and in vitro initial burst

Effect of L-arginine concentration in an internal water phase was also investigated at 2.7% sodium chloride in an external aqueous phase and a loading amount of 10% TAK-029. In vitro initial burst was remarkably reduced with increasing amounts of L-arginine and it was less than 10% of the initial TAK-029 content at 4% L-arginine (Fig. 2). But the drug entrapment was lowered at this 4% of L-arginine.

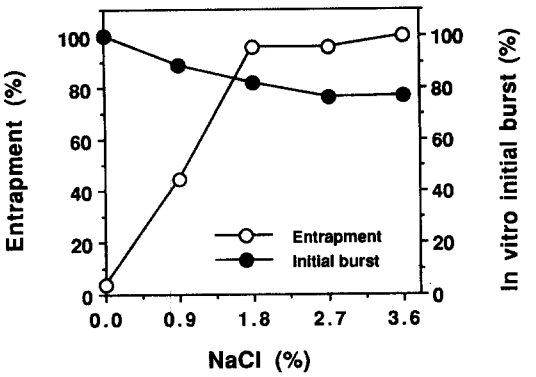

Fig. 1. Effect of sodium chloride concentration in external aqueous phase on drug entrapment and in vitro initial burst. Loading amount of TAK-029 was 10%.

3.3. Plasma levels of TAK-029 after subcutaneous injection of microspheres to rats

After subcutaneous injection of microspheres containing 0, 1 and 3% L-arginine with a loading amount of 10% TAK-029 in rats, plasma levels of TAK-029 were measured periodically for 4 weeks (Fig. 3A). The plasma levels 1 h after injection of microspheres with 1 and 3% L-arginine were 4090 ng/ml and 4013 ng/ml, which were 60% and 59% of that of microspheres without L-arginine (6797 ng/ml), respectively. Longer sustained plasma levels of TAK-029 were observed at higher concentrations of L-arginine in 2–4 weeks after injection. At a loading amount of 3.3% TAK-029, arginine also dose-dependently reduced initial burst of the drug from microspheres after subcutaneous injection in rats, while causing a slight but significant increase of plasma levels in 2–3 weeks (Fig. 3B). The plasma levels were 1479 and 1122 ng/ml 1 h after injection of microspheres containing 1 and 3% arginine, which were 28 and 22% of that (5195 ng/ml) of microspheres without arginine, respectively. Those initial bursts were lower than those of microspheres at a loading amount of 10% TAK-029.

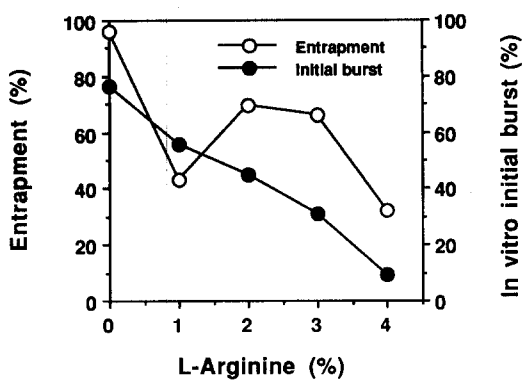

Fig. 2. Effect of L-arginine on drug entrapment and in vitro initial burst. Loading amount of TAK-029 was 10%. Sodium chloride concentration in external aqueous phase was 2.7%.

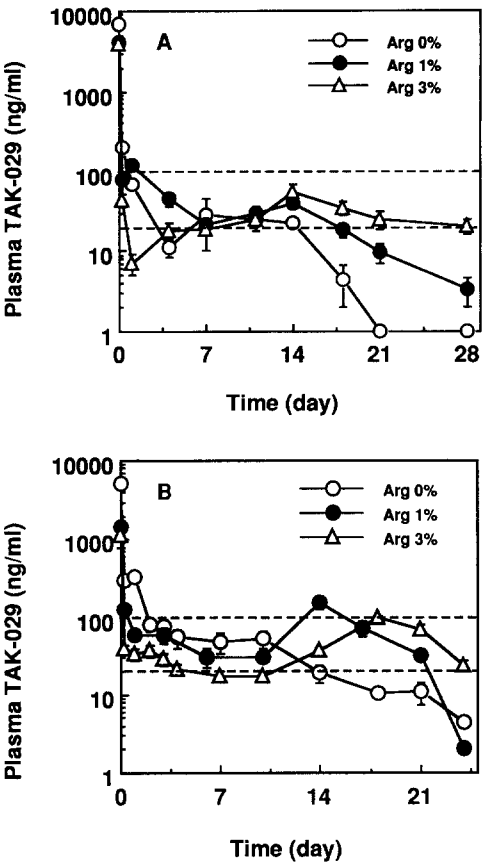

Fig. 3. Effect of L-arginine on plasma level profiles of TAK-029 after subcutaneous injection of PLGA microspheres to rats. Dose = 20 mg/kg. Data are shown as mean ± S.E. of four animals. Dotted lines represent the therapeutic range of plasma TAK-029 between 20 and 100 ng/ml. Loading amount of TAK-029 was 10% (A) and 3.3% (B).

3.4. Particle size distributions

Typical particle size distributions of microspheres with 0, 1 and 3% L-arginine are shown in Fig. 4. They ranged from 4 to 120 μm and the mean particle sizes by volume were 37, 33 and 27 μm, respectively.

3.5. Observation by scanning electron microscopy

3.5.1. Surface structure of microspheres

Microspheres with 0, 1 and 3% L-arginine all showed spherical shape and smooth surface without visible pores. There was no or little apparent difference among three types of microspheres (Fig. 5).

3.5.2. Inner structure of microspheres

Microspheres prepared at loading amount of 3.3% and 10% drug were also investigated for cross-sectional view by scanning electron microscopy (Fig. 6). Microspheres without L-arginine showed porous inner structure, but those became less porous with increasing amounts of L-arginine. Especially micropsheres obtained at 3.3% loading amount demonstrated little pore with rigid inner structure, while those at 10% loading amount gave large cracking.

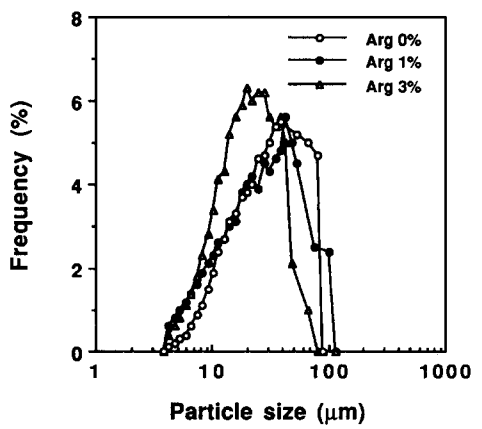

Fig. 4. Particle size distributions by volume of PLGA microspheres containing TAK-029 with 0, 1 and 3% L-arginine. Loading amount of TAK-029 was 3.3%.

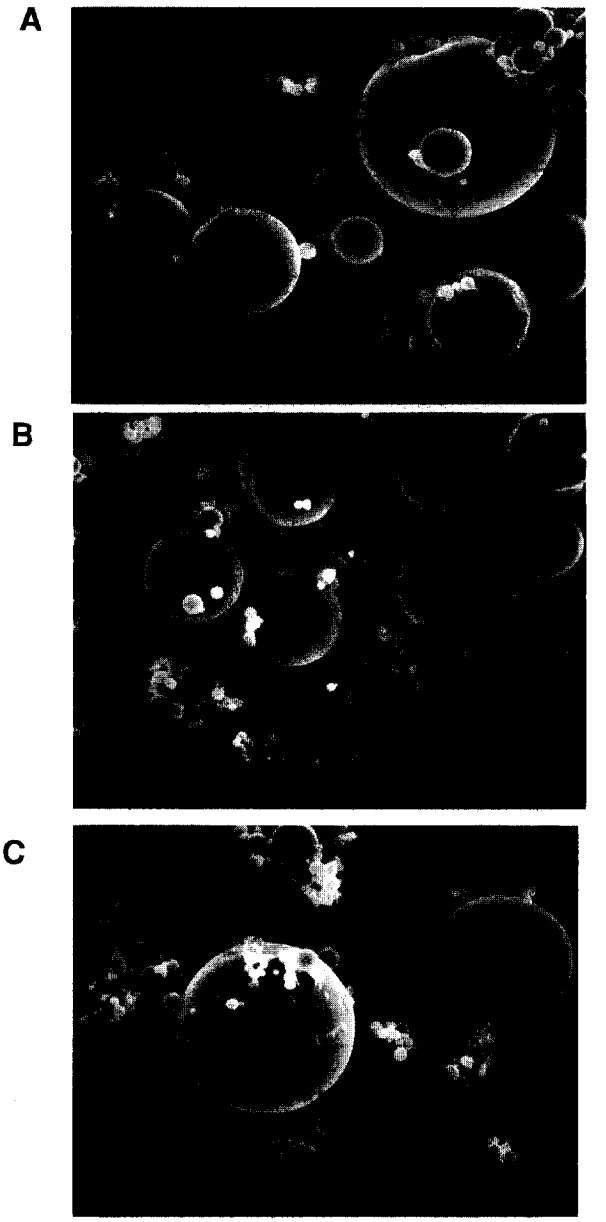

Fig. 5. Scanning electron micrographs of PLGA microspheres containing TAK-029 with 0% (A), 1% (B) and 3% (C) L-arginine. Loading amount of TAK-029 was 3.3%.

3.6. In vitro release profiles

At a loading amount of 3.3% TAK-029, in vitro

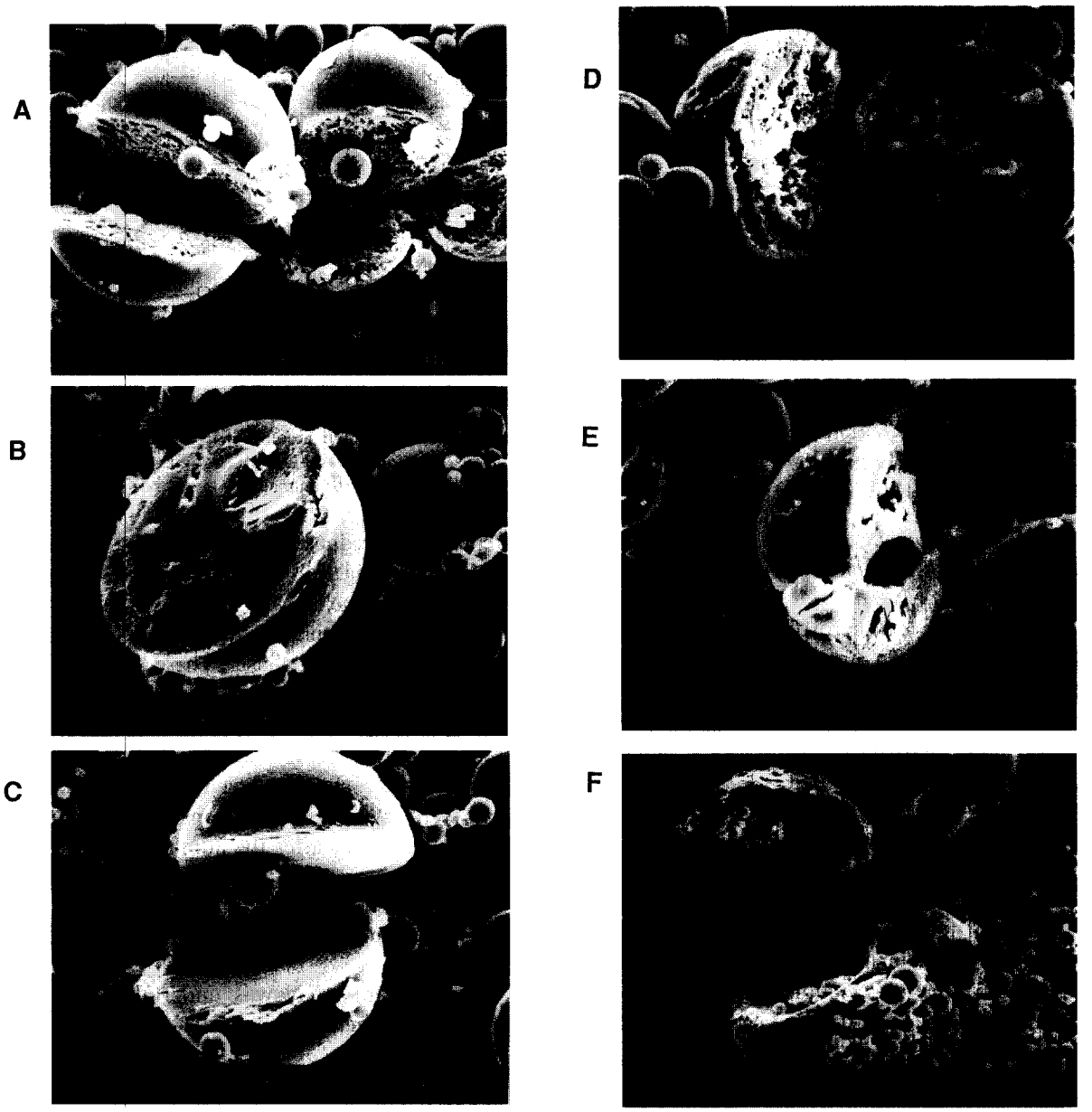

Fig. 6. Cross-sectional scanning electron micrographs of PLGA microspheres with 0% (A, D), 1% (B, E) and 3% (C, F) L-arginine. Loading amount of TAK-029 was 3.3% (A–C) and 10% (D–F).

initial burst of TAK-029 one day after suspending the microspheres in PBS-T was reduced from 64 to 23% by addition of 3% L-arginine (Fig. 7). Release rates between day 1 and 28 were almost the same, but the microspheres with 3% L-arginine released 37% of the initial content between day 28 and 35, while the ones without L-arginine released only 4%.

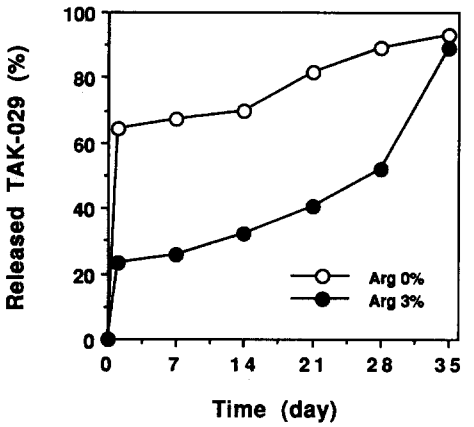

Fig. 7. Effect of L-arginine on the in vitro release profiles of TAK-029 from PLGA microspheres with a loading amount of 3.3% TAK-029. Data are shown as mean ± S.E. of three experiments.

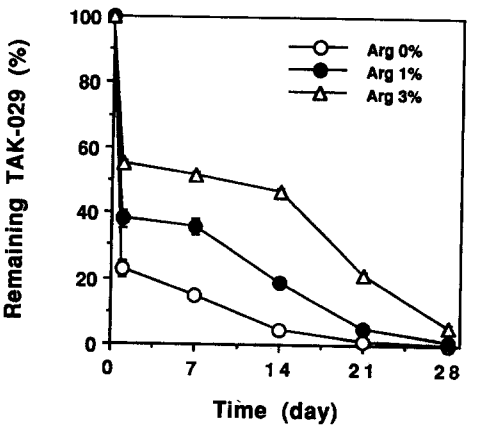

Fig. 8. Effect of L-arginine on remaining TAK-029 in microspheres with a loading amount of 10% TAK-029 after subcutaneous injection to rats. Dose = 20 mg/kg. Data are shown as mean ± S.E. of three animals.

3.7. Arginine content and glass transition temperature (T_g) of microspheres

Increased T_g of microspheres was obtained at higher contents of L-arginine, regardless of TAK-029 contents, accompanied with lower in vitro initial burst (Table 1). Addition of 3% L-arginine resulted in microspheres with 43.8°C of T_g and 31% of in vitro initial burst.

3.8. Effect of L-arginine on in vivo remaining of TAK-029 and PLGA in microspheres

Effect of L-arginine on disappearance of TAK-029 and PLGA in microspheres was investigated after subcutaneous injection to rats. At a loading amount of 10% TAK-029, in vivo initial burst was remarkably reduced with increasing amounts of L-arginine (Fig. 8). The microspheres containing 3% of L-arginine showed a triphasic release profile with initial burst phase, slow release (latent) phase and faster release phase. These release profiles corresponded well to the plasma level profiles shown in Fig. 3A. As for degradation of PLGA, reduction rate of the weight average molecular weight and remaining weight of the polymer in rats were retarded by additions of L-arginine especially 1–3 weeks after the administration (Fig. 9).

4. Discussion

The present study was undertaken to achieve high entrapment of a water-soluble non-basic

Table 1
Glass transition temperature (T_g) and L-arginine content of PLGA microspheres

TAK-029 (%)		L-Arginine (%)		T_g (°C)	In vitro initial burst (%)
Loading	Content	Loading	Content		
10	9.2	0	0	39.0	77
10	3.9	1	0.38	40.9	54
10	7.0	2	1.6	42.5	45
10	6.6	3	2.3	43.8	31

T_g of PLGA was 35.2°C.

drug, TAK-029 in PLGA microspheres and small initial burst of the drug from microspheres. It is generally difficult to achieve an efficient entrapment of water-soluble non-basic drugs into injectable PLGA microspheres with small initial burst by a solvent evaporation technique. During W/O/W emulsifying process in a solvent evaporation technique, dichloromethane diffuses from W/O droplets to external aqueous phase and simultaneously water in the external phase back-diffuses into the droplets, and finally PLGA precipitates to form microspheres. This water back-diffusion is supposed to increase in proportion to the difference in osmolarity between internal and external water phase, and more water back-diffusion would result in more leakage of the drug into external phase. Actually at a loading amount of 10% TAK-029, loss of the drug into external water phase was 71% without NaCl, and entrapment of TAK-029 in the resultant microspheres was 4%, respectively. The residual drug might be lost during washing procedures. The entrapment of the drug into microspheres increased with addition of sodium chloride into the external aqueous polyvinyl alcohol solution (Fig. 1), as previously reported (Herrmann et al., 1995; Uchida et al., 1996). TAK-029 was entrapped with efficiency of more than 90% by higher concentrations of NaCl than 1.8%. A W/O/W emulsion solvent evaporation technique gave microspheres with porous inner structure at a loading amount of 10% TAK-029, resulting in large initial burst (Fig. 6D–F). It was probably due to interconnecting aqueous pores formed in polymer matrix after the evaporation of water derived from the internal water phase to dissolve TAK-029 during the freeze drying process.

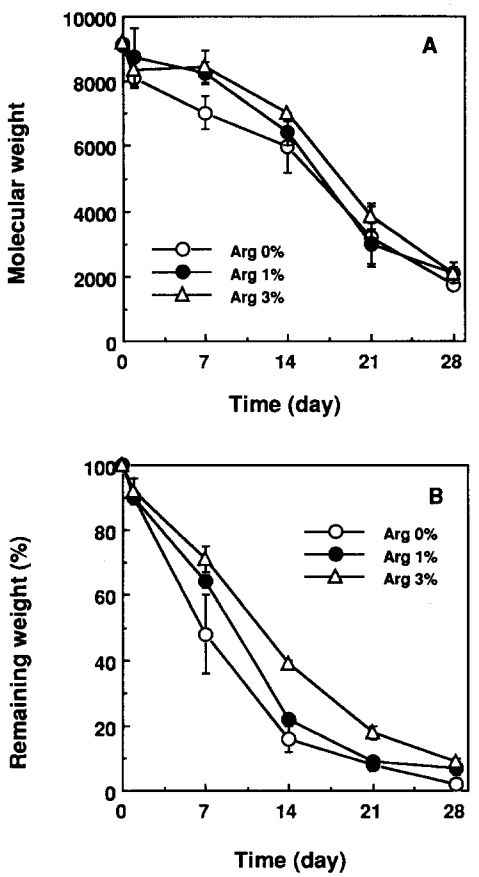

Fig. 9. Effect of L-arginine on weight average molecular weight (A) and remaining weight (B) of PLGA in microspheres with a loading amount of 10% TAK-029 after subcutaneous injection to rats. Dose = 20 mg/kg. Data are shown as mean ± S.E. of three animals.

Addition of L-arginine into the internal water phase dose-dependently reduced the initial burst of the drug from the microspheres both in vitro (Fig. 2) and in vivo (Fig. 3). Yoshioka et al. (1993) reported the effect of L-arginine on initial burst of water-soluble drugs from microspheres, however the mechanism has not been clarified. The present microspheres with different arginine contents demonstrated similar particle size distribution (Fig. 4) and surface structure (Fig. 5), indicating similar surface area. Therefore, reduced initial burst of microspheres with high arginine contents does not result from small surface area, but probably from hydrophobic diffusion barriers surrounding the drug core portions by a paired ion formation between arginine and carboxylic acid of PLGA as described in leuprorelin by Okada et al. (1994a,b). In the present study, T_g was raised with increasing amounts of arginine (Table 1), suggesting the formation of hydrophobic barriers or rigid structure of the polymer carbohydrate chains. Cross-sectional view of microspheres revealed porous inner structure without L-arginine (Fig. 6A and D), and rigid inner structure of those with L-arginine (Fig. 6C and F).

The hydrophobic diffusion barriers are supposed to cause the delay of water penetration, hydration and swelling of microspheres after subcutaneous injection. As a consequence, not only the initial burst but also the release rate of drug (Fig. 8) in accordance with polymer degradation (Fig. 9) were reduced in vivo.

When PLGA microspheres containing TAK-029 with 1 and 3% arginine were subcutaneously injected to rats, plasma TAK-029 levels were kept in the therapeutic range for 3 weeks with smaller initial burst, while causing a slight but significant increase in 2–3 weeks (Fig. 3). Sanders et al. (1984) reported triphasic release profiles of nafarelin acetate from PLGA microspheres (L/G = 50/50, intrinsic viscosity = 0.38 dl/g). In the primary release phase, the compound was lost by diffusion from the surface of the microspheres, and in the secondary phase subeffective release occurred by polymer hydrolysis with a decrease in its molecular weight, although it remained insoluble. In the tertiary phase, release resulted from dissolution of low-molecular weight fragments and erosion of the remaining polymer. In fact, they reported the initial increase of plasma levels immediately after injection (primary phase), the latent period between day 10 and 25 (secondary phase) and the increase in plasma levels between day 30 and 40 (tertiary phase) in rhesus monkeys and humans (Sanders et al., 1985, 1988). In this study, the triphasic release was also recognized in rats after subcutaneous injection of microspheres, although the tertiary phase appeared between day 10 and 20 since we used PLGA (L/G = 75/25, intrinsic viscosity = 0.13 dl/g) with a faster degradation rate. In the in vitro release studies using PBS-T (pH 7.4), the erosion (tertiary) phase appeared between day 28 and 35 (Fig. 7) probably because the degradation rate of the polymer was slower in the buffer of pH 7.4 than in the mass of microspheres at the injection site with an acidic condition caused by PLGA degradation products (Makino et al., 1985).

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