Utilization of an Amorphous Form of a Water-Soluble GPIIb/IIIa Antagonist for Controlled Release from Biodegradable Microspheres

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Purpose. We prepared injectable microspheres for controlled release of TAK-029, a water-soluble GPIIb/IIIa antagonist and discussed the characteristics of controlled release from microspheres.

Methods. Copoly(dl-lactic/glycolic)acid (PLGA) microspheres were used for controlled release of TAK-029 [4-(4-amidinobenzoylglycyl)-3-methoxycarbonyl-2-oxopiperazine-1-acetic acid]. They were prepared with a solid-in-oil-in-water (S/O/W) emulsion solvent evaporation technique using either a crystalline form or an amorphous form of the drug.

Results. An amorphous form of TAK-029 gave more homogeneous S/O dispersion and higher viscosity than its crystalline form when added to dichloromethane solution of PLGA, resulting in a high drug entrapment into microspheres and a well-controlled release of the drug. Additions of sodium chloride into an external aqueous phase and L-arginine into an oil phase also increased entrapment of the drug, and reduced initial burst of the drug from the microspheres. The microspheres demonstrated a desirable plasma level profile in therapeutic range (20–100 ng/ml) for 3 weeks in rats after single subcutaneous injection.

Conclusions. A well-controlled release of TAK-029, a water-soluble neutral drug, with small initial burst was achieved by utilizing its amorphous form as a result of possible interaction with PLGA and L-arginine.

KEY WORDS: amorphous; GPIIb/IIIa antagonist; controlled release; PLGA, microspheres.

INTRODUCTION

Injectable microspheres that have been prepared by using polylactic acid (PLA) and copoly(lactic/glycolic acid) (PLGA) have been widely studied for controlled release of many drugs including peptides and proteins. The advantages of a controlled release formulation include increased patient compliance and acceptance by reducing the number of subcutaneous injections, increased therapeutic benefit by eliminating fluctuations in drug concentrations in blood, and potentially lowering the total administered amounts of drug by reducing these fluctuations. For preparation of the microspheres containing water-soluble drugs, nonaqueous phase separation techniques (1,2) and spray drying techniques (3) have been adopted because of high-drug entrapment. In the former techniques, however, not only polymer solvent but coacervating and hardening agents were found to remain to a substantial extent in the microspheres (4). Those prepared by the latter techniques are subject to contamination with such debris as dust and fibers. On the other hand, solvent evaporation techniques have been successfully utilized for preparation of injectable microspheres containing water-soluble basic drugs such as leuprorelin (5,6) and thyrotropin releasing hormone (7) with an acceptable amount (< 50 ppm) of residual dichloromethane (8,9). Efficient entrapment of these basic drugs was probably due to the ionic interaction between the drugs and PLGA (10). For acidic or neutral drugs with high water-solubility, however, it is difficult in solvent evaporation techniques to achieve high entrapment and small initial burst of the drugs, because of drug leak to the external aqueous phase during emulsifying process (11–13).

TAK-029 [4-(4-amidinobenzoylglycyl)-3-methoxycarbonyl-2-oxopiperazine-1-acetic acid] (Fig. 1) is a highly potent antagonist to fibrinogen receptor GPIIb/IIIa (14), 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 atherosclerosis (15,16). 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 plasma levels above 100 ng/ml may cause prolongation of bleeding time. TAK-029 is highly water-soluble at > 200 mg/ml, and behaves as a zwitterion at neutral pHs with an amidino residue and a carboxylic residue. We considered that PLGA microspheres are one of the most desirable dosage forms for the drug to maintain the therapeutic range for more than two weeks following a single injection. The present paper describes the preparation of TAK-029-containing microspheres by an S/O/W emulsion solvent evaporation technique using either a crystalline form or an amorphous form of TAK-029 and the characterization of the microspheres in vitro and in vivo.

MATERIALS AND METHODS

Materials and Animals

TAK-029 was synthesized in Production Research Laboratories of our company. Copoly(dl-lactic/glycolic)acid (lactic/glycolic = 75/25 mole%, 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, sodium chloride, and mannitol were also supplied by Wako. Polyvinyl alcohol (Gosenol EG-40, molecular weight 79000) was commercially obtained from

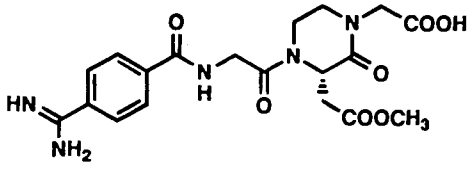

Fig. 1. Chemical structure of TAK-029)

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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, Inc. (Tokyo, Japan).

Preparation of Microspheres by an S/O/W Emulsion Solvent Evaporation Technique

An amorphous form of TAK-029 was obtained by freeze-drying, and a micronized crystalline form of the drug was obtained by micronizing its crystalline form with an A-O jet mill (Seishin Enterprise Co., Osaka, Japan). A weighed amount of the drug (450 mg) was dispersed in 4 ml of dichloromethane (DCM) containing PLGA (3960–4050 mg) with or without L-arginine (45–90 mg). The mixture was vigorously homogenized with a Polytron (Kinematica GmbH, Luzern, Switzerland) for one minute. This S/O dispersion was poured through a thin nozzle into 800 ml of a 0.1% polyvinyl alcohol aqueous solution, being cooled to 15°C, with or without sodium chloride (0.9–3.6%) under stirring with an Autohomomixer (Tokushu Kika Kogyo Co., Osaka, Japan), and the resulting mixture was stirred for a few minutes to make an S/O/W emulsion. To evaporate DCM, the S/O/W emulsion was further stirred gently with a propeller mixer for 3 hours at 800 rpm. After removing capsules larger than 125 μm by sieving, the resulting microspheres were collected by centrifuging for 5 minutes at 2000 rpm, rinsed with water three times and then freeze-dried.

Characterization of S/O Dispersions

A weighed amount (450 mg) of TAK-029 or mannitol was dispersed in 4 ml of DCM containing PLGA (4050 mg). L-Arginine (90 mg) was dissolved in 4 ml of DCM containing PLGA (3960 mg) as (PLGA + Arg) solution. Particle size distributions of solid drugs in S/O dispersions were measured using a laser diffraction particle size analyzer (SALD-2000A, Shimadzu Corp., Kyoto, Japan). Each viscosity of S/O dispersions and PLGA solutions was measured at 25°C using a rotary viscometer (Visconic ED, Tokyo Keiki Co., Tokyo, Japan).

Determination of TAK-029 by 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.

Extraction of TAK-029 from Microspheres

Microspheres (20 mg) were dissolved in 10 ml of DCM 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.

In Vitro Release Study

Microspheres (20 mg) were suspended in 10 ml of phosphate buffered saline (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 Industries Co., 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. In vitro initial burst means the percentage of TAK-029 released from microspheres during the first 24 hours.

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% trichloroacetic acid solution and kept in ice for 30 minutes. The mixture was centrifuged for 10 minutes at 12,000 rpm and the supernatant was diluted by 10 fold with PBS containing 0.2% bovine serum albumin. 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 hours 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 Inc., Burlingame, CA, USA) was added to the plate, followed by incubation for 2 hours at room temperature. After additional washing with PBS-T20, the plate was developed with 100 μl of 3, 3′, 5, 5′-tetramethyl benzidine dihydrochloride (TM BLUE) (Intergen-CDP, Milford, MA, USA) for 5 minutes 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 Inc., McLean, VA, USA).

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. 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.

RESULTS

Effect of Solid Forms of TAK-029 on Entrapment and in Vitro Initial Burst

Three types of microspheres were prepared by an S/O/W emulsion solvent evaporation technique using the crystalline, micronized, and amorphous forms of TAK-029 to study the influence on their entrapment and initial burst. A micronized form obtained from the crystalline one by a jet mill was found to be of a smaller size (5.2–6.3 μm) than original crystalline one (42–55 μm), but to be still crystalline from its X-ray powder diffraction pattern (X-ray diffractometer Model RINT1100, Rigaku Denki Co. Ltd., Tokyo, Japan) (Fig. 2). An amorphous form obtained by freeze-drying showed a similar particle size (4.1–6.7 μm) (Table I). Features of S/O dispersions and microspheres containing three forms of the drug were taken by a polarized differential interference microscope (Nikon MICROPHOT-FXA, Tokyo, Japan) (Fig. 3). The amorphous drug gave the most homogeneous S/O dispersion among three forms, and

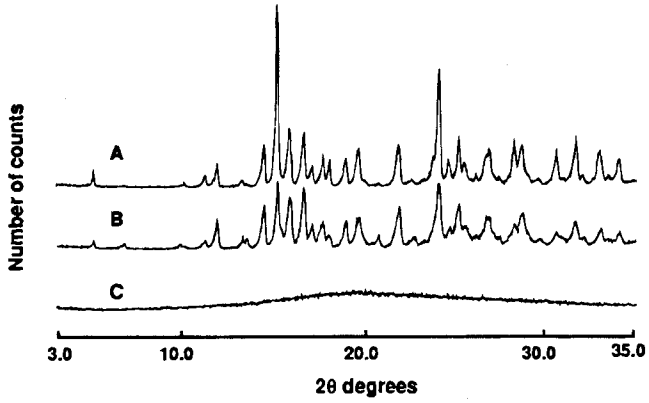

Fig. 2. X-ray powder diffraction patterns for crystalline (A), micronized (B), and amorphous (C) TAK-029.

was uniformly distributed throughout microspheres. At 2.7% sodium chloride in external aqueous phase and 2.0% L-arginine in an oil phase, entrapments of TAK-029 in microspheres prepared from crystalline, micronized crystalline (microcrystalline), and amorphous drug were 28%, 65% and 91%, respectively. In vitro initial bursts of TAK-029 from those microspheres were 90%, 87% and 12%, respectively.

Effect of Solid Forms of TAK-029 on Viscosity of S/O Dispersion

Three types of TAK-029 were dispersed in DCM solution containing PLGA. Amorphous TAK-029 raised the viscosity of the PLGA solution by 5.8 fold, while crystalline and microcrystalline forms raised the viscosity by less than two fold (Table I). Addition of 2.0% L-arginine also contributed to the increased viscosity, which was 44 fold higher in combination with amorphous TAK-029 than that of PLGA solution, while crystalline and microcrystalline forms did by less than seven fold. When mannitol was used instead of TAK-029, its amorphous form raised the viscosity by only 2.8 fold.

Effect of Sodium Chloride and L-Arginine on Entrapment and in Vitro Initial Burst

Sodium chloride was added to an external aqueous phase to increase its osmolarity and the effect was investigated for entrapment of amorphous TAK-029 into microspheres at a 10% loading amount and for its in vitro initial burst. Addition of sodium chloride ranging from 0.9 to 3.6% dose-dependently increased the drug entrapment and reduced the initial burst (Table II). L-Arginine was dissolved little in DCM alone, but in DCM containing PLGA, up to approximately 4%. Addition of L-arginine to an oil phase resulted in reduced initial burst with little effect on entrapment (Table II). At 2.0% L-arginine drug entrapment and initial burst were 91% and 12%, respectively.

In Vitro Release of TAK-029 from Microspheres

Microspheres were prepared using three solid forms of TAK-029 at a loading amount of 10% by an S/O/W technique using 2.7% sodium chloride and 2.0% L-arginine, and their release profiles were measured in an in vitro test (Fig. 4). Microspheres containing the amorphous drug demonstrated small initial burst of only 12% and continuous release for 5 weeks, while those containing the crystalline or microcrystalline released approximately 90% of the drug within the first 24 hours.

Plasma Levels in Rats After Subcutaneous Injection of Microspheres

Rat plasma levels of TAK-029 were determined for 28 days after subcutaneous injection of three kinds of microspheres described above (Fig. 5). Peak plasma levels were 3200, 1700, and 310 ng/ml one hour after injection of microspheres prepared using crystalline, microcrystalline, and amorphous TAK-029, respectively. Utilization of an amorphous form resulted in 10 fold less peak plasma level and brought it into therapeutic range between 20–100 ng/ml within 6 hours after injection. As for sustained release of the drug, an amorphous form also showed a better plasma level profile, giving 20–100 ng/ml for 3 weeks, while two other forms did little prolonged profiles.

DISCUSSION

The present study was undertaken to achieve high entrapment of a water-soluble non-basic drug, TAK-029 in PLGA microspheres and small initial burst of the drug from microspheres. In our previous paper those prepared by a W/O/W

Table I. Effect of Solid Forms of TAK-029 on Viscosity of S/O Dispersions

Formulation	Viscosity (cP)	Viscosity (fold)	Particle size[a] (μm)
PLGA solution	120	1.0	–
TAK-029(Crystalline)/PLGA solution	170	1.4	55
TAK-029(Microcrystalline)/PLGA solution	230	1.9	5.2
TAK-029(Amorphous)/PLGA solution	710	5.8	4.1
(PLGA + Arg) solution	630	5.2	–
TAK-029(Crystalline)/(PLGA + Arg) solution	820	6.7	42
TAK-029(Microcrystalline)/(PLGA + Arg) solution	760	6.3	6.3
TAK-029(Amorphous)/(PLGA + Arg) solution	5300	44.0	6.7
Mannitol(Crystalline)/PLGA solution	170	1.4	38
Mannitol(Amorphous)/PLGA solution	340	2.8	17

[a] Mean particle size of solid drugs.

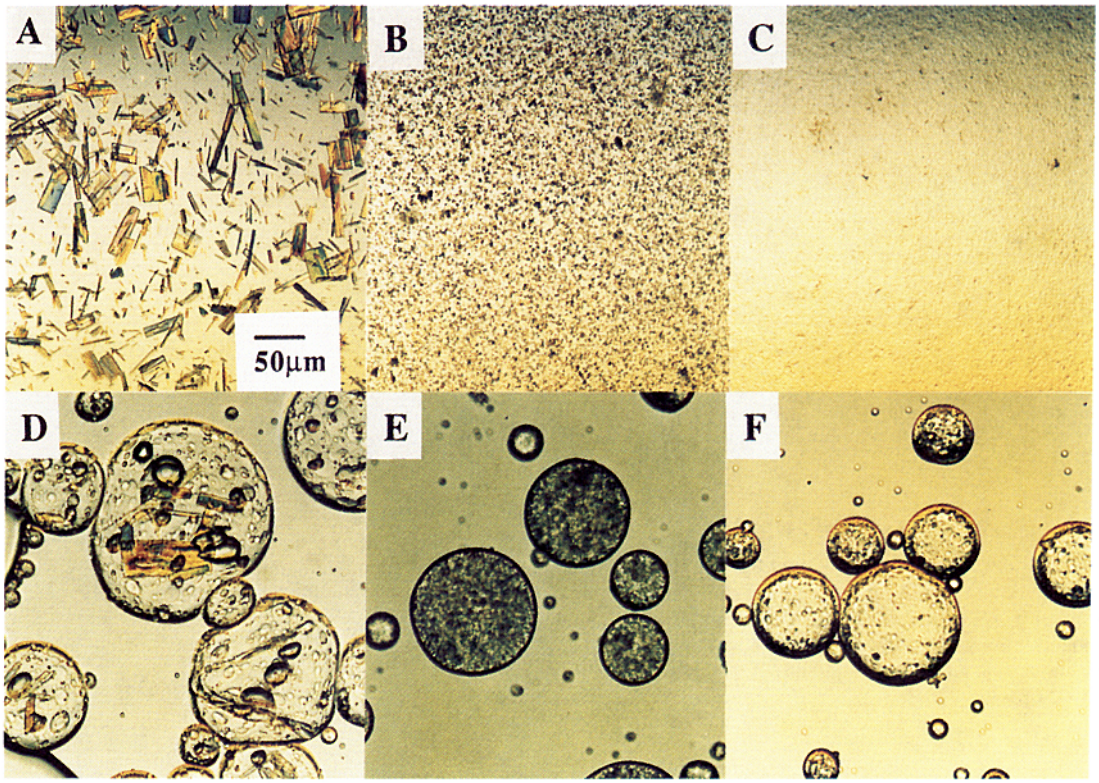

Fig. 3. Micrographs of S/O dispersions containing TAK-029 in crystalline form (A), microcrystalline form (B) and amorphous form (C). PLGA microspheres containing TAK-029 in crystalline form (D), microcrystalline form (E) and amorphous form (F).

emulsion solvent evaporation technique were investigated for the effects of sodium chloride and L-arginine on drug entrapment and initial burst of the same drug (17). The microspheres, however, showed large initial burst possibly due to interconnecting aqueous pores formed in polymer matrix during the evaporation of internal water. Here we have prepared microspheres by an S/O/W technique without internal water to avoid the formation of porous structure.

An S/O/W emulsion solvent evaporation technique was already reported for PLA microspheres containing cisplatin, a slightly water-soluble anticancer drug in a form of micronized crystal (18, 19). We have applied the method to the preparation of PLGA microspheres containing crystalline TAK-029, but the drug entrapment was very low. Then the use of amorphous TAK-029 led to more homogeneous S/O dispersion (Fig. 3C) and to higher entrapment (Table II). The homogeneous dispersion gave higher viscosity than that of the microcrystalline drug, although the drugs in both S/O dispersions showed similar particle size (Table I). When mannitol was employed instead of TAK-029, there was a slight difference in viscosity between crystalline and amorphous ones (Table I). These results suggest

Table II. Effects of Sodium Chloride and L-Arginine on Drug Entrapment and in Vitro Initial Burst

L-Arginine (%)	NaCl (%)	TAK-029 entrapment (%)	Initial Burst (%)
0	0	74	45
0	0.9	83	37
0	1.8	88	23
0	2.7	89	20
0	3.6	89	20
1.0	2.7	85	15
2.0	2.7	91	12

Note: Loading amount of amorphous TAK-029 was 10%.

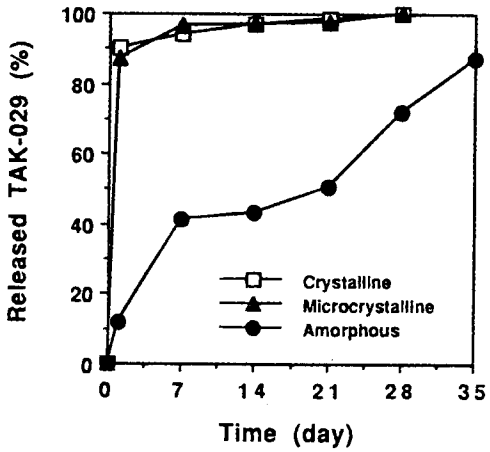

Fig. 4. In vitro release profiles of TAK-029 form PLGA microspheres with a loading amount of 10% TAK-029. Sodium chloride concentration in external aqueous phase was 2.7%. Loading amount of L-arginine was 2.0%. Data are shown as mean ± S.E. of three experiments.

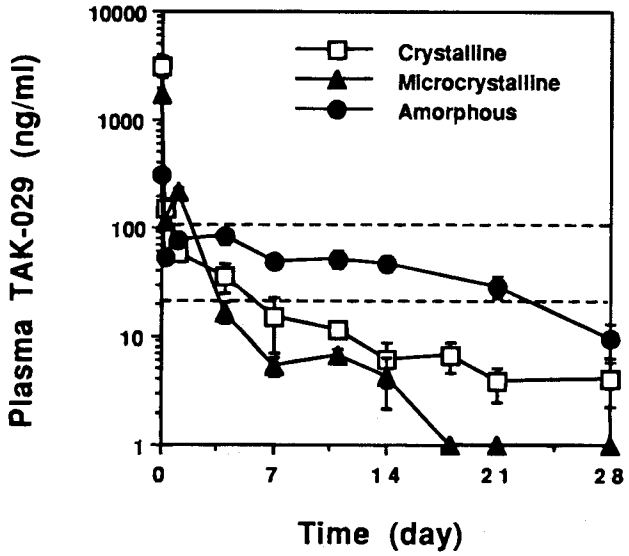

Fig. 5. Effect of solid forms of TAK-029 on the plasma level after subcutaneous injection of PLGA microspheres to rats. Dose = 20mg/kg. Sodium chloride concentration in external aqueous phase was 2.7%. Loading amount of L-arginine was 2.0%. Data are shown as mean ± S.E. of four animals. Dotted lines represent the therapeutic range of plasma TAK-029 between 20 and 100 ng/ml.

the increased viscosity of the S/O dispersion containing amorphous TAK-029 resulted from the molecular interaction between amorphous TAK-029 and PLGA or L-arginine. In fact, Hancock and Zografi described, in their latest review, that an amorphous state represented greater molecular motion resulting in greater chemical reactivity than a crystalline state, because of high internal energy (enthalpy) (20).

Yoshioka *et al.* reported the effect of L-arginine on initial burst of water-soluble drugs from microspheres (21) and we also described the addition of L-arginine reduced the initial burst of TAK-029 from microspheres prepared by a W/O/W technique (17). The present study indicated the possible ionic pair between L-arginine and PLGA because L-arginine only somewhat dissolved in DCM alone, but in DCM solution containing PLGA the dissolution was higher. The ionic pair formation resulted in 5.2 fold higher viscosity of PLGA solution, and surprisingly the combination of L-arginine and amorphous TAK-029 remarkably increased the viscosity by 44 fold (Table I). The appreciable interactions among TAK-029, PLGA and L-arginine seemed to form hydrophobic diffusion barriers leading to reduced initial burst of TAK-029 from microspheres with high L-arginine contents (Table II). As a result, the PLGA microspheres containing amorphous TAK-029 and L-arginine demonstrated desirable controlled release with small initial bursts in the in vitro and in vivo studies (Figs. 4 and 5).

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