Preparation of Three-Month Depot Injectable Microspheres of Leuprorelin Acetate Using Biodegradable Polymers[1]

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To obtain a three-month release injection of leuprorelin acetate, microspheres were prepared with copoly(DL-lactic/glycolic acid) or poly(DL-lactic acid) (PLA) using an in-water drying method, and drug release was evaluated. The content of water-soluble oligomers in the polymers was found to strongly affect the initial burst, and reducing the content to less than 0.1% was necessary to keep the first-day release below 10%. Drug loading of more than 15% also increased the initial drug release; the acceptable maximum loading was 12%. Elevation of the glass transition temperature of the microspheres was observed with an increase in drug loading. This suggests formation of a rigid structure, possibly with arrangement of the polymer around the drug cores like in a micelle. This structure provides a hydrophobic barrier against diffusion of the hydrophilic peptide, resulting in high trapping efficiency and long-term sustained release dependent on polymer erosion. The microspheres prepared with PLA having a m.w. of 12,000 to 18,000 provided linear sustained release and persistent serum levels of the drug in rats for over 3 months.

KEY WORDS: leuprorelin (leuprolide); three-month depot microspheres; poly(DL-lactic acid); water-soluble oligomers; glass transition temperature; serum drug levels in rats.

INTRODUCTION

Leuprorelin (leuprolide) acetate, a highly active agonist of luteinizing hormone-releasing hormone (LH-RH), potently inhibits the pituitary-gonadal axis upon chronic administration ("chemical castration") and is useful in the treatment of hormone-dependent diseases in males and females. To eliminate the inconvenience of daily s.c. injection of the conventional parenteral solution and to achieve greater efficacy, we developed one-month release injectable microspheres of the drug using a biodegradable polymer of copoly-(DL-lactic/glycolic acid) (PLGA) utilizing a novel in-water drying method (3–6). Our previous studies demonstrated that a single injection of this one-month depot provided sustained serum levels of the drug and persistently suppressed serum gonadotropin and testosterone levels and growth of reproductive organs for over 1 month in male rats and dogs (7–10) and growth of isografts of endometrium on the wall of the abdominal cavity in female rats (5). Clinical studies confirmed the expected therapeutic efficacy in patients with advanced prostate cancer (11), uterine leiomyomate (12) and endometriosis (13) upon chronic administration of this depot formulation. We further examined the possibility of developing a longer release depot formulation.

In the present study, to obtain a three-month depot, microspheres of leuprorelin were prepared using various polymers and the in-water drying method, and the drug release rate was evaluated by determining the amount of drug remaining at the injection site and serum drug levels in rats after s.c. injection.

MATERIALS AND METHODS

Animals and Materials

Male Sprague-Dawley rats were purchased from Clea Japan, Inc. (Tokyo). Leuprorelin acetate was synthesized at Takeda Chem. Ind. PLGA and poly(DL-lactic acid) (PLA) were purchased from Wako Pure Chem. Ind. (Tokyo).

Preparation of Microspheres

The microspheres of leuprorelin were prepared as previously described (4,9) except that gelatin was not used in the inner drug solution. In brief, in the case of PLA microspheres loading 12% drug, 550 mg of drug dissolved in 1 ml of distilled water (W_1) and 4 g of PLA dissolved in 7.5 ml of CH_2Cl_2 (0) were mixed and agitated vigorously with a homogenizer to form a W/0 emulsion. This emulsion was poured into 1 L of 0.25% polyvinyl alcohol (PVA) solution (W_2) under stirring. This $W_1/0/W_2$ emulsion was stirred gently for 3 hr to evaporate the organic solvent to obtain microspheres. The microspheres were sieved with a 74-μm screen to remove large particles and then centrifuged at 1,000 rpm for 5 min. The resulting microspheres were washed with water and lyophilized. PLGA(lactic/glycolic: 75/25) (weight average molecular weight, m.w.: 9,100–23,000), PLGA(90/10) (9,200–22,900) and PLA (4,700–162,100) were used. To determine the effects of drug loading on drug release, microspheres charging 9, 12, 15 and 18% drug were prepared using PLA-18,200 (m.w. of 18,200). The volume of the inner water phase and organic solvent and temperature of the W/0 emulsion and outer water layer were varied with the kind of polymer and % drug loading to maintain appropriate viscosity and obtain spherical and similar size microspheres.

Drug Release *in vitro*

The microspheres (50 mg) were dispersed in 10 ml of pH 7.0, 1/30 M phosphate buffer containing 0.02% Tween 80 and stirred with a rotator (RT-50, Taiyo Sci. Ind., Tokyo). The microspheres were collected periodically using a Millipore filter (0.8 μm) and extracted with 20 ml of pH 6.0, 1/30 M phosphate buffer containing 0.02% Tween 80 and 10 ml of CH_2Cl_2 to determine the drug content by HPLC (5).

Drug Release *in vivo*

The microspheres were s.c. injected into rats (6 weeks of age) at a site on the back at doses of 0.9 and 4.05 mg of the

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drug after being dispersed in 0.3 and 0.5 ml of vehicle, respectively. To determine the amount of drug remaining in the microspheres at the injection site, the microspheres and the thin layer of connective tissue surrounding them were excised periodically, homogenized in 10 ml of pH 6.0, 1/30 M phosphate buffered saline containing 0.02% Tween 80 (PBS) and extracted after adding 10 ml of each PBS and CH_2Cl_2 (5).

Serum Drug Levels in Rats After s.c. Injection

The microspheres prepared using PLA with a m.w. of 11,800 to 17,300 were injected s.c. into rats (10 weeks of age) at a dose of 4.5 mg of the drug, corresponding to a dose of about 100 μg/kg/day of the drug for 90 days (average body weight of 500 g at 16 weeks of age). About 1 ml of blood was collected periodically from the tail vein, and the serum was stored at less than −40°C until the day of RIA. Serum leuprorelin was determined by a double-antibody RIA system (5), in which the detection limit was 63 pg/ml.

Free Acid Content and Glass Transition Temperature of Polymers

To determine the effects of free acid content of the polymers on the drug release, 20 lots of PLA with a m.w. of 8,700 to 54,700 were used. PLA (150 mg) was dissolved in 5 ml of CH_2Cl_2 and extracted with 10 ml of distilled water. The acidic fraction in the aqueous layer (2.5 ml), which contains lactic acid and the water-soluble oligomers, was titrated with 0.001 N NaOH using phenol red, and the free acid content (%) was calculated as lactic acid monomer.

The glass transition temperature (Tg) of PLA-14,100, PLGA(74/26)-13,700 and the microspheres prepared with these polymers and containing 0 to 8% leuprorelin acetate was determined with a differential scanning calorimeter (DSC 7, Perkin Elmer, Norwalk, CT). About 10 mg of a sample was weighed, crimped into an aluminum pan and analyzed at a scanning rate of 10°C/min. The Tg was calculated using DSC 7 Automode software by extrapolating the linear portion of the thermograms above and below the glass transition point and determining the midpoint.

RESULTS AND DISCUSSION

We determined the *in vitro* and *in vivo* release of leuprorelin acetate from microspheres prepared with about 50 kinds of PLA and PLGA using the in-water drying method (3), modified to the extent that no gelatin is contained in the inner drug solution (W_1).

Drug Release *in vitro*

Although the data of drug remaining in the microspheres in the *in vitro* test fluctuated slightly and an initial lag time was often observed, a rough understanding of the release pattern afforded by each kind of polymer was achieved. The microspheres prepared with PLA with a small m.w. or PLGA(75/25)-(9,100–23,000) released the drug relatively rapidly; the release was finished within 6 to 8 weeks. The microspheres prepared with PLA with a high m.w. (54,700 and 162,100) synthesized by a ring opening method (14) showed a large initial burst, 44 and 58% during the first-day of incubation, respectively. This might be attributed to the fewer carboxylic anion terminals available to interact with the cationic drug core as compared with polymers prepared by direct condensation due to intramolecular ring formation. The microspheres utilizing PLGA(90/10) and PLA-(15,700–21,500) released the drug over 12 to 13 weeks.

Drug Release *in vivo*

Typical *in vivo* release profiles of leuprorelin microspheres in rats after s.c. injection are shown in Fig. 1. The release rate was controlled by the rate of bioerosion of the polymer. The microspheres using PLGA(75/25)–15,800 showed sustained release for 4 weeks. The release from the microspheres prepared with PLA-53,300 was too slow, as 16 weeks after injection the residual content was 40%, and that with PLA-4,700 was too fast for a three-month depot. The microspheres prepared using PLA-18,200, PLA-21,500 (data not shown) or PLGA(90/10)-19,000 provided continuous release of the drug over approximately 3 months without a lag time.

In the case of the one-month depot microspheres, *in vitro* release correlated well with *in vivo* release (15). The initial release is dependent mainly on drug diffusion through the swelled polymer matrix near the surface and the hydrated aqueous channels produced by the connected drug cores or evaporation of organic solvent and water in the inner drug layer. With the long-term depot, a long lag time (only 10% release in 8 weeks in the case of PLA-18,000 microspheres) followed by relatively rapid drug release was observed in the *in vitro* test. The reason for these differences is not clear but might be due to enhancement by acute cellular immune responses (16), i.e., attack by macrophages and giant cells, and enzymatic degradation (17). These polymers are fairly biocompatible (18), but cellular response is not completely avoided. This might stimulate the drug release and produce the linear release *in vivo*. Since the *in vitro* release is strongly affected by medium composition, i.e., buffer pH, osmolality, concentration and ion species, and the *in vivo* release is more complex due to the complex environment surrounding the microspheres, further investigation will be required to clarify their correlation. In this study, we

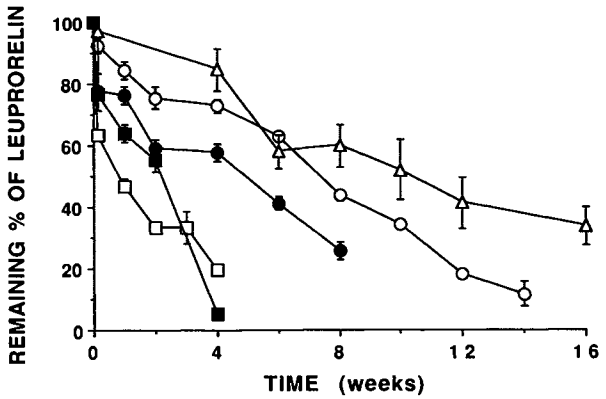

Fig. 1 *In vivo* release of leuprorelin from the PLA and PLGA microspheres in rats after s.c. injection. (Dose = 0.9 mg, mean ± SE, n = 5) □, PLA-4,700; ○, PLA-18,200; △, PLA-53,300; ■, PLGA(75/25)-15,800; ●, PLGA(90/10)-19,000.

abandoned the *in vitro* test and carried out the formulation study using the *in vivo* test.

Effects of Free Acid Content and Drug Loading on Drug Release

Leuprorelin remaining in the microspheres one day after s.c. injection versus free acid content in the 20 kinds of PLA-(8,700–54,700) used to prepare the microspheres is shown in Fig. 2. An increase in the free acid content caused an increase in the initial burst. The first-day burst was less than 10% when PLA containing less than 0.1% free acid was used. This water-soluble acid fraction designated as free acid is composed of heptamer or smaller oligomers of lactic acid. These oligomers are assumed to interfere with the formation of the hydrophobic barrier by arrangement of the polymer around the drug domains and increase the number of aqueous channels through the polymer barrier resulting in enhancement of the initial burst, "a tunnel effect."

The *in vivo* release profiles of the microspheres charged with different amounts of drug (9–18%) and prepared using PLA-18,200 containing less than 0.1% free acid are shown in Fig. 3. Trap ratio for the microspheres charged with 9 and 12% drug was 107.7 and 97.5%, respectively, but that at 15 and 18% was slightly lower: 95.3 and 95.6%, respectively. The release rates for the microspheres charged with 15 and 18% drug were rapid due to an increase in the number of aqueous channels formed by the hydrophilic drug. These phenomena were also observed in the case of leuprorelin one-month depot and TRH microspheres (19). Thus, it was indicated that drug loading should be limited to about 12%.

Glass transition temperature (Tg) of PLA-14,100, PLGA(74/26)-13,700 and the microspheres charged with 0 to 8% drug and prepared using these polymers is shown in Fig. 4. Trap ratio and remaining drug in the microspheres after one day are summarized in Table 1. Formation of microspheres with or without the drug caused a distinct elevation in Tg for both polymers. Moreover, Tg increased gradually with increasing drug loading. Trap ratio was somewhat low with a low drug charge, and maximum entrapment was observed between 5 and 8%. The residual content in the PLA microspheres one day after s.c. administration was not greatly influenced by the drug charge within the range examined. The residual amount in the PLGA microspheres after one day, however, increased with an increase in drug

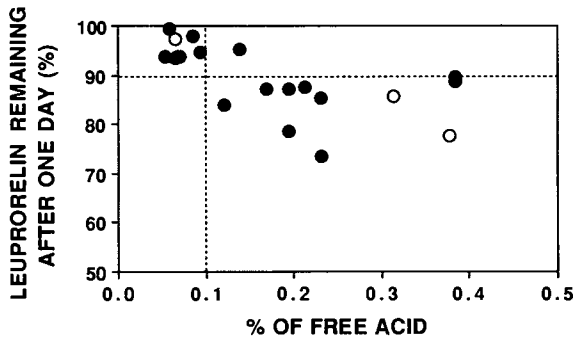

Fig. 2 Effect of water-soluble oligomers (free acid) in PLA on *in vivo* initial burst of leuprorelin microspheres. (Drug loading: ○, 9%; ●, 12%; 20 lots: PLA with a m.w. of 8,700 to 54,700)

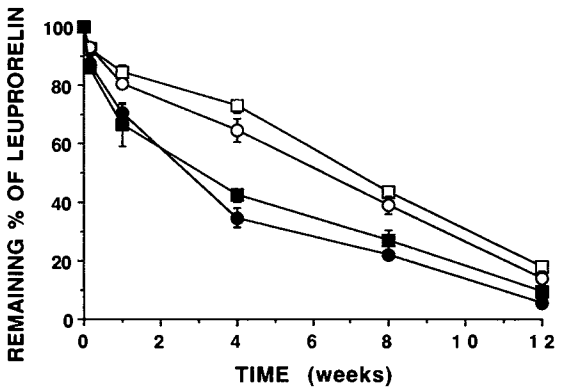

Fig. 3 Effect of drug loading on *in vivo* release of leuprorelin from the PLA microspheres in rats after s.c. injection. (Drug loading: □, 9.7%; ○, 11.7%; ■, 14.3%; ●, 17.2%; PLA-18,200, dose = 0.9 mg, mean ± SE, n = 5)

loading. These microspheres are polynuclei reservoir microcapsules in which drug cores are dispersed in the polymer matrix. These phenomena suggest the formation of rigid structure in the microspheres and that the polymer molecules are arranged around the drug cores similar to surfactant micelles due to the ionic interaction between the basic amino acids of the drug and the terminal carboxylic anions of the polymer. However, Tg of microspheres containing a lipophilic drug such as cyclosporin A which does not interact chemically with the polymer and forms a molecular dispersion in the polymer matrix decreases with drug loading (20). Thus, a barrier against the diffusion of hydrophilic drugs is constructed by the hydrophobic long alkyl chains of the polymers as illustrated previously (21). This ionic interaction between polymer and drug was confirmed by the chemical shift of the arginyl and histidyl protons of leuprorelin to a lower magnetic field in the NMR spectra of the $W_1/0$ emulsion. No diffraction peak, however, was observed in powder X-ray diffraction analysis of these microspheres having a high drug content (not published). The formation of such a rigid structure is also evidenced by the increase in viscosity of the $W_1/0$ emulsion with an increase in drug loading both in the case of leuprorelin and TRH (19).

The microspheres with leuprorelin loading of 0.5–2%

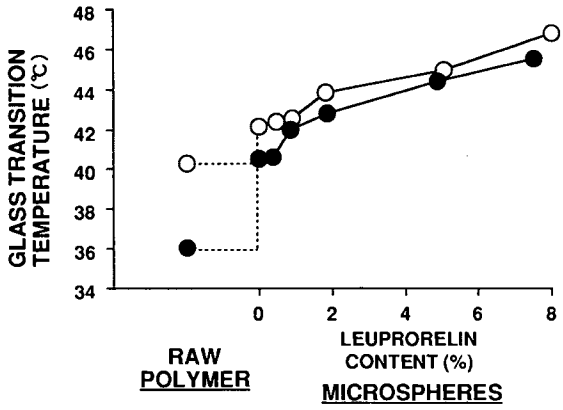

Fig. 4 Glass transition temperatures of the PLA and PLGA microspheres containing various amounts of leuprorelin. ○, PLA-14,100; ●, PLGA(74/26)-13,700.

Table I. Effect of Drug Loading on Trap Ratio in the Leuprorelin Microspheres and the % of Drug Remaining in the Microspheres After One Day

Polymer	Theoretical loading (%)	Observed loading (%)	Trapped (%)	Remaining after one day (%)
PLA-14,100	0.54	0.45	83.3	94.7 ± 1.1[a]
	1.06	0.90	85.3	87.4 ± 1.2
	2.06	1.81	88.1	93.4 ± 0.6
	5.08	5.05	99.6	93.5 ± 2.5
	7.73	8.00	103.5	94.5 ± 1.0
PLGA(74/26)-13,700	0.52	0.39	75.7	57.8 ± 1.8[b]
	1.04	0.86	82.8	63.9 ± 0.6
	2.06	1.85	90.0	77.7 ± 2.8
	5.00	4.87	97.3	82.9 ± 0.8
	7.32	7.53	102.8	83.9 ± 0.9

[a] *In vivo*, mean ± SE, n = 4–5, [b] *in vitro*, mean ± SE, n = 3.

had a low trap efficiency, and a rapid initial burst was seen with the PLGA microspheres. However, the effects of drug loading on microencapsulation, especially on initial burst, were less than those with TRH, likely resulting from the stronger cationic charge and larger molecular size of leuprorelin. Adding acids such as hydrochloric, tartaric and citric, which are more acidic than PLA and PLGA, to the inner drug cores caused a dramatically large initial burst from leuprorelin (to be published) and TRH (19) microspheres due to interference with the ionic interaction between peptides and polymers. This could explain why such water-soluble peptides are efficiently entrapped during the in-water drying process and why drug release from the microspheres is controlled mainly by degradation of the polymer for long periods of time avoiding a large initial burst. Tg of the microspheres, even those not containing any drug, was higher than that of the raw polymers. This can be attributed to arrangement of the polymers at the interfaces of the water layers of the inner water droplets without drug (W_1) and outer PVA solution (W_2) and the oil layer of the polymer CH_2Cl_2 solution (0).

Selection of Polymer for the Three-Month Depot Formulation

As PLA-18,200 and PLA-21,500 microspheres showed a release period slightly too long for a three-month depot, several lots of microspheres using PLA and PLGA(90/10) with a smaller m.w. were examined. The microspheres were prepared using PLGA(90/10)-(9,200–13,800) (6 lots), PLA-(9,100–13,000) (6 lots) and PLA-18,000 (4 lots), and *in vivo* release was examined. These microspheres provided linear sustained release for 12 or 14 weeks, and those constructed with smaller polymers had a tendency toward more rapid drug release. Mean release profiles of 4 lots of the microspheres prepared using PLGA(90/10)-(about 12,000) and PLA-(about 10,000 and 18,000) are plotted in Fig. 5. The microspheres prepared with PLGA-(90/10)-12,000 released

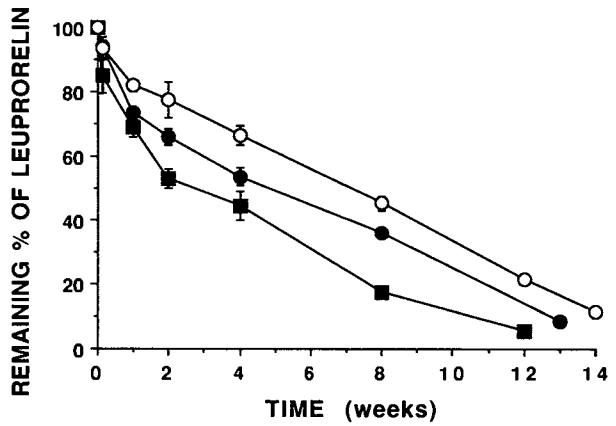

Fig. 5 In vivo release of leuprorelin from the PLA and PLGA microspheres in rats after s.c. injection. (Mean ± SE, n = 4 lots) ○, PLA-18,000; ●, PLA-10,000; ■, PLGA(90/10)-12,000.

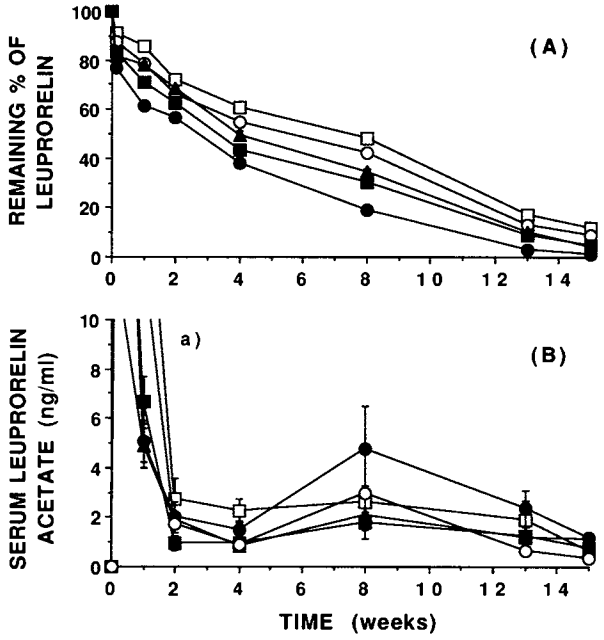

Fig. 6 Leuprorelin remaining at the injection site and serum levels of leuprorelin in rats after s.c. injection of the PLA microspheres. (Drug loading 12%, dose = 4.05 mg (remaining %) and 4.5 mg (serum levels), mean ± SE, n = 5) (A) PLA-: □, 17,300; ○, 16,500; ▲, 15,500*; ■, 14,100*; ●, 12,400*; (B) PLA-: □, 17,200; ▲, 15,500*; ■, 14,100*; ●, 12,400*; ○, 11,800. (* large scale products) a) C_{ld} (ng/ml): □, 31.1 ± 3.2; ○, 12.2 ± 0.9; △, 26.4 ± 2.6; ●, 32.4 ± 2.1; C_{lw} (ng/ml): □, 17.4 ± 1.8; ●, 12.8 ± 1.6.

the drug too fast during the initial 2 weeks, but those with PLA-10,000 and PLA-18,000 provided sustained release over 13 weeks at a fairly constant rate.

Finally, we determined the in vivo release of several kinds of microspheres prepared using PLA having a m.w. of 11,800 to 17,300, including those from large scale production (10 times with PLA-15,500, -14,100, and -12,400). The typical release profiles and serum drug levels are shown in Fig. 6. These microspheres provided linear sustained release dependent on the rate of polymer degradation for 3 months. The release rates (slope of each profile) among these microspheres were almost the same, but the initial burst was enhanced by a decrease in m.w. The serum drug levels after a single injection were well sustained over 13 weeks with all the microspheres: a plateau level of about 2 ng/ml following a large initial peak during the first week.

In summary, the results of screening biodegradable polymers, PLA and PLGA, for the three-month depot formulation indicated that microspheres prepared using PLA with a m.w. of 12,000 to 18,000 give linear sustained drug release for over 13 weeks. A similar sustained release pattern was confirmed by serum drug level assays following s.c. injection of these microspheres. The microspheres prepared using PLA with a m.w. of about 15,000, containing less than 0.1% water-soluble oligomers and loaded with 12% drug may be the most desirable formulation for the three-month depot injection.

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