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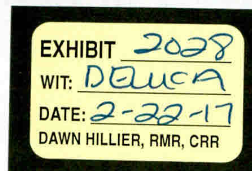

Short Communication

In Vitro Characterization and *in Vivo* Testosterone Suppression of 6-Month Release Poly(D,L-Lactide) Leuprolide Microspheres

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KEY WORDS: leuprolide; LHRH analogue; poly(D,L-lactide); microspheres; peptide stability; testosterone suppression.

INTRODUCTION

Controlled-release polymer delivery systems have been investigated to achieve the efficacy of biologically active agents and improve patient compliance by eliminating the need for frequent administration (1–5). Microsphere delivery systems fabricated from polyesters of lactide and glycolide were shown to improve the bioavailability of peptides, proteins, and DNA by protecting them from physical degradation and proteolysis in body fluids before release (6–11).

Leuprolide, a potent agonistic analogue of luteinizing hormone-releasing hormone, inhibits the secretion of pituitary gonadotropin when administered chronically in therapeutic doses (12,13). Microsphere depot formulations of leuprolide were developed successfully and marketed for long-term testosterone suppression. 1-, 3-, and 4-month release formulations of Lupron® depot, developed using a water-in-oil-in-water (w/o/w) emulsion method, currently are used for the treatment of hormone-dependent prostatic cancer, endometriosis, and precocious puberty (14–19).

A 4-month release poly(D,L-lactide) (PLA) microsphere delivery system using a solvent extraction/evaporation method have been developed recently (9). The microspheres prepared with PLA (molecular weight 11,000) provided sustained release of leuprolide and suppression of serum testosterone level for 4 months in rats. Compared to Lupron® depot, in which particles contain discrete internal pockets of drug, so-called microcapsules, the PLA microspheres were prepared from a clear homogeneous solution of polymer and drug so that the drug was molecularly distributed throughout the PLA matrix.

The goal of this study was to prepare and characterize a 6-month leuprolide microsphere formulation using a dispersion/solvent extraction-evaporation method. Microspheres were prepared with PLA polymers of m.w. 18,000–28,000 to obtain a more convenient and effective microsphere formulation for prostate cancer and endometriosis therapy.

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MATERIALS AND METHODS

Materials

Leuprolide acetate, [Des-Gly^{10}, D-Leu^{6}, Pro^{9}]-luteinizing hormone-releasing hormone ethylamide, was obtained from Bachem (Torrance, CA, USA). PLA polymers with a molecular weight of 18,000 (PLA 18k) and 28,000 (PLA 28k) were supplied by Birmingham Polymers (Birmingham, AL, USA) and Boehringer Ingelheim (Ingelheim, Germany), respectively. Polyvinyl alcohol (molecular weight 30,000–70,000; PVA) was obtained from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were obtained commercially as analytical grade reagents.

Preparation of Microspheres

Leuprolide PLA microspheres were prepared by dispersing a homogeneous solution containing polymer and drug into a PVA solution followed by solvent extraction/evaporation. Briefly, a solution of leuprolide in methanol was added to a 21–29% (w/w) solution of PLA polymer in methylene chloride. The resultant clear solution was then slowly dispersed in 0.35% aqueous PVA solution while mixing with a Silverson® L4R mixer (Silverson Machines Inc, East Longmeadow, MA, USA) at 7000 rpm using an in-line mixer (20). The solvents were removed by stirring, air sweep, and continuous phase replacement at 40°C for 1 h. The solidified microspheres were recovered by filtration and dried under vacuum at room temperature for 48 h.

Microsphere Characterization

Microspheres were sized by laser diffractometry using a Malvern 2600 laser sizer (Malvern 2600c Particle Sizer, Malvern, UK). The average particle size was expressed as the volume mean diameter V_{md} in microns. The morphology was examined by scanning electron microscopy (Hitachi Model S800, Japan) after palladium/gold coating of the microsphere sample on an aluminum stub. Peptide content was determined by high-performance liquid chromatography (HPLC) as follows: 10 mg of the microspheres was dissolved in 2 mL of methylene chloride. The peptide was extracted from the polymer solution by addition of 10 mL 0.1 M acetate buffer (pH 4.0) followed by agitation for 1 h. The peptide concentration was determined by HPLC using a Bondclone 10 C18 column (300 × 3.9 mm, Phenomenex, Torrance, CA, USA). Gradient elution was performed with 0.1% trifluoroacetic acid (A) and 90% acetonitrile, 0.1% trifluoroacetic acid (B) and increasing the amount of the solution B from 10 to 60% over 12 min at a flow rate of 1.5 mL/min. Leuprolide was detected at 215 nm.

In Vitro Release Study

Ten milligrams of the microspheres (n = 3) was incubated in 10 mL 0.1 M phosphate-buffered saline (PBS, pH 7.4) at 37°C. At each time point, the residual microspheres were recovered by centrifugation at 3000 rpm for 10 min and dissolved in 2 mL of methylene chloride. The peptide was extracted from the polymer solution by addition of 10 mL of

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Table I. Characterization of Leuprolide PLA Microspheres

Microsphere	PLA 28k low-drug load	PLA 28k high-drug load	PLA 18k
Polymer	PLA 28 000	PLA 28 000	PLA 18 000
Target drug load (%)	18.0	21.0	15.2
Drug content (%)	16.3	20.7	15.0
Drug incorporation efficiency (%)	90.6	98.6	98.7
Mean particle size (μm)	22.0	20.1	7.2

0.1 M acetate buffer (pH 4.0) followed by agitation for 1 h. The concentration of leuprolide was assayed by HPLC.

In Vitro Polymer Degradation

Thirty milligrams of the microspheres (n = 2) was incubated in 30 mL of 0.1 M PBS at 37°C. At each time point, recovered wet microsphere was weighed accurately (wet weight, W_w), dried for 48 h under vacuum at room temperature, and reweighed (dry weight, W_d). The mass remaining (MR) and the degree of hydration (DH) were calculated as follows:

$$MR\ (\%) = (W_d/W_o) \cdot 100 \qquad (1)$$

Where W_o is the initial mass at time zero.

$$DH = (W_w - W_d)/W_d \qquad (2)$$

Peptide Stability

Peptide was extracted from the PLA microspheres incubated in 0.1 M PBS for 161 days using the methylene chloride/0.1 M acetate buffer extraction method described previously. The stability was assessed by HPLC. The peptide eluted at the same retention time of standard leuprolide was collected and analyzed using an IonSpec HiResMALDI Fourier Transform mass spectrometer (IonSpec Co., Irvine, CA, USA).

In Vivo Testosterone Suppression

Male Sprague–Dawley rats (n = 6) weighing ~300 g were used to evaluate *in vivo* performance of leuprolide microspheres. The microspheres were injected subcutaneously at the back of the neck (18 mg/kg as leuprolide) after reconstitution in a suitable vehicle (1% carboxymethylcellulose and 2% mannitol, w/v). Blood samples were collected from the tail vein at specific time points. The blood samples were centrifuged in Microtainer® tubes (Becton Dickinson & Co., Franklin Lakes, NJ, USA) and serum was collected. Serum samples were frozen and stored at −20°C until analysis.

Serum testosterone levels were assayed using Active™ Testosterone RIA DSL-4000 kits (Diagnostic Systems Inc., Webster, TX, USA). The lower limit of detection for this assay was 0.08 ng/mL, and the intra- and interassay coefficients of variation were 10 and 9%, respectively. The cross-reactivity of the testosterone antiserum was less than 6%.

RESULTS AND DISCUSSION

Microsphere Characterization

The PLA 28k leuprolide microspheres were prepared with two different target drug loads, 18% and 21%. The actual drug contents were determined to be 16.3% and 20.7%, and the drug incorporation efficiency was 91% and 99% for the low and high target drug loads, respectively (Table I). The mean particle size was ~20 μm, which is suitable for intramuscular or subcutaneous injections. The PLA microspheres showed a spherical shape (Fig. 1, a and b) with some surface pores and scratch marks (lines of pores), which could be from the shear stress during microsphere preparation (Fig. 1b). Microspheres of PLA 18k were prepared with 15.2% target drug load. The actual drug content was found to be 15%, and the drug incorporation efficiency was 99%. The microspheres were spherical with relatively smooth surface morphology (Fig. 1c). The mean particle size was ~7 μm. The smaller particle size, compared to the PLA 28K microspheres, could be due to the lower viscosity of the polymer solution of the low molecular weight PLA polymer solution. For the preparation of long-term release microsphere formulations, maxi-

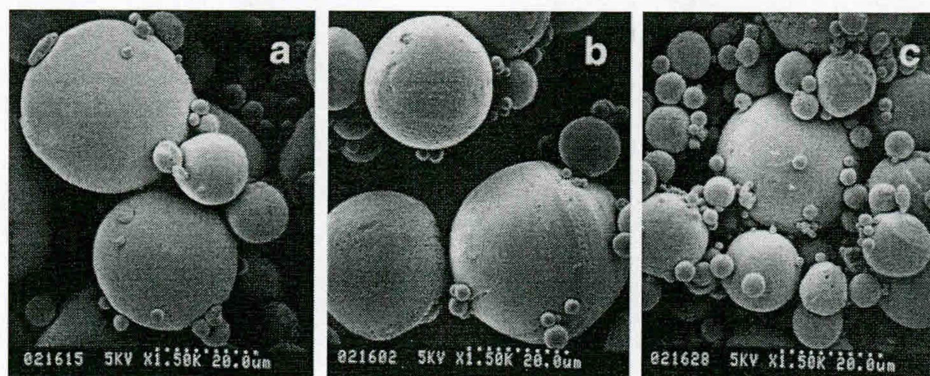

Fig. 1. Scanning electron microscopy of (a) leuprolide PLA 28k (Leup 16.3%) microspheres; (b) leuprolide PLA 28k (Leup 20.7%); and (c) leuprolide PLA 18k microspheres.

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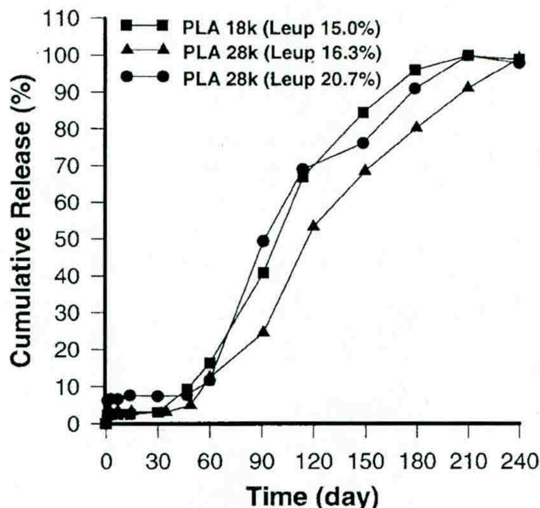

Fig. 2. *In vitro* release of leuprolide PLA microspheres in 0.1 M PBS (pH 7.4) at 37°C (n = 3).

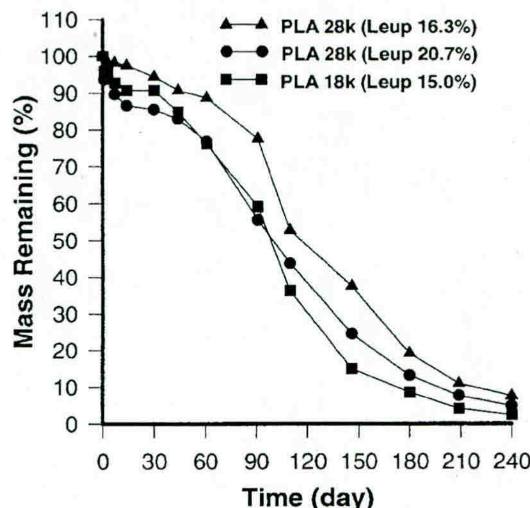

Fig. 4. Mass loss of leuprolide PLA microspheres in 0.1 M PBS (pH 7.4) at 37°C (n = 2).

mum drug load into minimum amount of polymer is desirable to reduce the dose of synthetic polymer to patients, while the microspheres contain the sufficient amount of peptide for long-term therapy. With a dispersion/solvent extraction–evaporation method, high drug incorporation efficiency was achieved at target peptide loads up to 21%.

In Vitro Release

The PLA microspheres showed a typical three-phase *in vitro* release. Three to seven percent initial release peptide was observed due to the fast diffusion of surface-associated peptide followed by a lag period of 30–45 days until the polymer hydrates and loses mass sufficient to initiate the erosion-controlled release. After the initial lag, a nearly linear and continuous release was observed over 5 to 6 months *in vitro*. The low-drug load (16.3%) PLA 28k microspheres showed ~3% initial release followed by a very slow release until day 45. The release increased after day 45 and a continuous release was observed between days 60 and 210 (Fig. 2). The high-drug load (20.7%) PLA 28K microspheres showed a faster and continuous release after 7% initial release. The higher initial and subsequent faster release might be due to the higher drug load. Peptide loading in the polymer matrix causes faster hydration and mass loss of polymer, leading to faster *in vitro* release (9). PLA 18k microspheres showed similar *in vitro* release to the high-drug load PLA microspheres. The microspheres showed ~3% initial release and almost no further release until day 30; a continuous release followed to 100% at day 210.

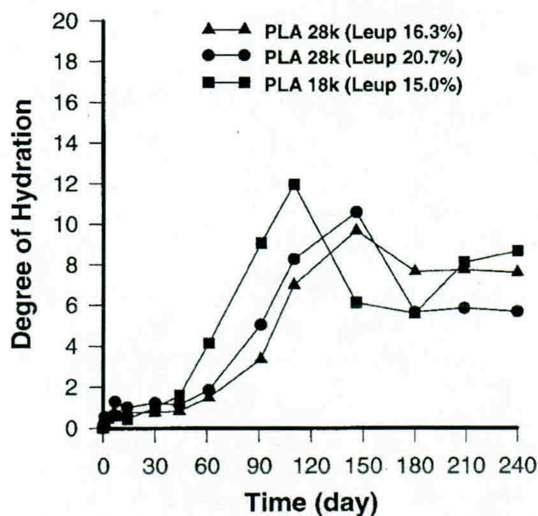

Fig. 3. Hydration of leuprolide PLA microspheres in 0.1 M PBS (pH 7.4) at 37°C (n = 2).

Hydration and Mass Loss

A low degree of hydration was observed with both PLA 28k microspheres during the first 45 days in 0.1 M PBS at 37°C (Fig. 3), which correlated well with the slow *in vitro* release observed for the first 45 days. The hydration increased slowly after day 45 and reached a maximum hydration at day 150. A faster and higher hydration was observed with PLA 18k microspheres. With PLA polymer, the decrease of hydration was observed after the maximum hydration due to the polymer degradation reaching a critical molecular weight and

Table II. Stability of Leuprolide Extracted from PLA Microspheres Incubated in 0.1 M PBS (pH 7.4) at 37°C

	Stability (%)	
Time (days)	PLA 28k (Leup 20.7%)	PLA 18k
1	100	99.5
3	99.4	99.5
7	99.4	99.4
14	99.4	99.4
30	99.2	99.6
45	99.5	99.2
60	99.6	99.1
90	97.5	97.6
110	95.9	92.6
161	91.9	88.1

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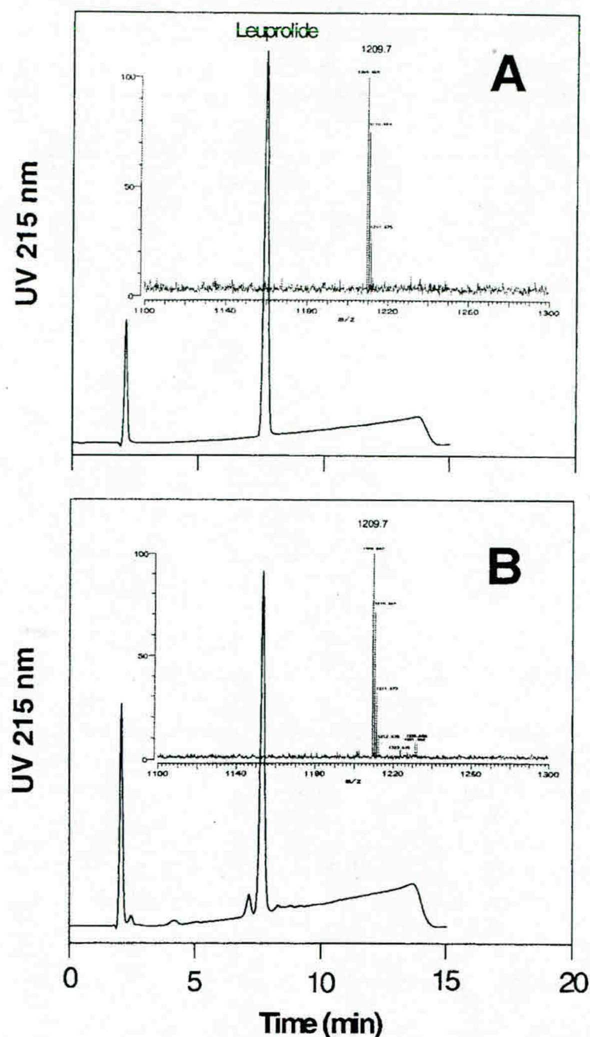

Fig. 5. HPLC chromatograms (large panels) and mass spectra (small panels) of standard leuprolide (A) and the peptide extracted from the PLA 28k (Leup 20.7%) microspheres incubated in 0.1M PBS (pH 7.4) at 37°C for 161 days (B).

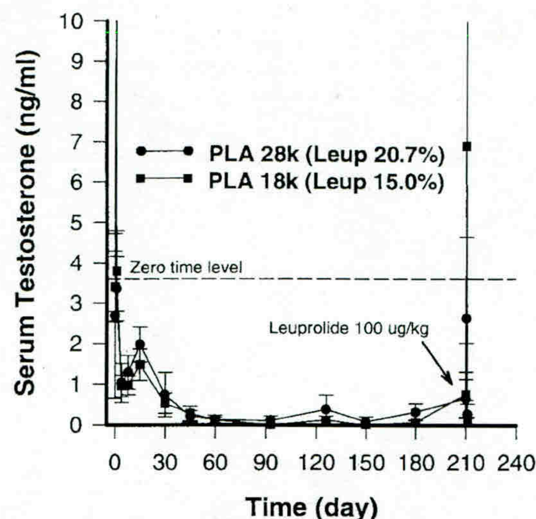

Fig. 6. *In vivo* serum testosterone concentrations after single administration PLA microspheres (dose = 18 mg/kg as leuprolide) in rats (n = 6). The arrow indicates the challenge of 100 μg/kg aqueous leuprolide at day 210. The zero time level represents the average serum testosterone level before the microsphere treatment in rats (n = 18).

loss of polymer matrix structure at which the capacity to retain water is decreased.

Four to seven percent initial mass loss was observed at the first day with high drug load PLA 28k (Leup 20.7%) and PLA 18k microspheres whereas PLA 28k (Leup16.3%) microspheres showed a lower initial mass loss of 2%, as shown in Figure 4. Both PLA 28k microspheres showed a slow mass loss for 45 days followed by a nearly linear mass loss up to day 150. The mass loss decreased between days 150 and 240 and the mass remaining was ~5% at day 240. The low-drug load microspheres showed higher mass remaining values than the high-drug load microspheres due to the low initial mass loss. However, after the initial mass loss, the mass loss profiles were similar. The PLA 18k microspheres also showed a slow mass loss for 30 days and then a nearly linear mass loss up to day 150 followed by a slow mass loss between day 150 and 240. The mass loss profiles of the PLA microspheres correlated well with the corresponding hydration and *in vitro* peptide release profiles.

Peptide Stability

As shown in Table II, over 99% of peptide remained as intact leuprolide in the PLA microspheres for 60 days before degrading slowly to 88–92% after 161 days. The large panel of Figure 5 shows the HPLC chromatograms of leuprolide (A) and the peptides extracted from the high-drug load PLA 28k microspheres incubated for 161 days (B). The major degradation product eluted prior to the intact leuprolide and minor degradation peaks were observed after the leuprolide peak. The small panels are the mass spectra of leuprolide (a) and the peptide from the PLA microspheres that eluted at the same retention time of intact leuprolide (b). This confirms that the major peptide peak from the PLA microspheres is intact leuprolide.

In Vivo Testosterone Suppression

Figure 6 shows the testosterone levels in rats after a single subcutaneous administration of the PLA leuprolide microspheres (dose = 18 mg/kg as leuprolide). Testosterone levels increased immediately after administration to 13 ng/mL due to the initial release of leuprolide from the PLA microspheres. After the initial elevation, the levels fell to ~1 ng/mL at day 4 followed by a slight increase to 2 ng/mL by day 14. The testosterone levels were suppressed to below 0.5 ng/mL at 30 days and the levels remained below 0.5 ng/mL through 180 days. The testosterone level elevated above 0.5 ng/mL at day 210 and a challenge of 100 μg/kg of aqueous leuprolide caused a peak of testosterone. This suggests that pituitary LHRH receptors were occupied no longer than 180 days by leuprolide from the PLA microspheres. A single injection of the PLA microspheres inhibited the pituitary-gonadal system in rates for 6 months and the *in vivo* efficacy correlated well with the duration of *in vitro* peptide release and the polymer degradation profiles.

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CONCLUSION

Six-month release microspheres of leuprolide were prepared successfully with PLA using a dispersion/solvent evaporation-extraction method. The *in vitro* behavior correlated well with the *in vivo* results. A single injection of the microspheres provided sustained testosterone suppression for 6 months in rats. The leuprolide PLA microspheres would be more convenient than 3- or 4-month formulation and potentially useful for improving compliance of the patients who need more reliable and effective hormone therapy.

ACKNOWLEDGMENTS

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