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Preparation, characterization and in vivo evaluation of 120-day poly(D,L-lactide) leuprolide microspheres

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Abstract

A 120-day poly(D,L-lactide) (PLA) microsphere delivery system for a luteinizing hormone-releasing hormone (LHRH) analogue, leuprolide, was prepared and evaluated. Leuprolide microspheres were prepared with PLA (m.w. 11 000 Da) by a dispersion/solvent extraction-evaporation method and characterized for drug load by HPLC, particle size by laser diffractometry and surface morphology by scanning electron microscopy. In vitro peptide release and polymer degradation were studied using a modified dialysis method. Serum peptide and testosterone levels were analyzed after subcutaneous administration using a rat model. Spherical microspheres with a mean diameter of 52 μm containing 13.4% peptide released 10% of the peptide within 24 h, followed by a linear release for 150 days. Serum leuprolide levels increased immediately after administration of the microspheres to 45.6 ng/ml, but then fell to 4.3 ng/ml at 15 days and ~2.0 ng/ml at 30 days where they remained for 120 days. The testosterone levels increased initially to 15 ng/ml and then decreased to below 0.5 ng/ml by day 4 where they remained for 120 days. In conclusion, a 120-day microsphere formulation of leuprolide was developed with excellent controlled peptide release characteristics and in vivo efficacy.

Keywords: Leuprolide; Luteinizing hormone-releasing hormone (LHRH) analogue; Poly(D,L-lactide); Microspheres; Testosterone suppression

1. Introduction

Controlled release dosage forms have been investigated to improve the efficacy of peptide drugs and eliminate the need for frequent administration. Biodegradable microspheres were shown to improve the bioavailability of peptides by protecting them from physical degradation and proteolysis in body fluids. Poly(D,L-lactide) (PLA) and poly(D,L-lactide-co-glycolide) (PLGA) are the most widely used and well-

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characterized materials for the preparation of biodegradable microspheres [1–8].

Leuprolide, the peptide drug used in this study, is a potent agonist of luteinizing hormone-releasing hormone (LHRH) currently used for the treatment of prostatic cancer, endometriosis and precocious puberty [9,10]. Utilizing an in-water drying method based on the w/o/w emulsion technique, 1-, 3- and 4-month release leuprolide microcapsules, Lupron®, have been developed [11–16]. In the in-water-drying method leuprolide acetate was dissolved in aqueous gelatin or water, dispersed into a polymer solution which was then emulsified into an external aqueous phase to form droplets. The microcapsules solidified after solvent removal and were freeze-dried. Several publications have described in vivo pharmacological and pharmacokinetic evaluations of these microcapsules [12–16].

In most literature reports of in vitro release, the microspheres were incubated in a certain volume of release medium, generally phosphate buffered saline (PBS) with a preservative, using glass or plastic test tubes at 37°C with optional agitation [17]. In these methods, the pH of the release medium consistently dropped due to the formation and accumulation of acidic polymer degradation products [18,19]. Continuous removal of the acidic polymer fragments and released drug by dialysis might be desirable to prevent the pH drop of the release medium. A dialysis in vitro release method for an LHRH antagonist, orntide, loaded PLGA microspheres has been developed by using a small dialysis unit and 0.1 M acetate buffer, pH 4.0 [20]. The in vitro release profile obtained with the dialysis method permitted a better correlation with in vivo release. However, only a little information is available on in vitro degradation kinetics and correlation between in vitro and in vivo release of long-term release peptide loaded PLA microspheres.

The goal of this study was to prepare and characterize a 4-month formulation of leuprolide microspheres using a dispersion method and to study in vitro drug release and in vivo efficacy. Also, an attempt to develop a modified dialysis method for testing in vitro release from biodegradable microspheres, which would exhibit a good in vitro–in vivo correlation, was undertaken.

2. Materials and methods

2.1. Materials

Leuprolide, [Des-Gly^{10}, D-Leu^{6}, Pro^{9}]-LHRH ethylamide, was obtained from Bachem (Torrance, CA, USA). Poly(D,L-lactide) (m.w. 11 000 Da, Resomer® R202H, PLA) was supplied by Boehringer Ingelheim (Ingelheim, Germany). Polyvinyl alcohol (m.w. 30 000–70 000; PVA) was supplied by Sigma (St Louis, MO, USA). Dialysis tubes (Tube-O-Dialyzer®) were purchased from Research Products International Co. (Mount Prospect, IL) and Spectrapor® CE dialysis membranes (MWCO 7000–300 000 Da) were supplied by Spectrum Medical Industries (Houston, TX). All other chemicals were obtained commercially as analytical grade reagents.

2.2. Preparation of microspheres

Leuprolide PLA microspheres were prepared by dispersing the homogeneous solution of polymer and drug into a PVA solution followed by solvent extraction/evaporation/dilution as previously described [21]. A solution of leuprolide in methanol was added into a 34% (w/w) solution of polymer in methylene chloride to form the clear solution. The resulting solution was then slowly injected into 0.35% aqueous PVA solution while mixed with a Silverson® L4R mixer (Silverson Machines, MA, USA) at 7000 rpm. The solvents were removed by stirring and continuous phase replacement at 38–40°C for 1 h. The solidified microspheres were recovered by filtration and dried under vacuum at room temperature for 48 h.

2.3. Particle characterization

2.3.1. Particle size distribution

Particles 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.

2.3.2. Surface morphology

The surface morphology was examined by scan-

ning electron microscopy (Hitachi Model S800, Japan) after palladium/gold coating of the microsphere sample on an aluminum stub.

2.3.3. Peptide content

A 10-mg amount of the microspheres was dissolved in 2.0 ml methylene chloride. The peptide was extracted from the polymer solution by addition of 10 ml of 0.1 M acetate buffer (pH 4.0) followed by agitation for 1 h. The peptide was assayed in the aqueous phase by HPLC method: Bondclone 10 C_{18} column (150×3.9 mm; Phenomenex, Torrance, CA, USA); mobile phase: 27% (v/v) acetonitrile, 0.1% trifluoroacetic acid; flow rate 1.0 ml/min; UV detection at 215 nm.

2.4. In vitro release study

In vitro release was carried out in 0.1 M PBS at 37°C using a modified dialysis method [20], which allowed control of the pH of the release medium and complete sample recovery. Dialysis membranes with various MWCOs and dialysis tubing with a large surface area were used to optimize the release method by assessing the permeability of the peptide through the membranes and the recovery of the peptide. A 50-mg amount of microspheres was suspended in 6 ml 0.1 M phosphate buffered saline (pH 7.4, PBS) containing 0.02% sodium azide in a 7-ml Tube-O-Dialyzer® tube (1.5 cm i.d.×5.5 cm length). The dialysis tubes were capped with a dialysis membrane-windowed screw cap (molecular weight cut off 300 000), placed in a 4-l dialysis tank filled with the same release medium and incubated at 37°C with continuous agitation. At each time point, remaining microspheres were recovered by filtration through a 0.8-μm filter. The amount of leuprolide remaining in the microspheres was determined by HPLC as described previously.

2.5. In vitro polymer degradation

2.5.1. Mass loss and hydration

At each time point, recovered wet mass 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:

$$MR\ (\%) = (W_d / W_0) \cdot 100 \quad (1)$$

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

2.5.2. Molecular weight (M_w) of polymer

The molecular weight distribution of PLA was determined by gel permeation chromatography (GPC) as described previously [22]. A Waters M-45 solvent delivery system with a Waters 990 Photodiode Array Detector was used. Two Ultrastyragel columns connected in series (7.8×300 mm each, one with 10^4-Å pores and one with 10^3-Å pores) were used. Samples, 1 mg/ml, were eluted with tetrahydrofuran at 1 ml/min. The weight average molecular weight (M_w) of each sample was calculated using monodisperse polystyrene standards, molecular weight 1000–50 000.

2.6. In vivo study

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 (12 mg leuprolide/kg) 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 samples were centrifuged in Microtainer® tubes (Becton Dickinson, Franklin Lakes, NJ) and serum was collected. Serum samples were frozen and stored at −20°C until analysis.

Serum leuprolide levels in rats were assessed using a radioimmunoassay (RIA) method as described previously [23,24]. The lower detection limit of the assay was 8 pg/ml. The intra- and interassay coefficients of variation were 6 and 9%, respectively.

Serum testosterone levels were assayed using Active™ Testosterone RIA DSL-4000 kits (Diagnostic Systems, Webster, TX). 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%.

3. Results and discussion

3.1. Microsphere characterization

Leuprolide microspheres were spherical with a relatively non-porous surface (Fig. 1, A). The average particle size was 51.7 μm, which is suitable for intramuscular or subcutaneous injections. The target load was 16.5% and the actual peptide content was determined to be 13.4%. Leuprolide encapsulation efficiency was 81.2%. The major difference between the microspheres prepared in this study and the microcapsules of 4-month Lupron® depot is the drug distribution in the polymer matrix. Lupron® microcapsules are prepared using a water-in-oil-in-water (w/o/w) emulsion technique and the particles contain discrete internal pockets of drug, hence called microcapsules. The microspheres in this study are prepared from a clear homogeneous solution of polymer and drug, and the drug is believed to be molecularly distributed in the PLA matrix.

3.2. In vitro release

Table 1 indicates that membranes with higher MWCOs showed higher rates of peptide permeability, faster equilibrium between the dialysis cells and the media and good peptide recovery. The dialysis tubing with a large surface area showed complete equilibrium after 48 h; however, it showed lower peptide recovery compared to the dialysis cells due to higher adsorption of the peptide on the large surface area. Based on these results, a dialysis membrane (MWCO 300 000) was selected for the in vitro release study. The dialysis method used in this study showed several advantages over the conventionally used test tube methods. This method eliminated: (a) the undesired loss of microspheres during sample preparation and handling; (b) pH changes of the release medium; and (c) shear stresses due to the centrifugation for sample recovery. Use of higher buffer capacity and frequent replacement of the release medium in the conventional tube method could also prevent a pH change. However, centrifugation is necessary to remove the supernatant without loss of microspheres for the conventional tube method. This will cause the packing of microspheres at the bottom of the tube that could affect the physical characteristics of the microspheres.

The in vitro release study of leuprolide microspheres is shown in Fig. 2. In the initial 15 days, 29% peptide released. Thereafter release was nearly linear through day 150 with ~0.5% released per day during this period. Release in the first 24 h is ~10%, probably due to the fast diffusion of the surface-associated peptide. The initial 24-h release is illustrated in the small panel. A 5% initial burst was observed in 30 min, followed by a decrease to ~2% in the next 5 h. Then a linear release ensued thereafter. The initial decrease in release in the first few hours has been a typical observation with leuprolide microspheres and the only reason which can be offered is that adsorption of released peptide occurs to the hydrated polymer surface in the early phase of release. Once the binding sites are saturated then the diffusional release proceeds.

3.3. In vitro polymer degradation

Both the peptide loaded and blank PLA microspheres showed slow mass loss in the first 50 days followed by linear profiles for 150 days (Fig. 3). The initial mass loss within 24 h was only 2.0%, confirming that the initial release was caused by diffusion of the peptide located on the surface of the microspheres. Blank microspheres showed lower mass loss than drug loaded microspheres. The peptide loaded and blank microspheres were slowly hydrated within 50 days (Fig. 4). Hydration rate increased between 50 and 90 days, with the peptide loaded microspheres showing a higher degree of hydration. The drug loading of PLA microspheres led to higher uptake of water into the polymer matrices and a faster mass loss. The molecular weight of residual polymers from both drug loaded and blank microspheres gradually decreased from 11 000 to 7000 Da after 90 days (Table 2). There was no significant difference in polymer degradation rate between drug loaded and blank microspheres. Blank and drug loaded microspheres showed relatively smooth surfaces with spherical shape at the beginning of the degradation study (Fig. 1). The drug loaded microspheres possessed very porous interior structures while the blank microspheres showed an extremely shrunken shape after 35 days. After 70 days, the drug

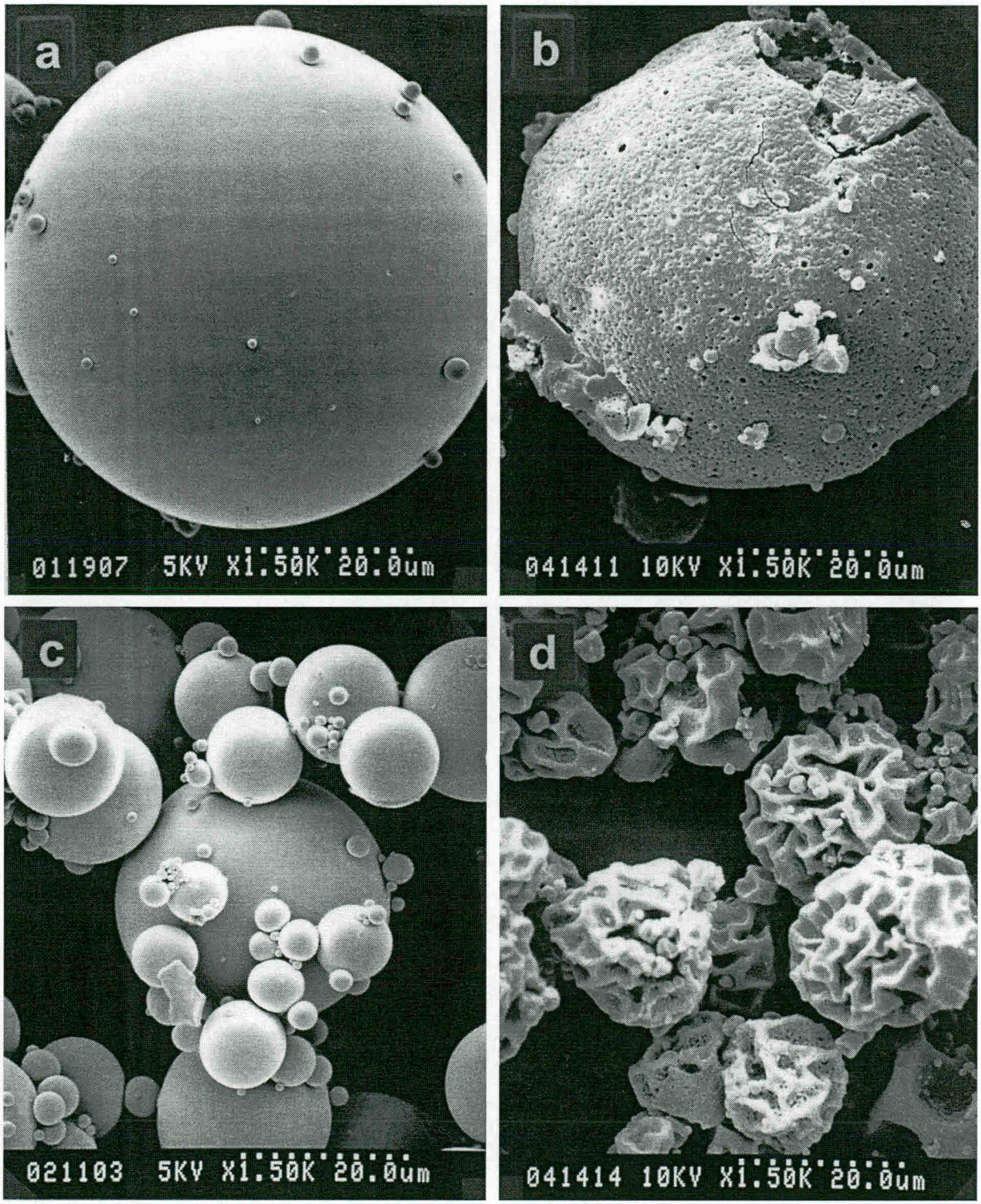

Fig. 1. Scanning electron microscopy of: (a) leuprolide microsphere; (b) leuprolide microsphere incubated in PBS at 37°C for 35 days; (c) PLA blank microspheres; and (d) PLA blank microspheres incubated in PBS at 37°C for 35 days.

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