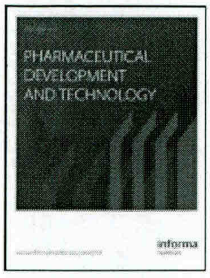

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RESEARCH ARTICLE

Enhancing Initial Release of Peptide from Poly(*d*,*l*-lactide-*co*-glycolide) (PLGA) Microspheres by Addition of a Porosigen and Increasing Drug Load

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ABSTRACT

*The objective of this study was to evaluate formulation variables such as drug load and addition of a porosigen in achieving an increased initial release of peptide from poly(*d,l*-lactide-co-glycolide) (PLGA) microspheres by altering carrier characteristics. Leuprolide acetate-loaded PLGA microspheres were prepared by a solvent-extraction–evaporation process and were characterized for their drug load (HPLC assay), bulk density (tapping method), size distribution (dynamic light scattering), specific surface area (Brunauer–Emmett–Teller [BET] analysis), surface morphology (scanning electron microscopy), in vitro drug release (at 37°C), and in vivo efficacy (suppression of rat serum testosterone). Increasing the drug load, and adding various amounts of calcium chloride to organic and aqueous phases of the emulsion during processing yielded particles with increased porosity, lower bulk density, higher specific surface area, and accordingly higher initial release. In an animal model, these formulations showed a faster onset of testosterone suppression compared to microspheres without higher drug load or calcium chloride. The approaches employed in this study were found to be effective in avoiding the therapeutic lag phase usually observed with microencapsulated macromolecular drugs.*

KEY WORDS: *Drug load; In vitro characteristics; In vivo efficacy; Leuprolide acetate; Peptide; PLGA microspheres; Porosigen.*

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INTRODUCTION

There is a need for developing sustained-release dosage forms for peptides and proteins, because these macromolecular drugs pose unique difficulties that include low oral bioavailability, instability in the gastrointestinal tract, dosage form design, and subsequent frequent and painful injections (1,2). Recent reports have shown that polymer-based systems can be useful in achieving sustained and controlled delivery with less frequent parenteral administration (3–8). Injectable systems of leuteinizing hormone releasing hormone (LHRH) analogues, based on poly(*d*,*l*-lactide-*co*-glycolide) (PLGA), were reported to be therapeutically advantageous and have been successfully commercialized in hormonal treatment of cancer (9–11). An advantage with these systems is that their biodegradation and drug release can be controlled by modifying polymer characteristics such as molecular weight, hydrophilicity, and comonomer (lactide/glycolide) ratio (12–15). In particular, compared to implants and nonbioerodible matrices, biodegradable and injectable microspheres obviate the need for surgical procedures during administration and subsequent removal.

Release of conventional low molecular weight drug molecules from polymer systems is primarily via a diffusional process (16). Macromolecular drugs such as proteins and peptides release via both diffusion and matrix degradation processes (17–19). Often, the initial diffusional release of proteins and peptides is not sufficient to elicit a sustained pharmacological response because diffusion of macromolecules through the polymer structure may be limited. This results in an unwanted therapeutic lag phase until the onset of polymer erosion and subsequent accelerated drug release. In addition to drug and polymer characteristics, diffusional release of drugs is controlled by carrier-related factors such as structure, size, surface area, porosity, and drug load (16,17,20,21). These factors can be controlled by formulation approaches and can be useful in altering the drug release to avoid the undesired therapeutic lag phase.

The purpose of this study was to evaluate preparation variables such as drug load and addition of a porosigen in achieving modified and, in particular, increased initial/diffusional release of a peptide from PLGA microspheres. LHRH-analogue-loaded microspheres were prepared by a solvent-extraction–evaporation process and physicochemical characteristics of microspheres were correlated with in vitro release profiles of peptide. These formulations were also compared for their efficacy in suppressing serum testosterone levels in rats. The peptide selected for these studies was the LHRH superagonist, leuprolide. Polymer selected was 50:50 PLGA with uncapped end groups to increase hydration. Calcium chloride was chosen as a porosigen to increase the porosity of peptide-loaded microspheres.

MATERIALS AND METHODS

Materials

PLGA (50:50) polymer, Resomer® RG503H (MW 28,032) was obtained from Boehringer Ingelheim (Wallingford, CT). LHRH analogue and leuprolide as an acetate salt was purchased from Bachem Inc. (Torrance, CA). Calcium chloride, dihydrate, was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of analytical reagent grade.

Preparation of Microspheres

Microsphere formulations in a typical batch size of 1.5 g were prepared by a solvent-extraction–evaporation method (22). Briefly, the preparation procedure was as follows. A methylene chloride (CH_2Cl_2) solution that contained approximately 16% w/w of polymer was mixed with a methanolic solution (CH_3OH) of peptide. The typical ratio of CH_3OH to CH_2Cl_2 was 0.24. The resulting mixture (dispersed phase [DP]) was then slowly added to 500 ml of 0.35% w/v poly(vinylalcohol) (PVA) aqueous solution (continuous phase, CP) maintained at 25°C with a water jacket. CP and DP were emulsified for 5 min using a homogenizer (Silverson L4R, Silverson Instruments Corp., MA) at 7000 rpm. The stirring rate was decreased to 500 rpm and the temperature of the emulsion was increased to 40°C to extract and evaporate the organic phase over 1 hr. The system was cooled to 25°C, and particles were recovered on 5 μm pore size solvent-resistant membrane filters and then dried overnight under vacuum at room temperature.

The target drug load for the formulations was 12.5% w/w, except for higher drug-loaded formulation (B), which had a set load of 20%. A slightly higher polymer concentration (18% w/w) in CH_2Cl_2 and 0.27 ratio of CH_3OH/CH_2Cl_2 were employed for the higher drug load formulation. For calcium-chloride-containing formulations (C–G), various amounts (Table 1) of salt were also dissolved in the CP and the CH_3OH component of the DP.

Process Characterization

The microsphere preparation process was characterized by determining microsphere yield, encapsulation ef-

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Table 1

Manufacturing Parameters of Peptide-Loaded RG503H Microspheres:[a] Addition of Porosigen

Formulation ID	Calcium Chloride (Molar) Concentration		
	Methanol	CP	Ratio (Methanol/CP)
A	–	–	–
B	–	–	–
C	0.268	0.100	2.68
D	0.268	0.060	4.46
E	0.155	0.060	2.58
F	0.117	0.060	1.95
G	0.117	0.045	2.60

[a]Target drug load for A, C–G is 12.5% w/w and for B, it is 20% w/w.

ficiency, and mass balance of peptide. The total microsphere yield was calculated gravimetrically on the basis of polymer/drug recovery. For the incorporation efficiency, peptide encapsulated in microspheres was expressed as a percentage of theoretical target load. The mass balance of the peptide was obtained as the sum of the peptide remaining in the CP (unincorporated) plus the amount incorporated in microspheres.

Characterization of Microspheres

Microspheres formulated as above were characterized for their drug content, bulk density, specific surface area, mean sizes, surface morphology, in vitro drug release, and in vivo efficacy.

Drug Content

Drug-loaded microspheres were quantitatively dissolved in CH_2Cl_2 and the peptide was extracted into acetate buffer (pH 4, 0.1 M) by shaking for 1 hr on a wrist-action shaker (Burrell, Pittsburgh, PA). The aqueous buffer phase was separated by centrifugation and extracted peptide was quantitated by modification of a reported reversed-phase HPLC method (23). This extraction procedure was repeated with fresh buffer and the combined peptide amounts from the two extractions were reported as the drug content, expressed as percent w/w of microspheres. Triplicate samples were used to determine the drug content and mean values. HPLC analytical conditions were as follows: chromatographic separation was achieved on a C_{18} μBondapak column (3.9 × 300 mm, Waters) by using a variable-wavelength detector at 220 nm, a gradient pump (both from Dionex Corp., Sunnyvale, CA), and an autosampler (Thermo Separation Products, Fremont, CA). The mobile phase was an isocratic mixture of HPLC grade water and acetonitrile in the ratio of 68:32 adjusted to pH 4.0 by adding approximately 0.1% trifluoroacetic acid. The flow rate was set at 1.1 ml/min.

Bulk Density

The dry microspheres were quantitatively transferred to a graduated test tube. The test tube was subsequently tapped 20 times from a vertical distance of approximately 0.5 in. and the occupied volume was recorded. The tapping process was repeated until the volume occupied by particles remained unchanged. The final volume was recorded as bulk volume, v_b, and the tapped bulk density (g/cm^3) was calculated as m/v_b, where m was the weight of microspheres employed.

Specific Surface Area

The specific surface area was determined using an ASAP 2000 surface area analyzer (Micromeritics, Norcross, GA) by Brunauer–Emmett–Teller (BET) transformation of the adsorption–desorption isotherms of Kr on the surface of the microspheres. The area values were normalized to the sample weight. The sample weight typically was in the range of 250–300 mg.

Size Distribution

Particle size distribution was determined by using a laser diffraction particle sizer (Malvern Instruments, Southborough, MA). The microspheres were suspended in 0.1% aqueous Tween 80 solution and either a 63-mm (for

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a size range of 0.5–118 μm) or a 100-mm (for a size range of 1.9–188 μm) focal length lens was employed to determine particle size, while the sample was stirred at about 100 rpm in the sample cell using a magnetic stirrer bar.

Surface Morphology

Particle surface morphology was examined by scanning electron microscopy (model S800, Hitachi, Japan) after the microsphere sample was coated with gold–palladium on an aluminum stub.

In Vitro Drug Release

Approximately 10 mg of peptide-loaded microspheres was incubated with 10 ml of 0.033 M phosphate buffer (pH 7) at 37°C in a temperature-controlled oven. Separate test tubes with equal amounts of microspheres were maintained for each time point of the release study. Aggregation and settling of microspheres were noticed during the release study. Test tubes were shaken twice weekly and 8 ml of supernatant was replaced with fresh buffer weekly to maintain sink conditions. At the sampling time, microspheres were separated by centrifugation. To minimize the loss of microspheres only 8 ml of the supernatant was removed and analyzed by HPLC for the drug content. The total peptide remaining in the microspheres and supernatant (2 ml that was left behind) was extracted into acetate buffer and quantitated by HPLC. On the basis of the supernatant analysis, peptide in the remaining 2 ml of supernatant was calculated and accounted for in the final calculations of peptide that remained in the microspheres. Compared to standards, no additional peaks were noted in the chromatographs obtained from the analysis of test samples.

Peptide release was determined on the basis of the drug remaining in the microspheres rather than on the released amount of peptide, because the peptide has limited stability in the releasing medium. Released drug was calculated as the difference between initially loaded drug and that remaining in the microspheres, and expressed as a percent of initially loaded amount.

In Vivo Evaluation

Male Sprague Dawley rats (Harlan Sprague Dawley, Inc., Chicago, IL) were employed ($n = 6$) in evaluating the formulation efficacy in suppressing serum testosterone. Animals were maintained as per the guidelines set forth in reference 24. All formulations were suspended in a mixture of 1% carboxymethylcellulose and 2% mannitol and injected into rats subcutaneously just below the neck region, at a drug dose of 100 μg/kg/day based on literature reports (25). A single injection was given to each animal immediately after an initial sample was collected from the tail vein. Additional samples were collected at 0.25, 1, 4, 8, 15, 25, 32, 33, 42, and 43 days after dose administration. On days 32 and 42, all the groups were challenged with 100 μg/kg of leuprolide acetate. Following regular sampling, additional samples were taken at 6 and 24 hr after the challenge. Samples were assayed in duplicate for testosterone analysis by radioimmunoassay using standard commercial kits.

RESULTS AND DISCUSSION

Process Characterization

The adapted manufacturing procedure resulted in a high microsphere yield (88–92%), high encapsulation efficiency (72–88%), and close to 100% peptide mass balance. Leuprolide acetate is highly water-soluble and was expected to have low microencapsulation efficiency by using the conventional oil/water (o/w) solvent-evaporation method due to rapid partitioning into the aqueous

Table 2

Characteristics of Peptide-Loaded Microspheres: Effect of Drug Load and Calcium Chloride

Formulation ID	Drug Content (%w/w)	Encapsulation Efficiency (%)	Surface Area (m^2/g)	Size (μm, 0.5, v^a)	Bulk Density (g/cm^3)
A	11.9	95.2	0.387	18.0	0.54
B	16.3	81.5	7.278	27.0	0.29
C	9.79	78.3	1.249	24.7	0.48
D	9.08	72.6	1.480	24.2	0.42
E	10.1	80.8	1.023	20.5	0.57
F	10.9	87.2	0.913	28.5	0.56
G	11.0	88.0	0.778	24.9	0.64

[a] *v*: Size distribution on a volume basis.

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