Biodegradation and biocompatibility of contraceptive-steroid-loaded poly (DL-lactide-co-glycolide) injectable microspheres: in vitro and in vivo study

Magharla Dasaratha Dhanaraju\textsuperscript{a,b,*}, Rajagopalan RajKannan\textsuperscript{c}, Devarajan Selvaraj\textsuperscript{c}, Rajadas Jayakumar\textsuperscript{c}, Chandrasekar Vamsadhara\textsuperscript{b}

\textsuperscript{a}Department of Pharmaceutics, GIET School of Pharmacy, NH-5, Rajahmundry 533 294, India
\textsuperscript{b}Institute of Pharmacology, Madras Medical College, Chennai 600 003, India
\textsuperscript{c}Bioorganic and Neurochemistry Laboratory, Central Leather Research Institute, Adyar, Chennai 600 020, India

Received 10 August 2005; revised 14 November 2005; accepted 30 January 2006

Abstract

\textbf{Purpose:} A controlled-release drug delivery of contraceptive steroids levonorgestrel (LNG) and ethinyl estradiol (EE) has been developed by successful encapsulation of LNG and EE in poly (lactide-co-glycolide) (PLG) microspheres.

\textbf{Materials and Methods:} Smooth, spherical, steroid-loaded PLG microspheres with a mean size of 10–25 \(\mu\text{m}\) were prepared by using the water/oil/water double-emulsion solvent evaporation method.

\textbf{Results:} In vitro release profiles showed an increased burst release of LNG/EE on Week 1; thereafter, the release was sustained. At the end of Week 7, the release of LNG/EE from 1:5 and 1:10 PLG microspheres was 75.64\% and 62.55\%, respectively. In vitro degradation studies showed that the PLG microspheres maintained surface integrity up to Week 8 and then eroded completely by Week 20. In an in vivo study, the serum concentration of LNG/EE in rats showed a triphasic release response, with an initial burst release of 8 ng/mL LNG and 14 pg/mL EE on Day 1; thereafter, a controlled release of the drugs to the systemic circulation was maintained until Week 15, maintaining constant drug levels of 2 ng/mL LNG and 3–4 pg/mL EE in the blood. Histological examination of steroid-loaded PLG microspheres injected intramuscularly into the thigh muscle of Wistar rats showed minimal inflammatory reaction, demonstrating that contraceptive-steroid-loaded microspheres were biocompatible.

\textbf{Conclusion:} This controlled-release and biocompatible nature of the PLG microspheres may have potential application in contraceptive therapy.

\(\text{\textcopyright\ 2006 Elsevier Inc. All rights reserved.}\)

\textbf{Keywords:} Poly (lactide-co-glycolide) (PLG) microspheres; Levonorgestrel (LNG); Ethinyl estradiol (EE); Biocompatibility; Biodegradability; Controlled drug delivery

1. Introduction

In recent years, there has been immense interest in using polymeric microspheres for the sustained or controlled release of protein and peptide drugs because of their ease of fabrication, relatively simple administration and versatility. In comparison to conventional dosage forms, biodegradable polymeric matrices provide improved delivery methods for small molecules, peptides, proteins and nucleic acids [1]. One of the most commonly used polymers is poly (lactide-co-glycolide) (PLG) because of its proven nature of releasing the drug at a relatively slow rate over a prolonged time. The rate of PLG microsphere degradation in achieving controlled release affords less frequent administration, thereby increasing patient compliance, reducing discomfort, protecting the therapeutic compound and maintaining constant blood levels of the drug within the body [2–5].

Contraceptive steroids levonorgestrel (LNG) and ethinyl estradiol (EE) are used in combination to depress the gonadotrophins follicle-stimulating hormone and luteinizing hormone, thus preventing ovulation. The oral use of LNG and EE is limited since they are not tolerated at higher doses. A long-term, systemic, controlled delivery of contraceptive steroids appears to be essential in the regulation of reproductive function, as well as in the case
of postmenopausal therapy [6,7]. However, the pharmacological approach to fertility control is still mainly by oral administration and transdermal delivery of contraceptive steroids. The main disadvantage of the oral combined pill is the requirement of daily ingestion and subsequent daily variations in blood concentration, leading to blood-drug-level-dependent unwanted side effects and a short biological half-life of the drug [8]. For transdermal delivery systems, variations in an individual’s skin permeability and poor patient compliance can result in insufficient or excessive mean serum concentrations. Side effects due to variations in the concentration of the drugs could be avoided by using a sustained or long-term controlled delivery system to ensure a longer period of drug availability in the blood at optimum concentration.

For long-term controlled delivery of contraceptive steroids, polymeric drug delivery systems have attracted considerable attention in the past several years [9–15]; currently, there is only a small number of commercially available products that utilize this technology. Progestasert®, a T-shaped device, was designed to provide a constant release of progesterone through a rate-controlling membrane of ethylene vinyl acetate, whereas Norplant® is a silicone-based device for the delivery of LNG. However, both polymers are nonbiodegradable, and the devices have to be removed after depletion of the drug [16]. To overcome this problem, biodegradable PLG microspheres were developed for implantation under the skin without special surgery [17]. The major advantage of biodegradable PLG microspheres is that they ensure a continuous delivery of the drug via diffusion to surrounding tissues or by polymer erosion and they enhance the bioavailability of compounds that are poorly soluble in body fluids. A constant release prevents cyclic variations in drug concentrations in the blood with time and offers maximum pharmacological efficiency at a minimum drug dose.

Recently, we reported on the development of the microsphere encapsulation of the lipophilic drugs LNG and EE using biodegradable PLG and poly (e-caprolactone) though the water/oil/water (W/O/W) double-emulsion solvent evaporation method, as described previously [18,19]. Briefly, a saturated solution of LNG (15 mg) and EE (3 mg) (in a 1:5 ratio) was mixed with ethanol/water (7:3), which was then emulsified in 10 mL of dichloromethane containing 180 mg of PLG polymer to form the W/O/W primary emulsion. The emulsion formed was stirred at 4000 rpm for 10 min and then added to an external phase containing 1% PVA solution to produce the W/O emulsion. The formed multiple emulsion was kept under constant stirring for 4 h at 600 rpm by magnetic spin bar assembly. Microspheres were separated by centrifugation at 2000 rpm for 10 min, washed thrice with phosphate buffer (pH 7.4) and then dried in nitrogen atmosphere.

2.2. Preparation of microspheres

The PLG microspheres containing both LNG and EE were prepared by the W/O/W double-emulsion solvent evaporation method, as described previously [18,19]. The PLG microspheres were prepared by the W/O/W double-emulsion solvent evaporation method, as described previously [18,19]. Briefly, a saturated solution of LNG (15 mg) and EE (3 mg) (in a 1:5 ratio) was mixed with ethanol/water (7:3), which was then emulsified in 10 mL of dichloromethane containing 180 mg of PLG polymer to form the W/O/W primary emulsion. The emulsion formed was stirred at 4000 rpm for 10 min and then added to an external phase containing 1% PVA solution to produce the W/O emulsion. The formed multiple emulsion was kept under constant stirring for 4 h at 600 rpm by magnetic spin bar assembly. Microspheres were separated by centrifugation at 2000 rpm for 10 min, washed thrice with phosphate buffer (pH 7.4) and then dried in nitrogen atmosphere.

2.3. Morphology of microspheres

The morphological features of PLG microspheres both initially and during the degradation process were carried out using scanning electron microscopy (SEM). The drug microspheres were sprinkled onto one side of a double-sided adhesive stub. The stub was then coated with conductive gold with Joel-JFC 1100E sputter coater and examined under a Joel-JFC 5300 scanning electron microscope (Joel Inc., Peabody, MA, USA) for qualitative assessment of microsphere morphology.

2.4. Drug content of microspheres

The content of LNG and EE loaded into PLG microspheres was determined by dissolving 100 mg of microspheres in 5 mL of dichloromethane. To do this, 5 mL of methanol was added, the solution was evaporated under vacuum to eliminate dichloromethane and the polymer was allowed to precipitate. The drugs dissolved in methanol were filtered using a 0.1-μm Millipore [Millipore (India) Pvt. Ltd., Peenya, Bangalore, India] filter assembly, suitably diluted and subsequently injected into a Hypersil C18 (250×4.6-mm) column (Thermo Electron Corp., San Jose,
Drug content was determined by a previously reported procedure [20]. The mobile phase used was a combination of acetonitrile/methanol/water in a ratio of 3.5:1.5:4.5 at a flow rate of 2 mL/min; the eluted sample was detected at 215 nm using Shimadzu high-performance liquid chromatography (HPLC) LC 10AT-vp (Shimadzu Corporation, Kyoto, Japan).

2.5. In vitro degradation studies

One hundred milligrams of PLG microspheres containing steroidal contraceptives LNG and EE was placed in test tubes containing phosphate-buffered saline (PBS) buffer (pH 7.4). The test tubes were kept in an incubator shaker maintained at 37±1°C. The buffer medium was renewed every week. After predetermined periods, samples were taken out by centrifugation of the buffer, then washed with distilled water and dried under vacuum at room temperature.

2.6. In vitro drug release studies

Release studies of LNG and EE from PLG microspheres were carried out under physiological conditions by simulating the in vitro environment. Fifteen milligrams of drug equivalent microspheres was weighed and added to 50 mL of PBS in an Erlenmeyer flask. The flask was agitated at 50 rpm at 37±1°C in an incubator shaker. A sample (1 mL) was taken at different intervals up to 5 months and replaced with fresh medium. The amount of released drug was estimated from the sample by HPLC [20].

2.7. In vivo drug release study

Colony-inbred female rats of Wistar albino strain were used for the in vivo drug release study. Twelve rats weighing between 170 and 200 g were randomized into two groups of six animals each and were evaluated and used for the contraceptive efficacy of LNG/EE-loaded PLG microspheres. The rats were maintained in a room at 25±3°C. The animals were exposed to a 12-h dark/light cycle, fed ad libitum with commercial pellet diet (Hindustan Ltd., Bangalore, India) and given free access to water. Sterile microspheres containing 5 mg of LNG and 0.69 mg of EE drug equivalent dose per kilogram of body weight, and pure contraceptive agents of 5 mg of LNG and 0.69 mg of EE were injected intramuscularly into the thigh muscle after reconstitution in a suitable vehicle (2 mL of physiological saline containing 0.1% Tween-80). Blood samples were collected from orbital venous plexus punctures at different time intervals up to 5 months.

Fig. 1. Scanning electron micrograph of LNG/EE-loaded PLG microspheres.

Fig. 2. Scanning electron micrograph of PLG microspheres retrieved from phosphate buffer medium (pH 7.4) on: (A) postdegradation Week 2, (B) postdegradation Week 8 and (C) postdegradation Week 20.
centrifuged at 3000 rpm for 10 min, and serum was collected and stored frozen at −20°C until analysis. Drug concentrations in blood serum were determined after suitable extraction and dilution with mobile-phase solvent using the HPLC technique.

2.8. In vivo biocompatibility and stability study

Eighteen rats (n=6) weighing between 170 and 200 g were used for the in vivo compatibility and stability study. The in vivo biocompatibility and stability of the LNG/EE-loaded PLG microspheres were examined after implanting the microspheres into the thigh muscle of Wistar rats via intramuscular injection; 5.69 mg/kg body weight of drug equivalent dose of sterilized microspheres was suspended in 2 mL of physiological saline containing 0.1% Tween-80 and injected using an 18-gauge needle. The injected microspheres, along with their surrounding tissues, were excised on Weeks 1, 8 and 20 postimplantation after anesthetizing the animals with an overdose of pentothal sodium (80 mg/kg body weight). Control tissues were taken from the thigh muscle of the opposite leg. Retrieved samples were processed for histological examination.

2.9. Histological examination

Tissue samples were fixed in 10% phosphate-buffered formaldehyde solution and embedded in paraffin. The samples were then sectioned at a thickness of 7 μm using an automatic microtome, followed by staining with hematoxylin and eosin (H&E). The stained sections of each test sample were examined by light microscopy (Polyvar 2 photomicroscope; Leica, Bensheim, Germany) for tissue inflammatory reaction and were photographed.

2.10. Tissue processing for immunohistochemistry

The tissues were immersed for 24 h at 4°C in 10% phosphate-buffered formaldehyde fixative and rinsed in cold...
PBS, and specimens were covered with 20% sucrose in PBS and allowed to stand at 4°C overnight. The tissues from all groups were processed for immunofluorescent localization of tissue antigens. The specimens were embedded in Optimum Cutting Temperature (Tissuetek; Sakura Finetek, Torrance, CA, USA) embedding medium, frozen and sectioned at 16 μm. Serial transverse and longitudinal sections around the injected muscle area were taken and permeabilized with 0.3% Triton X-100 in PBS for 2 h and then incubated for 24 h at 4°C with the primary antibody goat polyclonal IL-1α. Slides were then washed with PBS and incubated with rabbit antigoat IgG secondary antibody conjugated with FITC at the recommended dilution (1:500) for 1 h at room temperature. The samples were further washed with PBS, and coverslips were mounted with bicarbonate-buffered glycerol (pH 8.6) and viewed with a Zeiss Axioscop 2 fluorescent microscope (NAG f. HBO50; Carl Zeiss, Jena and Oberkochen, Germany). The following control procedures were applied to all stainings: tissues from the control and microsphere-injected area underwent the same immunohistochemical protocol, but with omission of the primary antibody and replacement of the primary antibody with normal goat serum with an additional control [21].

3. Results

3.1. Morphology of microspheres

The PLG microspheres containing LNG and EE, which were prepared by the W/O/W double-emulsion solvent evaporation technique, were spherical, individual and non-porous, with mean particle sizes from 10 to 25 μm (Fig. 1). The microspheres obtained from both 1:5 and 1:10 drug/polymer ratios were free-flowing and had adequate syringability when mixed with vehicle for in vivo administration.

3.2. In vitro degradation studies

The surface morphology of LNG/EE-loaded PLG microspheres before and after degradation was compared as a measure of in vitro degradation (Fig. 2). The surface morphology of PLG microspheres was unchanged up to the end of Week 1 (Fig. 2A), indicating the crystalline behavior of the matrix. After Week 8, PLG microspheres collapsed and were found to be highly porous in nature (Fig. 2B). Finally, on Week 20, it was observed that the PLG microspheres had eroded completely (Fig. 2C).

3.3. In vitro release studies

The release profiles of LNG and EE from PLG polymeric microspheres showed an initial burst release on Week 1, followed by sustained release of the drugs. The cumulative release of LNG/EE from 1:5 and 1:10 (drug/polymer) PLG microspheres at the end of Week 7 was 75 (64%) and 62 (55%), respectively (Figs. 3 and 4).

3.4. In vivo drug release studies

After injecting drug-loaded PLG microspheres intramuscularly, the serum concentrations of the LNG and EE in rats showed a triphasic release response (Figs. 5 and 6). Initially, 8 ng/mL LNG in serum on Day 1 was attributed to the higher amount of drug release from the microspheres. Thereafter, the release of LNG in the blood was estimated to maintain a constant level of 2 ng/mL throughout the study. The initial release of EE was 14 pg/mL, and the system was capable of constantly delivering 3 pg/mL EE in the blood.

3.5. In vivo biodegradation of microspheres

After muscular implantation in rats, the morphology of the microspheres changed progressively with time and

Fig. 7. Scanning electron micrograph of PLG microspheres retrieved from an implanted site on: (A) postdegradation Week 1, (B) postdegradation Week 8 and (C) postdegradation Week 20.
finally disintegrated. On Week 1, PLG microspheres retained good sphericity, similar to that of the microspheres before implantation (Fig. 7A). On Week 8, the PLG microspheres were noticeably degraded into smaller fragments (Fig. 7B). The biodegradation of the PLG microspheres retrieved after Week 20 was more significant compared to that retrieved on Week 8, indicating that the microspheres were degraded into fine fragments with greater size reduction (Fig. 7C).

3.6. Histological examination

The levels of macrophage infiltration were studied histologically. The tissues injected with LNG/EE-loaded PLG microspheres stained with H&E and retrieved after Weeks 1, 8 and 20 showed differential macrophage response at different time intervals (Fig. 8). Histological analysis of the normal tissue showed the least macrophage infiltration (Fig. 8A), whereas the drug-loaded PLG microspheres injected after Week 1 (Fig. 8B) showed heavy macrophage infiltration around the muscle at the injection site. These levels of macrophage infiltration became reduced after Week 8 (Fig. 8C) and almost disappeared after Week 20 of microsphere injection (Fig. 8D).

3.7. Immunohistochemistry

The level of inflammatory cytokines was determined by immunostaining for IL-1α (Fig. 9). After Week 1, the expression of IL-1α was significant in the case of animals injected with PLG microsphere formulations (Fig. 9B), in accordance with histological analysis. The presence of IL-1α confirmed the increased macrophage infiltration around the injection site. Conversely, after Week 8 of injection, a moderate amount of IL-1α was observed, which indicates that the production of inflammatory cytokines at the injection site declined (Fig. 9C). The immunofluorescent images taken of tissue samples after Week 20 of PLG microsphere injection showed little or no reaction to the antibody against IL-1α. When compared with those of Weeks 1 and 8, it was clearly observed that the production of inflammatory cytokines at the injection site had ceased, as the macrophage infiltration decreased at the injection site (Fig. 9D).

4. Discussion

The W/O/W double-emulsion solvent evaporation method was used to prepare PLG microspheres in order to obtain spherical LNG/EE-loaded PLG microspheres with a narrow size distribution from 10 to 25 μm. The surface morphology of PLG microspheres in in vitro degradation studies revealed that, up to Week 1, the microspheres remained unchanged, indicating the crystalline behavior of PLG matrices [22]. By Week 8, the microspheres had disintegrated into smaller particles, and the surface of the spheres had collapsed and were highly porous, signifying that the PLG polymer was gradually hydrolyzed but had not yet
decreased sufficiently in molecular weight to allow an increased diffusional release of the drug. The PLG microspheres had eroded completely by Week 20, releasing the remaining drug, because the molecular weight of the polymer and also the amount of drug present in the polymer matrix were sufficiently low to allow its solubilization in the simulated medium (aqueous environment) [23].

The in vitro release profiles of LNG and EE from 1:5 and 1:10 (drug/polymer) PLG microspheres showed an initial burst release on Week 1 followed by a sustained release of the drugs. A reason for the observed initial burst release could be the unstable nature of inner water emulsion droplets during solvent evaporation, leading to coalescence and probably causing the drug to locate at the surface of polymeric microspheres [24]. However, the initial burst effect of all formulations was well below 20% because of extensive washings of the microspheres, which removed the surface-free, poorly entrapped and surface-associated drug crystals of the PLG microspheres. LNG and EE are lipophilic in nature, showing lesser tendencies to migrate toward the aqueous medium; therefore, some of the initial releases were due to simple partition diffusion of the drugs through intact polymeric spheres. The drug release rate from PLG matrices has been controlled by both the diffusion rate of the drug in the matrices and the degradation rate of matrices [25]. Discharges of the remaining amount of the drug from the polymer matrix after Week 1 were dependent on the rate of polymer erosion [26]. At the end of Week 7, the cumulative release of LNG/EE from 1:5 and 1:10 (drug/polymer) PLG microspheres was 75.64% and 62.55%, respectively. This shows that the degradation rate of PLG polymers was very slow in an aqueous medium because of hydrophobicity, thereby assisting a controlled release of the drug [27].

The serum concentration of LNG and EE in rats showed a triphasic release response, with an initial burst release of 8 ng/mL LNG and 14 pg/mL EE on Day 1 due to the release of the steroid adsorbed on the microsphere surface. This may be attributed to a higher volume of distribution and to an increased plasma protein binding affinity of the drug. It was followed by a second sustained release phase from Week 5, which was initiated because of steroid diffusion through the pores or channels formed in the polymer matrix, and a third phase until Week 15 by bulk erosion at the polymer matrix. This slow advancement in hydrolytic degradation of the PLG microspheres led to the controlled release of the drugs to the systemic circulation. These parameters acted as a reservoir and aided in the maintenance of constant drug levels of 2 ng/mL LNG and 3–4 pg/mL EE in the blood until Week 15. After Week 15, the drug levels were less than the minimum amount for maintaining contraception.

The morphology of the microspheres changed progressively with time and finally disintegrated after muscular

![Fig. 9. Immunohistochemical analysis of tissues explanted from the PLG microspheres injection site by fluorescence microscopy: (A) normal thigh muscle, (B) postimplantation Week 1, (C) postimplantation Week 8 and (D) postimplantation Week 20 (original magnification, ×200). (++) Expression of IL-1α.](image-url)
implantation in rats. Once implanted, a biodegradable drug delivery device should maintain its mechanical property until it is no longer needed and then be absorbed and excreted by the body. On Week 1, PLG microspheres retained good sphericity similar to that of the microspheres used before implantation. Simple chemical hydrolysis is the prevailing mechanism for polymer degradation at the initial stage, after which surface degradation occurs, creating pores on the surface of the spheres by hydrolysis. Water penetrates into the bulk of the device, preferentially attacking ester bonds and converting long polymer chains into shorter water-soluble fragments, with reduction in molecular weight followed by metabolism of the fragments, resulting into their monomers. On Week 8, PLG microspheres were degraded into smaller fragments; after Week 20, the microspheres were degraded into fine fragments with reasonable size reduction. This ensured that the PLG microspheres had a slow degradation rate and can be used as a promising device for long-term delivery of contraceptive steroids.

PLG microspheres (average size, 30 μm) generally induced a mild foreign body reaction and were reported to be biocompatible [28]. The volume of microspheres injected into the tissue may be considered as an open porous implant, which induces an inflammatory response characterized by the infiltration of macrophages, neutrophils, fibroblasts and some lymphocytes and by the formation of fibrin, giant cells and new blood vessels [29–32]. Tissue reaction to the PLG microsphere injection site after Week 1 showed heavy macrophage infiltration around the injection site. Concurrently, a moderate inflammatory sites was enhanced.

The macrophage infiltration and the levels of inflammatory cytokine IL-1α at the microsphere injection site almost disappeared after Week 20, indicating that the release was almost complete, resulting in greater degradation of microspheres and enhanced scavenging by the host defense mechanism.

The data obtained in this study suggest that the LNG/EE-loaded PLG microspheres prepared by the W/O/W double-emulsion solvent evaporation method can be used as an intramuscularly injectable drug delivery carrier, in consideration of their biodegradation, biocompatibility and particle size. The biodegradable property of PLG polymers makes this delivery system a potential carrier for long-acting controlled drug delivery. Furthermore, the longer duration of LNG and EE levels in the blood for contraceptive action with controlled-release characteristics finds potential application in contraceptive therapy.

Acknowledgments

We are grateful to Dr. T. Ramasami (Director, CLRI) for granting permission to publish this work. We are also thankful to Dr. C.V. Gokularathnam (Department of Metallurgical Engineering, IITM, Chennai) for helping with SEM analysis.

References


