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Controlled release of levonorgestrel from biodegradable poly(D,L-lactide-*co*-glycolide) microspheres: In vitro and in vivo studies

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Abstract

Poly(D,L-lactide-*co*-glycolide) (PLG) biodegradable microspheres containing a contraceptive drug, levonorgestrel (LNG), were prepared using both the solvent evaporation method and a modified solvent extraction–evaporation method. The microspheres prepared with the solvent evaporation process had porous surfaces with low product yields and poor encapsulation efficiencies. On the other hand, the microspheres prepared using the modified solvent extraction–evaporation method were nonporous with encapsulation efficiencies close to 100%. In vitro drug release showed the nonporous microspheres had a lower initial burst and a slightly prolonged duration of release than those porous microspheres. In vivo release kinetics of the low burst microspheres were determined by measuring LNG plasma levels after a single intramuscular injection to female rats. At a LNG dose of 41.1 mg/kg, average plasma LNG levels were 6–10 ng/ml in the first 24 h and subsequently remained above 1 ng/ml until 126 days. The duration above the minimum effective LNG plasma level of 0.2 ng/ml was 168 days. By comparison, a similar dose of LNG microcrystals used as control produced a much higher plasma level of 15–21 ng/ml in the first day followed by a fast and continuous decline of LNG levels with a duration of only about 35 days.

Keywords: Levonorgestrel; Poly(D,L-lactide-*co*-glycolide); Microspheres; In vitro release; In vivo release; Rats

1. Introduction

Long acting contraceptives based on biodegradable microspheres have been in development for the past several decades. The advantages of the microsphere delivery system are its long duration of action, ease of

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administration through regular intramuscular or subcutaneous injections and its biodegradability. Additionally, the biodegradable microspheres can provide a relatively constant rate of drug release, thus improving the drug efficacy and reducing side effects. Beck et al. (1979a) first reported progesterone-containing microspheres prepared with D,L-polylactic acid (PLA). These microspheres had a large initial burst and a duration of release of about a month. Later, another progestin, norethisterone, was encapsulated in PLA microspheres by the same group (Beck et al., 1979b). The PLA microspheres could continuously release norethisterone for 6 months after a single intramuscular injection in female baboons. Further research of the norethisterone microspheres yielded a second generation of microspheres with improved drug release and biodegradation characteristics and a 90-day duration of action (Beck et al., 1983). The 90-day norethisterone microspheres have since been tested in phases II and III clinical trials and have been shown to be safe and efficacious (Grubb et al., 1989; Singh et al., 1997).

It is our belief that a longer contraceptive duration of 6 months may offer additional benefits of convenience and compliance; however, relatively few 6-month injectable contraceptive formulations have been reported. Beck et al. (1985) once reported a LNG microsphere that released LNG continuously for more than 8 months in female baboons, but no further progress has been reported. Recently, a poly(ε-caprolactone) (PCL) microsphere containing both LNG and ethinylestradiol was reported to release both drugs for more than 150 days in vitro (Dhanaraju et al., 2003). Our purpose is to develop a 6-month injectable contraceptive that should also biodegrade completely by 6 months or slightly longer than 6 months once injected. The selection of LNG as the encapsulating drug is mainly based on its long history of extensive use as an oral contraceptive and its use in implantable delivery systems (Kang and Tan, 2004). LNG also has an established minimum effective plasma concentration of 0.2 ng/ml for controlled release implant (Fotherby, 1995). Additionally, it is more potent than norethisterone, thus, even for a 6-month formulation, the dose will be small and should be easily injected. Here, we report a new LNG microsphere made with a modified solvent evaporation–extraction encapsulation process that shows continuous LNG release for up to 6 months after a single intramuscular injection in female rats.

2. Materials and method

2.1. Materials

Poly(D,L-lactide-*co*-glycolide) (PLG) with a lactide/glycolide ratio of 90:10 was synthesized by the Chemical Laboratory of Zhejiang Academy of Medical Science, Zhejiang, China. The polymer has an inherent viscosity of 1.39 dl/g in $CHCl_3$ at 30 °C and average molecular weight (Mw) of 1.77×10^5 Da as measured by gel-permeation chromatography using polystyrene standards (GPC, Waters Model 510). USP grade levonorgestrel (LNG) was purchased from Peking Pharmaceutical Co., China. Poly(vinyl alcohol) (PVA-124, 98–99% hydrolyzed, viscosity 54–66 cps) was purchased from Shanghai Chemical Reagent Company, China. Medium viscosity USP grade sodium carboxymethylcellulose (CMC–Na) with a viscosity of 600–1400 cps was purchased from Zhangjiagang Sanhui Chemical Industry Co. Ltd., China, for the preparation of the reconstitution solution for LNG microspheres and LNG microcrystals. All other chemicals were of analytical grade and were used without further purification.

2.2. Preparation of microspheres

LNG powder was ground in an agate mortar with a pestle and sieved through a 325 mesh (44 μm) screen. The sieved LNG microcrystals were used to prepare all the microspheres in this study and were also used to prepare the LNG microcrystal suspension. Two oil-in-water (w/o) emulsification–solvent evaporation encapsulation processes were used in this study that were adapted from previous reports (Beck et al., 1983; Shiga et al., 1996). In Method A, 2.0 g PLG was dissolved in 5 ml dichloromethane in a vial. Exactly, 500 mg of LNG microcrystals were then added to the polymer solution and sonicated to form a homogeneous suspension. The polymer–drug suspension was gradually added drop by drop into a 450 ml beaker (8 cm diameter) containing 250 ml of 5% PVA-124 aqueous solution, maintained at room temperature (∼25 °C) and stirred at 800 rpm by a Heidolph RZR-2000 stirrer equipped with a 6 cm diameter plastic paddle. The resulting emulsion was stirred continuously at 800 rpm at room temperature and ambient pressure for about 9 h to allow the evaporation of dichloromethane

and to let the droplets harden into microspheres. The microsphere-containing solution was then centrifuged at 4000 rpm for 10 min. The PVA solution was poured off and the microspheres were washed twice with distilled water and dried at room temperature under vacuum for 24 h. In Method B, the organic solvent, dichloromethane, was replaced with 5 ml of a solvent mixture of acetone and chloroform (at 3:1 volume ratio) to dissolve the polymer. Emulsification and solvent evaporation were carried out at 0–5 °C using an ice bath instead of room temperature. The solvent evaporation time was also extended from 9 to 18 h. All other conditions were the same as in Method A.

2.3. Microsphere characterization and determination of drug loading

Scanning electron microscopy (SEM) was used to observe the shape and the surface characteristics of the microspheres. Particle size and size distribution of the microspheres were measured by a TA-II Coulter Counter (Coulter Electronics Ltd., Luton, UK). LNG drug loading in the microspheres was determined in triplicate by extracting and quantifying the encapsulated LNG. Briefly, approximately 10 mg of dried microspheres was weighed into a 25 ml volumetric flask and then 20 ml of ethanol was added and refluxed at 80 °C for 4 h. The flask was then cooled to room temperature and ethanol was added to bring the volume to 25 ml. The resultant solution was filtered and diluted to proper concentration with ethanol. LNG content was determined by measuring the UV absorbance of the diluted solution at 248 nm using a Beckman DU65 spectrophotometer. The same amount of blank microspheres without LNG was used as blank control.

2.4. Determination of residual chloroform in microspheres

The level of residual chloroform in the microspheres was determined by a headspace gas chromatographic technique. Approximately, 200 mg of microspheres was weighed in a headspace vial and 5 ml of *N*,*N*-dimethylformamide was added to dissolve the microspheres. The vial was sealed immediately. A headspace autosampler HP7694 and a HP6890 gas chromatograph equipped with a FID detector and an HP-INNOWax capillary column were used. Injector temperature was set at 200 °C and detector temperature was 250 °C. Initial column temperature was maintained at 50 °C for 15 min and then raised to 200 °C at 60 °C/min. The sample vial was heated to 80–85 °C for 30 min before 1 ml of the headspace sample was injected into the column. Calculation was based on a standard curve constructed with standard chloroform solutions. Three samples from three different batches of microspheres made with Method B were analyzed for their chloroform content.

2.5. In vitro drug release

In vitro release studies were performed in an aqueous solution containing 25% (v/v) ethanol. Approximately, 10 mg of microspheres were accurately weighed into a 4 cm × 5 cm paper tea bag (donated by Tea Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou, China). The bag was then sealed with staples and placed into 50 ml of releasing medium in an amber jar. The jar was placed on an orbital environmental shaker (Lab-Line Instruments Inc.) at 37 °C and 100 rpm. At various time points, the releasing medium was emptied from the jar and replaced with 50 ml of fresh and preheated releasing medium. The releasing solution samples were filtered with a 0.45 μm syringe tip filter and LNG concentrations were determined by measuring absorbance at 248 nm using a Beckman DU65 spectrophotometer. Release studies were run on five samples from each microsphere batch.

2.6. In vivo drug release in rats

Only the Method B microspheres were used in the in vivo release study. Female Sprague–Dawley rats weighing between 165 and 195 g were used. These rats were randomly divided into two groups with six rats in each group, one group for injection of LNG microcrystals at a dose of 45 mg/kg and the other group for microspheres at a dose equivalent to the LNG microcrystal group. Both microspheres and LNG microcrystals were suspended in 0.4 ml 0.9% saline solution containing 2% (w/w) sodium carboxymethylcellulose (CMC–Na) and 1% (w/w) Tween 20. The suspension was injected into the rat quadriceps muscle using a 23-gauge needle. The residual amount of LNG in the syringe and needle was measured and subtracted from the injection

dose to obtain the actual dose of injection. The actual dose of LNG was 35.0 mg/kg for the microcrystals and 41.1 mg/kg for the microspheres. Blood samples were taken at 1 day before injection and then at 1, 2, 4, 8 and 24 h post injection. After Day 1, blood samples were again taken at 2, 3, 5, 7, 14, 21, 28, 35, 42, 49, 56, 70, 84, 98, 112, 126, 140, 154, 168, 182 and 196 days after injection. About 1 ml of blood sample was drawn from the tail vein at each time point. All blood samples were collected in heparinized tubes and were immediately centrifuged. Plasma samples were obtained and stored in a −20 °C freezer until analysis.

LNG concentrations in rat plasma samples were measured using a radioimmunoassay (RIA) method (Ahsan, 1990). The LOQ of the method was 10 pg/tube and the average inter-day and intra-day precision was less than 6.5%. Average extraction recovery was greater than 90% for the method.

3. Results and discussion

3.1. Characterization of the PLG (90:10) LNG microspheres

Microspheres prepared using dichloromethane as the organic solvent (Method A) had low yields and varied drug loadings (Table 1). Some microspheres also appeared to be porous with large holes (Fig. 1). The low yield was mostly due to coalescence of the o/w emulsion on the stirring rod during solvent evaporation. In order to improve drug loading and microsphere yield, another encapsulation process was developed (Method B). In the new process, chloroform was selected instead of dichloromethane and acetone was added in the organic phase in order to speed up microsphere solidification by solvent extraction. A ratio of 3:1 acetone:chloroform was found to be optimal for this new process. The new process required a low evaporation temperature (0–5 °C) and a prolonged evaporation time in order to achieve good product yield. The microspheres thus prepared had a very smooth and nonporous surface as shown in Fig. 2. Product yield, encapsulation efficiency and drug loading were all improved with this new method (Table 2). Encapsulation efficiency was improved to nearly 100%. It was noted that variation in product yields was still high (32–61%) probably due to small batch size although each batch was made exactly the same. Still, drug loading, surface characteristics and size were very reproducible with Method B.

Table 1
Product yield, drug loading and encapsulation efficiency of LNG microspheres prepared with the solvent evaporation method (Method A)

Batch	Yield (%)	LNG loading (%)	Encapsulation efficiency (%)
LT #1	49.0	15.32	76.2
LT #2	38.2	20.21	101.1
LT #3	32.3	16.44	82.2

Table 2
Product yield, drug loading and encapsulation efficiency of LNG microspheres prepared with the modified solvent extraction–evaporation method (Method B)

Batch (%)	Yield (%)	LNG loading	Encapsulation efficiency (%)
LT #1	58.1	20.91	104.5
LT #2	61.6	20.02	100.1
LT #3	32.3	19.92	99.6
LT #4	45.6	19.92	99.6
LT #5	40.8	18.44	92.2

As shown in Figs. 1 and 2, the surface characteristics of microspheres were quite different for the two microspheres. In Method B, the organic phase contained 75% acetone which was a better solvent than dichloromethane for LNG. Although LNG was not completely dissolved in that polymer solution, the polymer solution was only slight milky without visible LNG particles. The microspheres thus had no visible LNG crystals on the surface of the microspheres. In addition, the method employed the low vapor pressure chloroform as part of the solvent and a low evaporation temperature of 0–5 °C. The rate of chloroform removal after the dissipation of acetone was slow because of low evaporation temperature, leaving enough time for the solvent inside the polymer droplet to migrate to the surface of the droplet. The microspheres were thus smaller, denser and nonporous. Jalil and Nixon (1990) have made similar findings that the temperature of solvent evaporation affected microsphere morphology. If the solvent was removed too rapidly at a high temperature, highly porous microcapsules were formed that demonstrated a “honey comb” internal structure when freeze fractured. In addition, the organic solvent selected for polymer dissolution could have significant

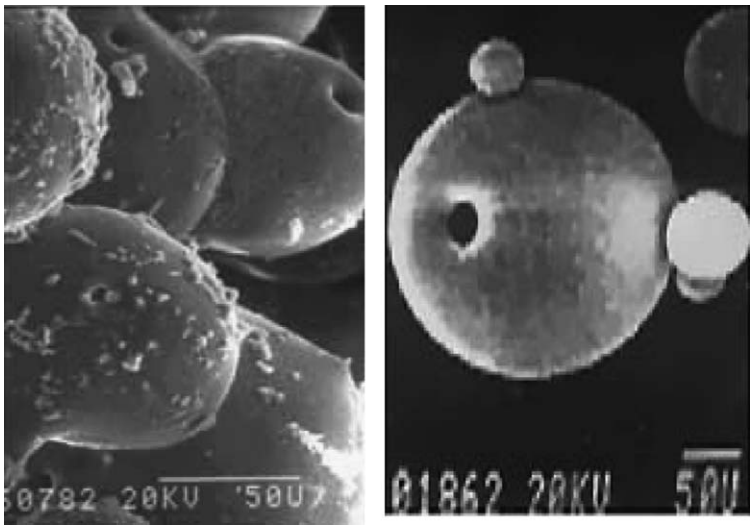

Fig. 1. SEM photographs of PLG (90:10) LNG microspheres prepared by solvent evaporation at room temperature (Method A).

effect on physical characteristics and release behaviors of microspheres as well (Birnbaum et al., 2000).

The particle sizes and size distribution of the microspheres were as measured by a TA-II Coulter Counter. Fig. 3 shows the size distribution of microspheres prepared using Method B. Almost all microspheres had sizes ranging from 10 to 105 μm in diameter and about 73% of the microspheres were in an even narrower size range of 25–80 μm. These sizes can easily pass through a 23-gauge needle for intramuscular injection.

3.2. Residual chloroform in microspheres

The microspheres prepared with Method B were analyzed for residual chloroform using a GC method. Residual chloroform was determined to be 8, 12 and

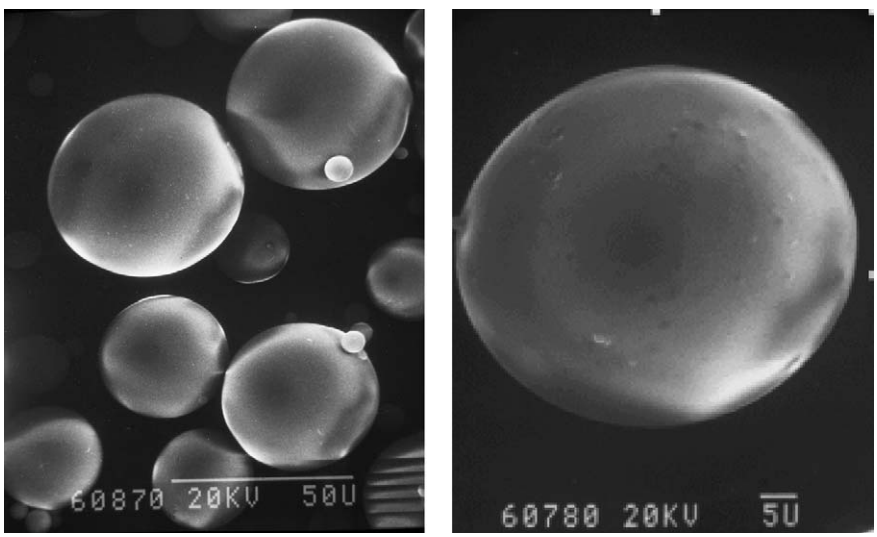

Fig. 2. SEM photographs of PLG (90:10) LNG microspheres prepared by solvent extraction and evaporation at 0–5 °C (Method B).

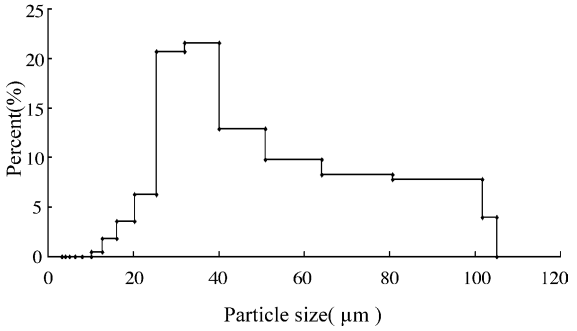

Fig. 3. Size distribution (in diameter) of the microspheres prepared with Method B as measured by the TA-II Coulter Counter.

7 ppm for the three different batches of microspheres. The values are well below the ICH chloroform limit of 60 ppm.

3.3. In vitro drug release

In vitro drug release was performed at 37 °C using a 25% ethanol aqueous solution in order to achieve sink condition. Fig. 4 shows the percentage of LNG released over time for both PLG (90:10) microspheres. Fig. 5 shows the daily LNG release over time. The data show that initial fast release (burst) phase lasted about 4 days for both microspheres (Fig. 5). The highest rate of release from both microspheres was reached at 12 h. Microspheres prepared with Method A had much higher initial burst than those microspheres prepared with Method B. At 4 days, the accumulated LNG

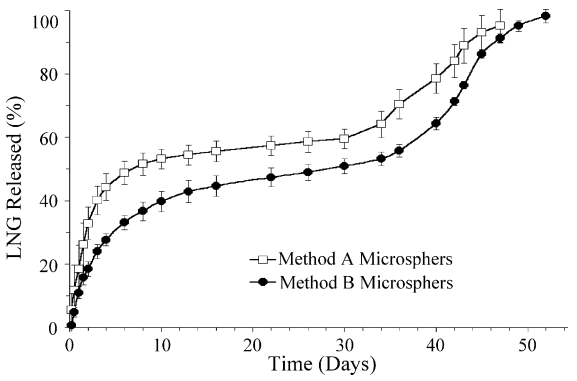

Fig. 4. Percent of LNG released over time from the PLG (90:10) microspheres in the 25% (v/v) ethanol aqueous solution at 37 °C and 100 rpm. Each data point represents the average of five measurements.

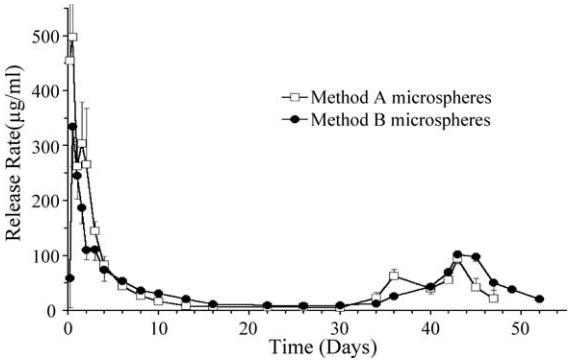

Fig. 5. Daily amount of LNG released over time from the PLG (90:10) microspheres in the 25% (v/v) ethanol aqueous solution at 37 °C and 100 rpm. Each data point represents the average of five measurements.

release was 44.3 and 27.7% for Methods A and B microspheres, respectively. After the initial burst, both microspheres released LNG at fairly constant rates that lasted about 30 days followed by a second burst that lasted another 18 days until complete drug exhaustion. During most of the release period, daily release from Method B microspheres was much higher than those from Method A microspheres, reflecting the advantage of lower initial burst. The Method B microspheres also had smaller fluctuations of daily LNG release and a slightly extended duration. Because the Method A microspheres had porous surfaces (Fig. 1), it was not surprising that the microspheres had a higher initial burst.

LNG is practically insoluble in water, so a solubilizing agent has to be used in order to maintain sink condition during the in vitro release test. Several solubilizing agents have been used by various groups in their LNG release media. Chien et al. (1989) used a saline solution containing 40% (v/v) PEG400 as the release medium for various LNG transdermal contraceptive patches. Gao et al. (1995) employed a dissolution medium consisting of 20% propylene glycol and 80% Sorenson's buffer to study the release of LNG from biodegradable and injectable gel formulations. Recently, a 1:750 benzalkonium chloride aqueous solution was used to determine the LNG release rates from silicone elastomer tubing implants (Nash et al., 2004). In the current study, a 25% ethanol solution was selected partially due to analytical considerations. Our purpose of the in vitro release test was to screen and select the best LNG

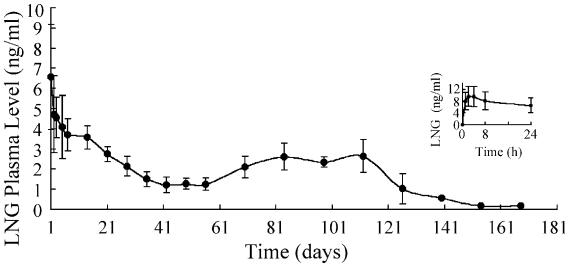

Fig. 6. LNG plasma levels after a single intramuscular injection of the LNG microspheres prepared with Method B at a dose of 41.1 mg/kg LNG in rats. Each point represents the mean ± S.D. of six animals.

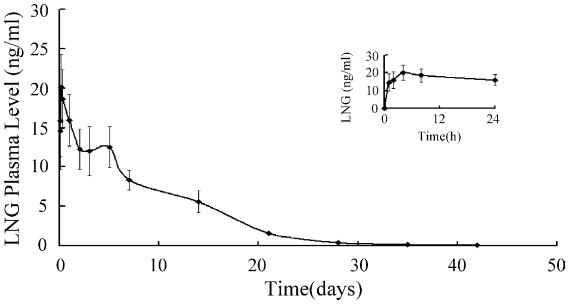

Fig. 7. LNG plasma levels after a single intramuscular injection of LNG microcrystals at a dose of 35.0 mg/kg LNG in rats. Each point represents the mean ± S.D. of six animals.

microsphere for further study. As shown in Fig. 5, the current method was able to discriminate LNG release profiles from the two microspheres, especially during the initial burst phase.

3.4. In vivo drug release in rats

Only microspheres prepared with Method B were selected for in vivo release study in female SD rats because of its low initial burst and a longer duration of release. A similar dose of LNG microcrystals was administered to another group of animals as control. Fig. 6 shows the LNG plasma concentration–time profile after intramuscular injection of LNG microspheres. The plasma LNG concentration raised quickly to the maximum concentration (C_{max}) of 10.1 ng/ml at 4.67 h (t_{max}). The plasma LNG level then decreased steadily to 1.4 ng/ml by 42 days and was maintained at that level without much variation until 55 days. The level then started to rise slowly again, reaching a second maximum plasma level of 3.0 ng/ml on Day 112, followed by a rapid decrease to 1.2 ng/ml by Day 126. The decline extended further to around 0.2 ng/ml by Day 168 and 0.1 ng/ml by Day 182. Thus, the duration of LNG release from microspheres was about 6 months. In contrast, the LNG microcrystals had a much higher maximum concentration (C_{max}) of 21.1 ng/ml and a short duration of only 35 days as shown in Fig. 7. In addition, the area under the curve (AUC) was 338.4 day ng/ml for the microspheres and only 191.4 day ng/ml for the LNG microcrystals. The relative bioavailability of the microspheres was thus 151% higher than the LNG microcrystals. It should be noted that the low relative bioavailability of LNG may not indicate incomplete absorption of the LNG microcrystals at the injection site because LNG pharmacokinetics in female rats is dose dependent (Naqvi et al., 1984).

Despite the apparent differences of in vitro and in vivo release environments, the overall in vitro and in vivo release patterns were still similar. Both release profiles showed an initial burst phase and a later second burst release phase. The initiation of the second burst release phase in vivo was somewhat later than in vitro. This second burst release was most likely the result of polymer degradation and erosion. In the in vitro environment, once the molecular weight of the polymer decreased to a certain level, the microspheres started to swell somewhat, as observed in current in vitro release test, thus accelerating the drug release from the microspheres. The second burst in vitro was strong and brief due to the swelling. It is expected that in vitro drug release is different than the in vivo release environment, where due to the constant removal of the degraded soluble oligomers, the microspheres most likely could not swell, but rather, just kept shrinking in size as polymer degraded. Therefore, the second burst phase lasted much longer.

Several examples have shown that the in vivo drug release from microspheres in animals correlated very well with drug release in humans. Leuprorelin microspheres have been studied in rats and humans and these studies have shown similar release patterns and efficacies in both rats and humans (Okada et al., 1996; Periti et al., 2002). The release of octreotide acetate from PLG microspheres has been tested in rabbits and humans. And as expected, similar release patterns from rabbits and humans were observed (Lancranjan et al., 1996; Comets et al., 2000). In the current study, LNG

release from the microspheres in rats has shown to be fairly constant, especially in the first 126 days, it may be reasonably anticipated that the microspheres will provide a fairly constant rate of LNG release in humans and provide stable contraceptive effect for several months. Based on the extensive data available with the LNG implantable devices, a daily LNG dose of 30–50 μg could provide adequate contraceptive effect (Sivin, 2003). The LNG dose currently used in rats is probably too high to be used in humans.

Both norethisterone and levonorgestrel have been incorporated in long acting contraceptive delivery systems, such as microspheres, implants and intrauterine devices. An injectable, 90-day biodegradable microsphere system with norethisterone has been studied in several clinical trials (Grubb et al., 1989; Singh et al., 1997). Clinical studies show that the norethisterone microsphere can provide a safe, efficacious and acceptable contraception. In comparison, no biodegradable LNG microspheres have been reported in any clinical studies. The LNG microspheres reported in this paper clearly show that such a microsphere formulation can provide sustained and steady LNG plasma levels lasting 4–6 months with a single intramuscular injection. The advantages of the current LNG microspheres will be a longer duration than the norethisterone microspheres and a much smaller injection dose, thus further improving the convenience and acceptability of such a contraceptive method. It is recognized that just like the LNG implant and the norethisterone microspheres, those progestin-only systems could cause menstrual irregularity and amenorrhea (Kang and Tan, 2004; Singh et al., 1997). Nevertheless, the LNG microspheres could be mixed with sustained release estrogen microspheres, which may be prepared separately and the combined LNG and estrogen microspheres preparation should theoretically be able to minimize those side effects.

4. Conclusions

We have demonstrated that biodegradable microspheres containing LNG can be successfully prepared using the modified solvent extraction–evaporation method at low temperature. This modified preparation method produced LNG microspheres with a smooth nonporous surface. Moreover, the encapsulation process was reproducible and was able to generate microspheres with the expected drug loading and similar size distributions. In vivo drug release showed the microspheres, once injected intramuscularly in rats, produced fairly steady and long lasting plasma LNG levels for 168 days. The microspheres may thus be used as a long acting injectable and biodegradable contraceptive.

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