In Vivo Evaluation of Biodegradable Progesterone Microspheres in Mares

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INTRODUCTION

Progesterone is an endogenous hormone which plays a major role in the regulation of the reproductive cycle and fertility. An important veterinary application of progesterone is in the synchronization of estrous behavior and the prevention of pregnancy loss in mares (1). The current regimen involves intramuscular administration of drug dissolved in an alcoholic–oil solution, either daily (200 mg) or twice a week (1 g), for 6 to 8 months. This has resulted in several occurrences of abscesses, inflammation, infection, and even permanent scars. The drug causes pain at the site of injection and hence is very distressing to the animal (2). Development of a parenteral delivery system that can maintain therapeutic levels of this drug over an extended period would be beneficial.

Polymeric microspheres are being developed for the parenteral delivery of a variety of therapeutic agents. Encouraging results with some biodegradable microsphere systems have led to their clinical evaluation for the delivery of contraceptive agents (3). The release of drug from such nonporous microspheres is governed by its diffusion through the microsphere mass and/or erosion of the microsphere matrix (4,5). Several compounds, including progesterone, have poor diffusivity through the carrier matrix, hence a high surface-to-volume ratio for delivery would be desirable. A solvent-extraction-precipitation technique, which produces microspheres with a high surface area (6–8), has been investigated to prepare progesterone-loaded poly *l*-lactide (PLA) microspheres.

The purpose of the present work was to develop a delivery system which would maintain progesterone serum levels of 2 to 6 ng/ml for 10 to 14 days after intramuscular administration in mares. Using PLA, porous biodegradable microspheres have been prepared by the solvent-extraction-precipitation technique, and the bioavailability evaluated and compared with the routinely used dosing strategy in ovariectomized and/or anovulatory mares.

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MATERIALS AND METHODS

Materials

Micronized progesterone (Lot 320CP) was a gift from UpJohn Co.; PLA (Lot 73960, MW 50,000) was obtained from Polysciences; methylene chloride, glycerin, and isopropanol were from Fisher Scientific; and methylcellulose (400 cp) was from ICI America Inc. All chemicals and reagents were of analytical grade or better and used as obtained. Membrane filters (DA 0.65 μm) were obtained from Millipore Corp.

Preparation of Microspheres

The process for preparing progesterone/PLA microspheres is illustrated in Fig. 1 (6–8). In brief, a solution of drug and PLA in methylene chloride was dispersed in a continuous phase of glycerin. The dispersion was stirred at 3000 rpm for 10 min at 8–10°C. The temperature was then rapidly increased to 50°C and held for about 1 min. The microspheres were solidified by extraction of methylene chloride with 5–10% aqueous isopropanol using a Vibromixer for 2–10 min. The microspheres were collected by filtration using a Millipore Type DA 0.65-μm filter, washed with aqueous isopropanol, and dried under vacuum for 24 hr. The process was performed under a sanitized fume hood with due caution to minimize any physical, chemical, or biological contamination. The glassware and processing equipment were sanitized with 70% isopropanol and heat-sterilized wherever possible before use.

Characterization of Microspheres

Particle Size. The size distribution of the microspheres was determined using a Malvern Laser Particle Sizing System. A small amount of microspheres was suspended in 0.05% Tween 80 by mild sonication and transferred to a counting chamber. The size distribution was obtained by laser defraction and analyzed by a computer.

Total Drug Content. A known amount of progesterone microspheres was dissolved in 0.5 ml methylene chloride and the polymer was precipitated from the solution with 9.5 ml absolute alcohol. The contents were centrifuged (10,000 rpm for 2 min) to remove the polymer, and the supernatant was analyzed for drug concentration using a Perkin–Elmer UV spectrophotometer at 235 nm.

Microbiological Screening of the Microspheres. The microspheres were tested for microbial contamination. Typically, a small amount of microspheres (~5 to 10 mg) was incubated in soybean casein digest broth for 7 days at 37°C. In most cases the broth demonstrated an inherent turbidity after incubation of microspheres. In order to distinguish between the microbial growth and the suspension characteristics of the sample, the turbid broth was further tested for possible contamination using agar slants as well as agar plates. A set of positive controls and the microsphere samples were incubated at 37°C for 48 hr before being inspected for bacterial growth.

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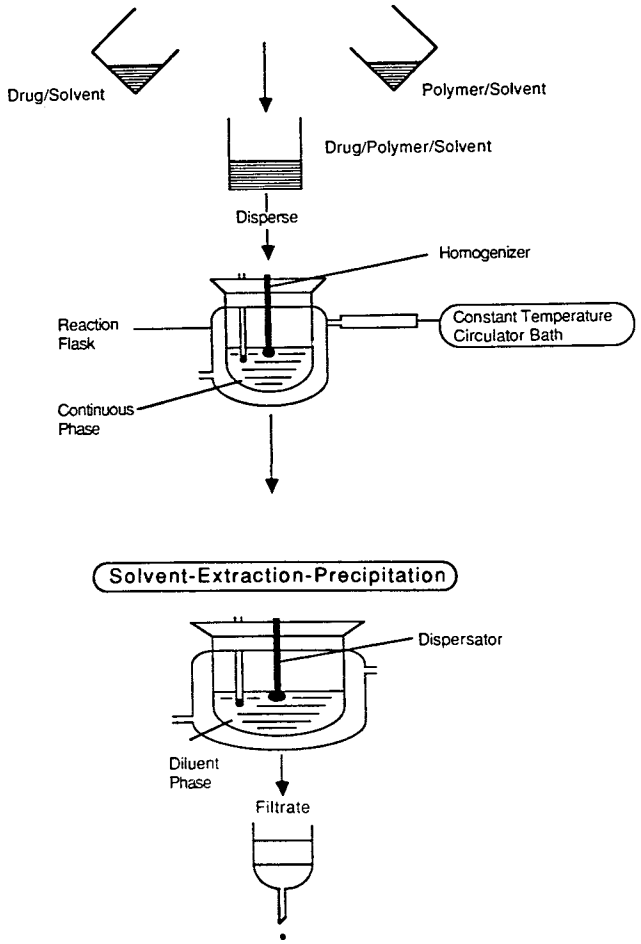

Fig. 1. A schematic representation of the microsphere preparation process.

Protocol for *in Vivo* Evaluation

In vivo evaluation of the progesterone microspheres was carried out in ovariectomized and ovary-bearing mares. Three ovariectomized mares (*R*ebel, *W*anda, and *T*raveller) and one anovulatory ovary-bearing mare (*B*obby) were housed at the Bluegrass Embryo Transfer Laboratories and used for the studies over a period of 1 year. Table I summarizes the experimental design and the treatment of each mare. Progesterone in different dosage forms was administered intramuscularly in the neck region and blood samples were collected from the jugular vein at predefined time intervals for 14 days.

Study 1. This study was designed to assess the effect of the formulation type on the serum progesterone profile. Mare R1 (mare *R* used in Study *1*) received 1 g of progesterone dissolved in 50 ml of 30% (v/v) benzyl alcohol in corn oil. Mare W1 received a suspension of the same dose of progesterone with 0.5 g of bovine serum albumin (BSA) in 50 ml of 1.5% methylcellulose. Mare T1 received 5 g of 20% (w/w) progesterone microspheres (equivalent to 1 g of progesterone) suspended in 50 ml of 1.5% methylcellulose.

Study 2. This study was designed to assess the effect of the dose of progesterone in the microspheres on the pharmacokinetic parameters. Mare R2 received 1 g of progesterone as in Study 1. Mare T2 received 4 g of 35% (w/w) progesterone microspheres (equivalent to 1.4 g of progesterone) suspended in 45 ml of 1.5% methylcellulose.

Study 3. This study was designed to assess the effect of microsphere drug loading on the pharmacokinetics of progesterone. Mares B3, W3, and T3 were administered 2.8 g of 50% (w/w) progesterone microspheres (equivalent to 1.4 g of progesterone) suspended in 25 ml of a 0.5% methylcellulose.

Table I. Protocol for *in Vivo* Evaluation

Study no. and mare[a]	Microsphere dose (g)	Load (%)	Drug dose (g)	Formulation type[b]	Volume (ml)
1					
R1	-	-	1.0	SOLN	50
W1	-	-	1.0	SUSP	50
T1	5.0	20	1.0	MS-1	50
2					
R2	-	-	1.4	SOLN	45
T2	4.0	35	1.4	MS-1	45
3					
B3	2.8	50	1.4	MS-2	25
T3	2.8	50	1.4	MS-2	25
W3	2.8	50	1.4	MS-2	25

[a] Mares R, W, and T are ovariectomized. Mare B is ovary-bearing but anovulatory at the time of use.

[b] SOLN, progesterone in corn oil with 30% benzyl alcohol; SUSP, free progesterone in 1.5% methylcellulose with BSA; MS-1, progesterone microspheres in 1.5% methylcellulose; MS-2, progesterone microspheres on 0.5% methycellulose.

Serum Progesterone Concentration

The concentrations of progesterone in serum were obtained by a solid-phase radio-immunoassay. The progesterone antibody was immobilized on the walls of a polypropylene tube to which serum samples (100 μl) and ^{125}I-labeled progesterone were added. The tubes were incubated for 3 h at room temperature. The solution was decanted to terminate the competitive binding and read directly in a gamma

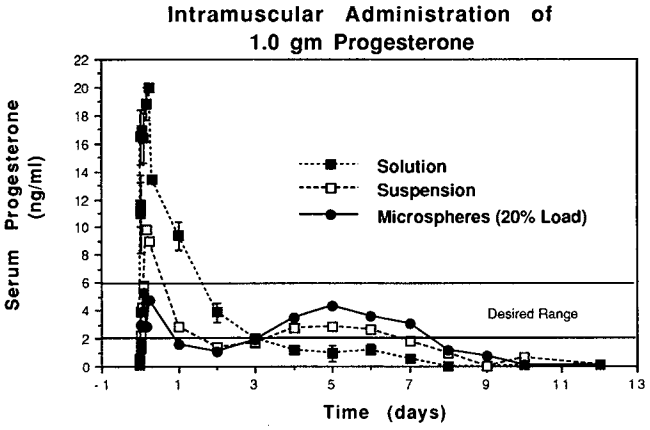

Fig. 2. A plot of serum progesterone concentrations in ovariectomized mares following i.m. administration of 1.0 g of drug as a solution in oil, a suspension in methylcellulose, and a microsphere formulation.

Table II. Summary of Pharmacokinetic Parameters

	Solution (1.0 g)	Suspension (1.0 g)	Microspheres		
			1.0 g (20%)[a]	1.4 g (35%)	1.4 g (50%)
Mare:	R1, R2	W1	T1	T2	B3, T3, D3
C_{max} (ng/ml)	19.2 (0.8)[b]	9.8	5.3	5.3	6.4 (0.2)
AUC_{0-288} (ng · hr/ml)	692.3 (78.2)	546.1	571.1	855.5	1050.7 (82.9)
RBA[c]	1.00	0.79	0.83	0.8	1.08

[a] Microsphere drug loading.
[b] Standard deviation in parentheses.
[c] Relative bioavailability.

counter. The minimum detection limit of the assay was approximately 0.5 ng/ml.

Data Analysis

The serum progesterone concentrations were monitored over a period of 12 days (288 hr) following administration. The serum concentrations were transformed using the trapezoidal method to determine the area under the serum progesterone concentration–time curve (AUC). The maximum drug levels (C_{max}) were also obtained from the serum concentration data.

RESULTS AND DISCUSSION

Microsphere Preparation and Characterization

The solvent-extraction-precipitation technique of preparing microspheres is based on emulsification of a dispersed phase containing drug and polymer in a nonsolvent continuous phase followed by the addition of a diluent phase which extracts the dispersed phase and washes off the continuous phase without dissolving the drug. The processing parameters may be varied to yield microspheres with different characteristics (6–8). The microspheres prepared were small in size, with mean diameters of 14 to 25 μm.

In vitro drug release from the microspheres could not be assessed accurately due to difficulty in formulating a suitable release medium. Micronized progesterone did not suspend well in any hydroalcoholic system which is routinely employed in dissolution of water-insoluble compounds. Thus a series of *in vivo* studies was undertaken to aid in further refinement of the microsphere characteristics and formulation based on the pharmacokinetics of progesterone after microsphere delivery. The microsphere formulation was modified based on the results of each *in vivo* study, as represented in Table 1.

The microbiological testing of the microspheres using soybean casein digest broth and agar slants did not indicate any biological contamination of the formulations. The type of materials used and the precautions taken during manufacturing appear suitable to produce product without any discernible bacterial contamination.

Suspending vehicles including corn oil, saline–Tween 80, and human serum albumin failed to produce uniform microsphere suspensions which would pass through an 18-G needle. However, the microspheres suspended well in 1.5% aqueous methylcellulose; further optimization allowed the use of 0.5% methylcellulose which passed through a 21-G needle. Free progesterone, however, did not suspend very well in methylcellulose. The addition of bovine serum albumin increased the suspendibility of micronized progesterone in methylcellulose.

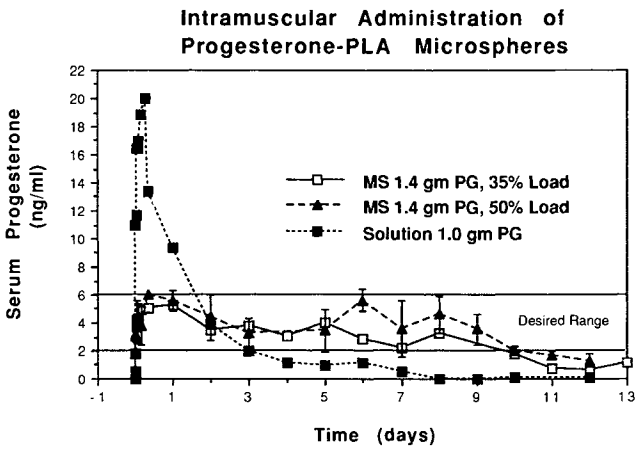

Fig. 3. A plot of serum progesterone concentrations following 1.0 g of drug as a solution in oil and 1.4 g of drug in microspheres of a different load.

In Vivo Evaluation of Progesterone Formulations

The serum progesterone concentrations following administration of 1.0 g drug as an oil solution, an aqueous suspension of free progesterone, and a suspension of progesterone microspheres are presented in Fig. 2. The calculated pharmacokinetic parameters are summarized in Table II (R1, R2, W1, T1). The microsphere form of progesterone resulted in a fourfold reduction in the peak serum concentration compared to the solution and a twofold reduction compared to the progesterone suspension at the 1.0-g dose. The AUC_{0-288} was higher when the drug was administered as a solution than in a suspension or a microsphere formulation. The relative bioavailability (RBA) of the suspension was 79% and that of the microspheres was 83%, which may be attributed to the incomplete release of progesterone from the microspheres or the powder form. A bimodal release is seen with the progesterone suspension and microspheres at the 1.0-g dose. The secondary release may be a result of microspheres degradation or the phagocytosis of particles by cells of the immune system. Our previous studies in cultured mac-

Table III. AUC Distribution Over Time and Serum Concentrations for Progesterone Formulations

AUC	Solution (1.0 g)	Suspension (1.0 g)	Microspheres	
			1.0 g (20%)[a]	1.4 g (50%)
0–288 hr	692.3	546.1	571.7	1050.7
0–72 hr	586.6 (82%)[b]	237.0 (43%)	150.2 (26%)	391.0 (37%)
72–288 hr	123.7 (18%)	309.1 (57%)	421.5 (74%)	659.7 (63%)
2–6 ng/ml	103.2[c]	—	115.5	210.9

[a] Microsphere drug loading.
[b] Percentage of AUC_{0-288}.
[c] AUC between 2 and 6 ng/ml serum levels.

rophages show that lactide/glycolide polymer microspheres begin degrading rapidly after phagocytosis (9). More recent *in vivo* studies in rats indicate that, after subcutaneous administration, tissue histiocytes phagocytose microspheres within 1 week (unpublished results). Thus, any residual drug would be released upon microsphere degradation. For particles of progesterone, the interaction with phagocytic cells may result in solubilization and subsequent release.

The serum progesterone concentrations following microsphere delivery at the 1.0-g dose declined to below minimum therapeutic levels between day 1 and day 3 and after 7.5 days. The desired effective duration would be 10–14 days, which may be achieved by increasing the progesterone dose. However, difficulties were encountered while administering the microsphere formulation due to its large volume (50 ml) even at the 1.0-g dose in mare T1. Therefore, it was advantageous to reduce the amount of microspheres administered by increasing the drug loading.

The second study was carried out with microspheres containing 35% (w/w) progesterone at a dose equivalent to 1.4 g progesterone. A total of 4.0 g of microspheres was administered in 45 ml of vehicle. The serum profile is shown in Fig. 3 and the pharmacokinetic parameters are presented in Table II (T1, T2). At this dose there was a lower intermediate decline in serum concentrations than that seen with the 1.0-g dose, and the serum levels of 2–6 ng/ml were maintained for about 10 days.

The injection volume of 45 ml in Study 2 was also difficult to administer. By reducing the methylcellulose concentration from 1.5 to 0.5% and improving the technique of preparing the suspension, a more uniform and concentrated suspension of microsphere resulted. The loading of progesterone was further increased to 50% (w/w), which resulted in a significant reduction in the microsphere dose (from 4.0 to 2.8 g) and the volume of suspending medium (from 45 to 25 ml) in the final study.

The effect of increasing drug load to 50% is also shown in Fig. 3, and the pharmacokinetic parameters are summarized in Table II (B3, T3, W3). A slightly higher C_{max}, serum levels, and AUC_{0-288} were seen at the 50% load than for the same dose of progesterone in microspheres with a 35% drug load. The microsphere drug loading appeared to have some effect on the progesterone bioavailability relative to the oil solution. With microsphere loading of 20%, the RBA was 0.83 (mare T1), which increased to 0.88 for 35% loading (mare T2). Increasing the microsphere drug loading to 50% (mares B3, T3, W3) caused a further increase in the RBA to 1.08. This may be attributed to a relatively rapid and more complete release of progesterone due to decreased polymer content of the microsphere matrix at higher drug load. In spite of the apparent drug loading dependent changes in the bioavailability of progesterone from the microspheres, the serum progesterone levels were not substantially different in mares receiving microspheres of different drug loading at the 1.4-g progesterone dose.

Table III shows the distribution of AUC_{0-288} over the initial period (AUC_{0-72}) and the later period (AUC_{72-288}). It is interesting to note that 82% of the area following solution administration and 43% of the area following suspension administration were contributed by the profile during the first 3 days due to the high initial peak. On the other hand, the serum progesterone profile during the first 3 days for the microsphere formulations with 20% drug load contributed only 26% and formulations containing 50% drug load contributed only 37% of their AUC_{0-288}, indicating a more uniform release of progesterone. Further, analyzing the AUC fractions for contribution to the therapeutic window (serum levels of 2 to 6 ng/ml; AUC_{2-6}), we found that a progesterone dose of 1.4 g in microspheres provided AUC_{2-6} of 210.9 ng · hr/ml, 1.0 g in microspheres provided AUC_{2-6} of 115.5 ng · hr/ml, and 1.0 g in alcohol–oil solution provided AUC_{2-6} of 103.2 ng · hr/ml. AUC_{2-6} is an important parameter since it is the actual fraction of AUC that is therapeutically important. Serum levels greater than 6 ng/ml do not provide any additional benefit and may be toxic, and those below 2 ng/ml are not therapeutically effective. Thus, progesterone in microspheres provides more exposure in the therapeutic window than progesterone in alcohol–oil solution.

The results suggest that 1.4 g of progesterone in microspheres at a drug load of 50% may be successfully used to maintain therapeutic levels known to be sufficient to prevent pregnancy loss in mares for a period of 10–12 days, whereas a solution form of 1.0 g provides levels above therapeutic levels for 2 days and below therapeutic levels after 3 days. Thus, although not completely optimized, the progesterone microsphere delivery system is a viable alternative to the more frequent injections of an oil solution.

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