J. Pharm. Pharmacol. 1989, 41: 439–444
Received July 4, 1988

Controlled Release of LHRH Agonist, Leuprolide Acetate, from Microcapsules: Serum Drug Level Profiles and Pharmacological Effects in Animals

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Abstract—The pharmacokinetic behaviour of leuprolide acetate from a controlled release parenteral dosage form has been studied in rats and dogs. The release of the drug in rats after a single subcutaneous injection exhibited pseudo-zero-order kinetics for one month in doses ranging from 0·0135 to 1·35 mg/rat; the release rate at a dose of 1·35 mg/rat was 2·8% of dose/day; after intramuscular injection the response was similar. In rats, the serum leuprolide acetate levels increased sharply immediately after injection by either route as a consequence of the initial release of the drug; subsequently, the levels attained a plateau for two weeks. The serum level profiles in dogs showed essentially the same pattern as those in rats. When the dosage form was injected into rats, the serum testosterone level (a pharmacological index) sharply peaked, abruptly decreased to below the normal level, and then was sustained at a suppressed level for over six weeks at a dose of 1·35 mg/rat (equivalent to 3 mg kg^{-1}) and higher, while the serum testosterone level after an injection of 0·0135 and 0·135 mg/rat was not sufficiently suppressed. The profiles in dogs showed essentially the same pattern as those in rats. With multiple administrations (once every 4 weeks), serum testosterone levels in dogs did not show any sharp rise after the second and third injections. Changes in rat reproductive organ weights agreed well with the serum testosterone profile in the suppression. The results demonstrate that this dosage form releases the drug at a constant rate for one month and has a long-acting potency.

Leuprolide acetate, a highly potent LH-RH agonist ([D-Leu^6, des-Gly-NH_2^{10}, Proethylamide^9]—LH-RH) is being used for the treatment of prostatic cancer (Wojciechowski et al 1986) and it has potential in the treatment of hormone-dependent disease such as endometriosis (Okada et al 1988). However, such treatment requires repeated once-daily injections for a long time. An injectable prolonged-release microcapsule dosage form using a polymer has been designed to allow the drug to be released at a constant rate for a month. The polymer, the wall substance of the microcapsules, is degraded biphasically and eliminated completely from the injection site within two months. Copoly(lactic/glycolic) acid (PLGA) was selected as the wall substance for microencapsules, because it is well known to be biocompatible and biodegradable (Brady et al 1973; Wise 1984). We have previously shown that the in-vitro release profile of the drug from microcapsules prepared with PLGA of average molecular weight 14000 and copolymer ratio of 75/25 (abbreviated as PLGA(75/25)-14000 hereafter) was optimum (Ogawa et al 1988a), and that the in-vivo release profile from the microcapsules injected subcutaneously in rats was comparable with the in-vitro release (Ogawa et al 1988b). We have therefore undertaken pharmacokinetic studies to clarify the long-acting effect of the drug after a single administration of the microcapsules containing leuprolide acetate to rats and dogs. We have also evaluated their pharmacological efficacy by measuring serum testosterone levels after single and multiple injections. In addition, the relationship between the in-vivo release of the drug, and the serum level profile, serum testosterone levels, and weight losses of the reproductive organs after a single injection of the microcapsules has been examined.

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Materials and Methods

Chemicals

Leuprolide acetate (Lot M548-131) synthesized in the Chemical Development Laboratories of Takeda Chemical Ind. Ltd. (Osaka, Japan) was used. PLGA(75/25)-14000, synthesized without a catalyst by the polycondensation process, was purchased from Wako Pure Chemical Ind. (Osaka); purified gelatin was from Nitta Gelatin Co. (Osaka) and Gosenol EG-40 of polyvinyl alcohol from Nihon Synthetic Chemical Ind. Ltd. (Osaka). Other chemicals were of reagent grade.

Preparation of microcapsules

PLGA(75/25)-14000 microcapsules containing leuprolide acetate were prepared by an in-water drying method as described by Ogawa et al (1988c). About 500 mg of the drug and 80 mg of gelatin were dissolved in 1 mL of distilled water as an inner water phase. The solution was gradually poured with stirring into a PLGA(75/25)-14000 (400 mg) methylene chloride solution (5·5 mL) to make a w/o emulsion. The emulsion obtained was cooled and poured into 400 mL of a 0·1% aqueous solution of polyvinyl alcohol to make a (w/o)/w emulsion. The oil phase was hardened to obtain microcapsules by evaporation of methylene chloride. These were lyophilized to powder, and the microcapsules obtained were fairly spherical with a mean diameter about 20 μm. The drug content in the microcapsule was about 10%.

Animal experiments

Male Sprague-Dawley rats supplied from Clea Japan, Inc.

were maintained in an air-conditioned room at 24±1°C under illumination for 14 h each day and they were allowed free access to food (CE-2, Clea Japan, Inc.) and water throughout the studies. The test samples (microcapsules) redispersed with a diluent (an aqueous solution containing 1% sodium carboxymethyl cellulose, 0·2% Tween-80, 0·14% methyl *p*-hydroxybenzoate, 0·014% propyl *p*-hydroxybenzoate, and 5% sorbitol) were injected subcutaneously into the nuchal region, or intramuscularly into the thigh. The rats were killed by aortic exsanguination at various times after administration of the microcapsules and the injection site excised. The microcapsules remained in a cyst at the injection site, therefore all those remaining were recovered. The leuprolide acetate content in them and in the serum, and also testosterone in the serum, were measured. In addition, tissue, both seminal vesicles, the ventral and dorsal prostate and the right testis, were excised and placed in separate pre-weighed tubes and each weighed.

The following three experiments were executed. (i) The leuprolide acetate remaining, serum drug and testosterone levels, and the change in the weight of reproductive organs were measured at each specified time after a single administration of the Lot 300M microcapsules at a dose of 1·35 mg/rat (almost same as 3 mg kg^{-1}) to groups of five rats aged 10 weeks (one group for each specified time). (ii) The leuprolide acetate remaining, the serum testosterone levels, and the weight changes of the organs were measured at each specified time after a single administration of the Lot 104 microcapsules to groups of five rats aged 6 weeks (one group for each specified time) at three different doses (0·9 mg/rat, 3 mg kg^{-1}). (iii) The serum testosterone levels, and the weight changes of the organs were measured after a single administration of the Lot 104 microcapsules to groups of five rats aged 10 weeks (one group for each specified time) at three different doses (1·35 mg/rat, 3 mg kg^{-1}).

With each beagle dog ca 10 kg, the test samples were injected subcutaneously into the upper part of foreleg or intramuscularly into the thigh, and blood was withdrawn from the cubital vein at various times.

The blood samples from rat and dog were centrifuged after clotting, and the serum collected and kept frozen until assayed for leuprolide acetate and testosterone concentrations.

Determination of leuprolide acetate

The method by which the remaining leuprolide acetate in the microcapsules was determined was described by Ogawa et al (1988c). Briefly, the remaining microcapsules were dissolved in a mixture of 10 mL of methylene chloride and 20 mL of phosphate buffer, pH 6·0, and the leuprolide acetate extracted into the buffer was assayed by an HPLC procedure with an ultraviolet detector. The leuprolide acetate in serum was determined by essentially the same radioimmunoassay as reported by Yamazaki & Okada (1980); it is a double-antibody method that uses [^{125}I] leuprolide acetate. Serum drug levels were determined in duplicate and the sensitivity was 5–10 pg/tube. Serum testosterone was assayed after extraction with ethyl ether according to the radioimmunoassay procedure by using a commercially available kit of Testosteron-H-3 (Green Cross Co. Ltd.).

Results and Discussion

Release of leuprolide acetate from microcapsules at the injection site in rats

In the in-vitro experiments of Ogawa et al (1988a), leuprolide acetate was released from PLGA(75/25)-14 000 microcapsules at a zero order rate over 35 days. Fig. 1 demonstrates the in-vivo release patterns of the drug from the microcapsules (Lot 300M). The curves show a small initial release followed by pseudo-zero-order kinetics for 4 to 5 weeks. There were no significant differences in drug release between subcutaneous and intramuscular injection methods. The following equation was obtained by a single linear regression analysis of the observed data:

$$R\ (\%\ \text{of drug remaining}) = 2{\cdot}8\ T\ (\text{time in day}) + 82{\cdot}2$$

The correlation coefficient was 0·990. The results indicate that the release of leuprolide acetate from the microcapsules at the injection site in rats followed zero-order-kinetics of (2·8% of dose) day^{-1} after the initial release of 17·8%.

Fig. 2 shows the time course of the remaining leuprolide acetate in the microcapsules (Lot 104) at the injection site after a single subcutaneous injection of three different doses of the drug to rats. The percentage of leuprolide acetate remaining was similar for each dose.

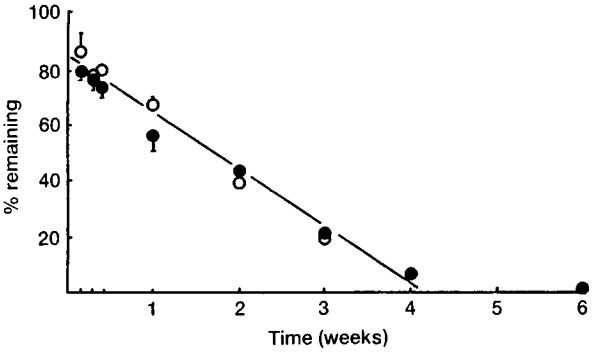

FIG. 1. Remaining leuprolide acetate after subcutaneous and intramuscular injection of the PLGA microcapsules in a group of five rats for each specified time. Each point represents the mean of the results for five rats with the standard error. Key: ○, s.c.; ●, i.m.

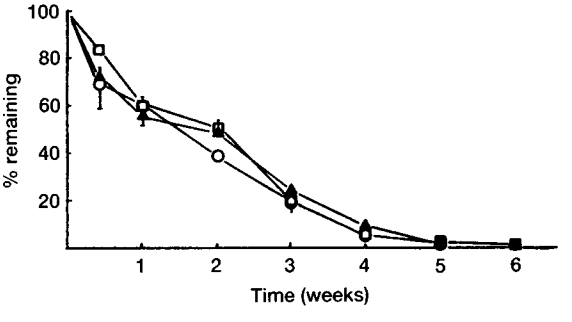

FIG. 2. Remaining leuprolide acetate after subcutaneous injection of PLGA microcapsules at three different doses in a group of five rats for each specified time. Each point represents the mean of the results for five rats with the standard error. Key: ○, 0·9 mg/rat; ▲, 3 mg/rat; □, 9 mg/rat.

Serum leuprolide acetate level profiles after a single administration of PLGA(75/25)-14 000 microcapsules

Fig. 3 shows the mean serum level profiles of leuprolide acetate after a single subcutaneous or intramuscular injection of the microcapsules (Lot 300M) to five rats for each group. The initial release of the drug from the microcapsules administered produced a sharp increase in the serum level; subsequently the levels after intramuscular injection were reasonably sustained at about 4 ng mL^{-1} for 2 weeks and those after subcutaneous injection were between 2·6 and 5·9 ng mL^{-1}. After week 2, they gradually declined until about week 6. The half-life of the β phase of leuprolide acetate from rat serum, after intravenous administration of the drug in an aqueous solution, was 30 min. Compared with the rapid elimination rate, it is considered that the serum levels between 2·6 and 5·9 ng mL^{-1} after a single administration of the microcapsules represent a definite level of ca 4 ng mL^{-1}. These results agreed well with those related to the in-vivo release. The profile after subcutaneous injection was comparable to that after intramuscular injection.

Since the release of leuprolide acetate from microcapsules was reasonably well controlled, i.e. the microcapsule drug delivery system released the drug at a constant rate, we can predict the serum level of the drug at the steady state using constant infusion kinetics. As long as the system obeys linear kinetics, the relation is represented as follows:

$$C_{ss} = R_I/V \cdot k_{el} = R_I/CL_{total}$$

$$CL_{total} = \text{dose/AUC}$$

where C_{ss} represents the steady state drug level, and R_I, V and k_{el} represent the constant infusion rate, distribution volume, and elimination rate constant, respectively; CL_{total} represents the total body clearance of the drug. By multiplying the release rate derived from the slope of the regression line in Fig. 1 ((2·8% of dose) day^{-1}) by 1·35 mg/rat (the corresponding dose), a value of 37·8 $\mu g\ day^{-1}$/rat is obtained for R_I for both subcutaneous and intramuscular injections. CL_{total} can be obtained from the AUC value and the dose after subcutaneous injection of an aqueous leuprolide acetate solution to rats; CL_{total} was 10 100 mL day^{-1}/rat for the mean AUC value of five rats after intravenous injection of an aqueous leuprolide acetate solution at a dose of 45 μg/rat. Consequently, the C_{ss} value of 3·74 ng mL^{-1} was obtained by calculation for the 1·35 mg/rat dose of the drug in microcapsules, while serum leuprolide acetate level observed at the steady state was about 4 ng mL^{-1} (Fig. 3). The predicted value, C_{ss}, agreed fairly well with the observed level. The serum level profiles of the drug at different doses exhibited essentially the same pattern (data were not shown). They rapidly declined during the 24 h after the high levels produced by the initial release, subsequently the levels of each dose were maintained at a steady level depending on the dose until week 3, although a small trough occurred at week 1 and a small rise at week 2.

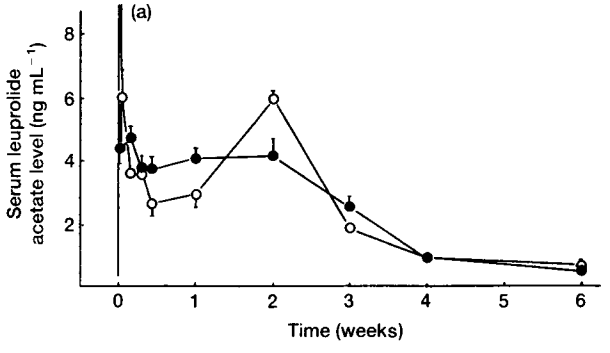

FIG. 3. Serum leuprolide acetate levels after subcutaneous and intramuscular injection of PLGA microcapsules in a group of five rats for each specified time at a dose of 1·35 mg/rat. Each point represents the mean of the results for five rats with the standard error. (a): Serum level at 3 h; ○, 19·4 ± 4·9; ●, 20·6 ± 5·6. Key: ○, s.c.; ●, i.m.

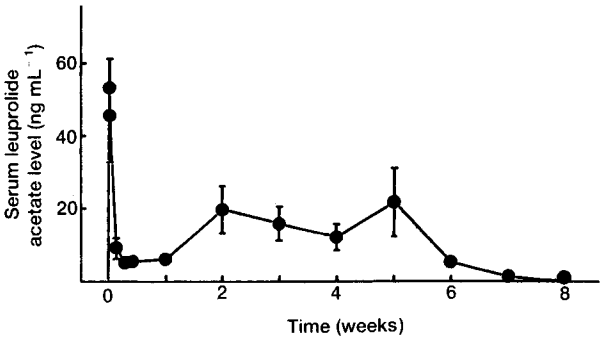

FIG. 4. Serum leuprolide acetate levels after intramuscular injection of PLGA microcapsules in five dogs at a dose of 3 mg kg^{-1}. Each point represents the mean of the results for five dogs with the standard error.

Fig. 4 shows the time course of the mean serum leuprolide acetate levels in five dogs after a single intramuscular injection of the microcapsules (Lot 104) at a dose of 3 mg kg^{-1}. The time curves exhibited essentially the same pattern as that seen in rats. The serum leuprolide acetate levels dipped slightly immediately after the high levels produced by the initial release, then gradually increased between day 3 and week 2. Subsequently they remained at a plateau from week 2 to week 5, thereafter they decreased until week 8.

Sanders et al (1985) reported that serum level of nafarelin, an LH-RH agonist, in a male cynomolgus monkey following a single injection of PLGA(50/50) microspheres containing 0·94% nafarelin, after a long and deep trough, increased sharply at day 28, attained a peak at day 40, and thereafter sharply decreased to day 44. There are differences between the microcapsules prepared by Sanders et al and those prepared by us: the copolymer ratio and molecular weight of PLGA, the type of LH-RH analogue used, the amount loaded, and the preparation method used (they adopted the phase separation technique). Therefore, it is difficult to compare directly the release profiles of the drug from the two types of microcapsules. The probable cause of the difference might be the amount of the drug loaded and the preparation method, because all the serum level profiles of nafarelin reported (Sanders et al 1984, 1985) exhibited a high peak at about the middle of the release period. This might be because in the microcapsules by the phase separation method, the drug was not uniformly distributed. On the other hand, the release kinetics of leuprolide acetate from the microcapsules prepared by the in-water drying method did not show a plain peak and a deep trough. The drug was definitely released

from an early to a late stage, and this might be because the in-water drying method distributed the drug more uniformly throughout the microcapsules as a monolithic type than the phase separation method.

Serum testosterone level profiles after a single administration of PLGA(75/25)-14 000 microcapsules

Fig. 5 shows the mean serum level profiles of testosterone after a single subcutaneous or intramuscular injection of the microcapsules (Lot 300M) to groups of five rats (one group for each bleeding time). Serum testosterone levels increased greatly just after administration, an initial rise caused by the stimulatory effects of the drug, they then decreased sharply to below the normal control level due to the antigonadal effects (Johnson et al 1976), and were sustained at a suppressed level for over 6 weeks. There were no significant differences in the hormone levels produced after subcutaneous and intramuscular injections. These results agreed well with those related to the serum level profile described above. The time course of serum testosterone levels obtained in this dose-determining study is shown in Figs 6, 7. In the doses ranging from 13·5 μg/rat to 1·35 mg/rat, the degree of suppression of serum testosterone increased with an increase

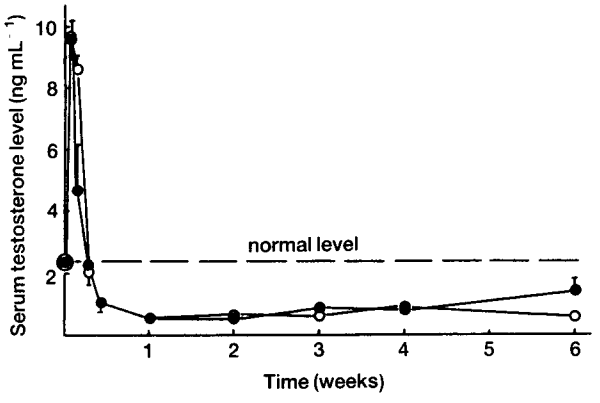

FIG. 5. Serum testosterone levels after subcutaneous and intramuscular injection of PLGA microcapsules in a group of five rats for specified time at a dose of 1·35 mg/rat. Each point represents the mean of the results for five rats with the standard error. Key: ○, s.c.; ●, i.m.

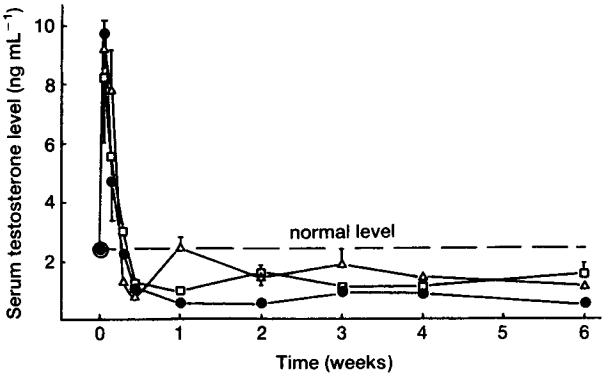

FIG. 6. Serum testosterone levels after intramuscular injection of PLGA microcapsules in a group of five rats for each specified time at three different doses. Each point represents the mean of the results for five rats with the standard error. Key: △, 13·5 μg/rat; □, 135 μg/rat; ●, 1·35 mg/rat.

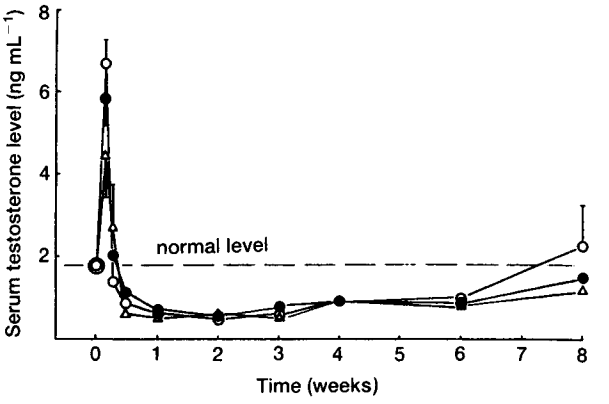

FIG. 7. Serum testosterone levels after subcutaneous injection of PLGA microcapsules in a group of five rats for each specified time at three different doses. Each point represents the mean of the results for five rats with standard error. Key: ●, 0·9 mg/rat; ○, 3 mg/rat; △, 9 mg/rat.

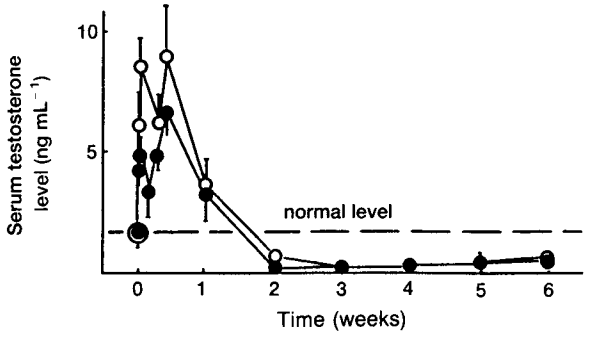

FIG. 8. Serum testosterone levels after subcutaneous and intramuscular injection in five dogs at a dose of 3 mg kg^{-1}. Each point represents the mean of the results for five dogs with standard error. Key: ○, s.c.; ●, i.m.

in dose (Fig. 6), while above the dose of 1·35 mg/rat, essentially superimposable patterns were obtained (Fig. 7). The serum testosterone level seemed to recover to normal after a gradual rise between weeks 6 and 8, and at the initial rise it was independent of the dose, being about 10-fold higher than normal. It appears that the minimum dose providing the maximum activity of leuprolide acetate in the rat was 1·35 mg/rat (3 mg kg^{-1}).

As shown in Fig. 8, the time courses of testosterone levels after a single administration of the microcapsules (Lot 104) to dogs showed essentially the same pattern as seen in rats and no significant differences were observed between the two injection routes. Serum testosterone levels from hour 3 to week 1 after dosing were fairly high during the initial rise, they were then subsequently sustained at a suppressed level from week 2 to 6.

Serum testosterone level profiles in dogs after multiple administrations of PLGA(75/25)-14 000 microcapsules

The microcapsules (Lot M07) were injected subcutaneously every four weeks in dogs at a dose of 1·5 mg kg^{-1} and then the serum testosterone levels were assayed. As shown in Fig. 9, the mean hormone levels in the serum were as high as the initial rise until day 7, they then decreased to low levels which

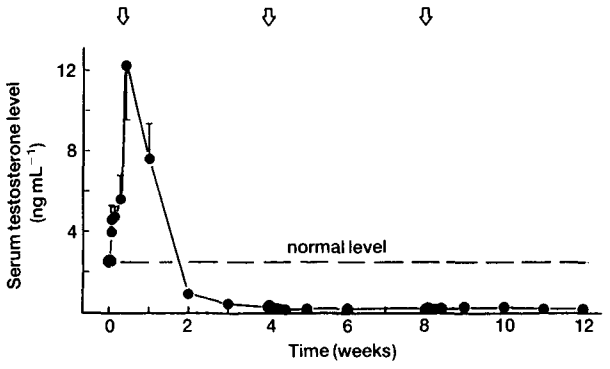

FIG. 9. Serum testosterone levels after three replicates of subcutaneous injections of PLGA microcapsules in five dogs at a dose of 1·5 mg kg^{-1}. Each point represents the mean of the results for five dogs with standard error. The arrows represent administration of the microcapsules.

were maintained until week 12. No elevation of serum testosterone level was exhibited after additional intramuscular injections at weeks 4 and 8. The above results, i.e. the absence of an "acute on chronic effect", shows that the microcapsule dosage form is useful for LHRH therapy.

The serum testosterone level profile in rats after multiple administration of the microcapsules exhibited a similar pattern to that in dogs (data were not shown).

Change in the weight of rat reproductive organs after a single administration of PLGA(75/25)-14000 microcapsules

The time course of the ratio of organ weights (the ratio of organ weight from treated rats to that from normal control) after subcutaneous and intramuscular injection of the microcapsules (Lot 300M) to rats is shown in Fig. 10. The relative weight of the prostates and seminal vesicles increased slightly on day 3; this response was considered to result from the initial rise caused by a transient stimulation of gonadal function. Thereafter, the relative weight of each organ decreased on week 1 due to an antigonadal effect, subsequently, between week 2 and 6, the relative weights were maintained at about the level of week 1. The relative weight response at each specified time was similar for both injection routes. The time course of weight changes of reproductive organs in the dose-determining studies were shown in Figs 10, 11. The curves obtained at doses below 1·35 mg/rat exhibited an approximate proportionality to the dose (Fig. 10), while doses higher than 1·35 mg/rat resulted in essen-

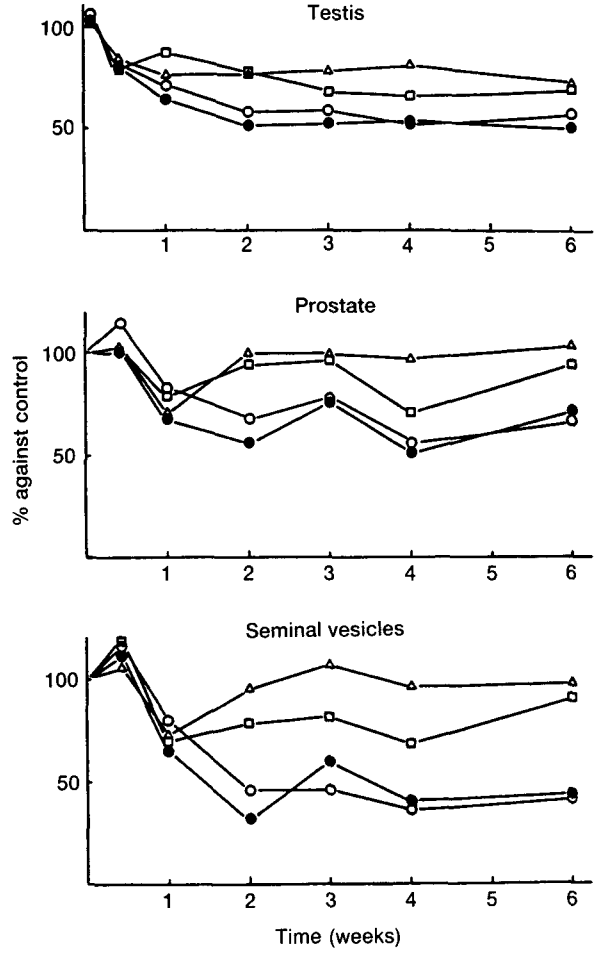

FIG. 10. Change in weight of rat reproductive organs after subcutaneous and intramuscular injection of PLGA microcapsules in a group of five rats for each specified time at a dose of 1·35 mg/rat and lower doses. Each point the mean of the result of five rats. Key: ○, s.c. at 1·35 mg/rat; ●, i.m. at 1·35 mg/rat; △, i.m. at 13·5 μg/rat; □, i.m. at 135 μg/rat.

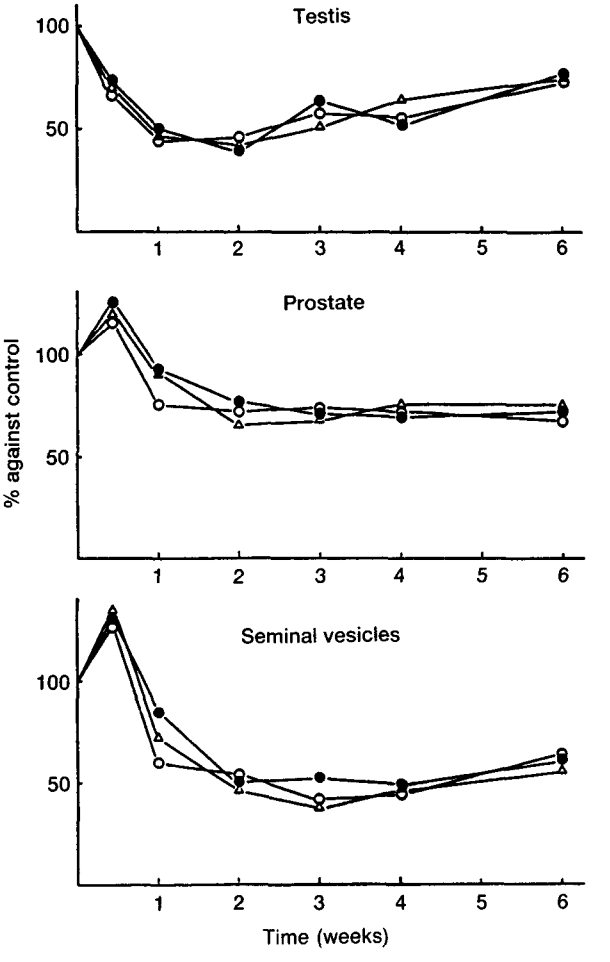

FIG. 11. Change in weight of rat reproductive organs after subcutaneous injection of PLGA microcapsules in a group of five rats for each specified time at three different doses in rats. Each point represents the mean of the results for five rats. Key: ●, 0·9 mg/rat; ○, 3 mg/rat; △, 9 mg/rat.

tially superimposable patterns (Fig. 11). These results agreed well with those related to the suppression of the serum testosterone level.

The results demonstrate that this dosage form releases the drug at a constant rate for one month and has a long-acting potency. This approach should increase the utility of LH-RH analogues for treatment of prostate carcinoma, other hormone sensitive neoplasms, and hormone dependent diseases such as precocious puberty and endometriosis.

Acknowledgements

The authors are indebted to Dr Hatanaka for supplying leuprolide acetate. We would like to thank Dr Y. Sugino for his encouragement. We wish to thank Dr J. R. Miller and Mr K. Tanaka for their linguistic advice.

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