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ScienceDirect
International Journal of Pharmaceutics 328 (2007) 42–48
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Phase-sensitive polymer-based controlled delivery systems of leuprolide acetate: In vitro release, biocompatibility, and in vivo absorption in rabbits

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Received 30 March 2006; received in revised form 27 June 2006; accepted 26 July 2006
Available online 1 August 2006

Abstract

Leuprolide acetate (LA) is a synthetic analog of gonadotropin releasing hormone. It is effective in prostrate cancer treatment only when its desired concentration in blood is maintained for longer duration. Therefore, the purpose of this study was to investigate the in vitro release, biocompatibility, and in vivo absorption of LA from phase-sensitive polymer delivery systems capable of delivering it at a controlled rate for longer duration. Phase-sensitive formulations were prepared by dissolving DL-polylactic acid (DL-PLA) in a mixture of organic solvents, benzyl benzoate (BB) and benzyl alcohol (BA). LA was incorporated into the polymer solution by homogenization. In vitro release was studied into 15 ml of releasing media contained in a vial which was maintained at 37 °C in a reciprocal shaking water bath. The amount of LA in the released samples was analyzed by stability indicating HPLC method. The biocompatibility of polymer formulations was investigated by in vitro 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In vivo absorption of LA from delivery systems was studied in rabbits. Blood samples were analyzed for LA and testosterone contents by commercially available immunoassay kits. In vitro release studies showed a greater release of LA from formulations containing a greater proportion of BA (hydrophilic fraction) in the solvent mixture. In vitro biocompatibility study showed significantly ($p < 0.05$) higher cell viability in growth media diluted with polymer extract than the control. In vivo absorption of LA and its effect on testosterone level in rabbits from the delivery system showed a sustained plasma level of LA up to 12 weeks which suppressed the testosterone plasma concentration to castration level beginning from 14th day until 90 days. Thus, phase-sensitive polymer delivery systems of LA were biocompatible and delivered LA at a controlled rate both in vitro and in vivo to keep testosterone plasma concentration at a castration level up to 3 months.

Keywords: Leuprolide acetate; Phase-sensitive polymer; Controlled release; Biocompatibility; In vivo absorption; Rabbits

1. Introduction

Leuprolide acetate (LA) is a synthetic nonapeptide analog of naturally occurring gonadotropin-releasing hormone which possesses greater potency than the natural hormone. Its chemical name is 5-oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-D-leucyl-L-leucyl-L-arginyl-*N*-ethyl-L-prolinamide acetate. LA desensitizes and downregulates pituitary luteinizing hormone-releasing hormone (LHRH) receptors, thus reducing luteinizing hormone (LH) synthesis and release (Briken et al., 2003). This, in turn, decreases testosterone levels to those observed in treated and orchiectomiesed patients. Moreover, LA in contrast to diethylstilbestrol does not increase cardiovascular events. Therefore it has been accepted as a safe hormonal therapy for prostate cancer (Perez-Marrero and Tyler, 2004; Marks, 2003). Currently, LA is also being evaluated in Phase II clinical trials for the treatment of Alzheimer's disease (Casadesus et al., 2004; Meethal et al., 2005).

LA has no oral bioavailability and relatively short half-life which reduces testosterone level only when administered on a continuous basis (Periti et al., 2002; Croom and Perry, 2003). Therefore several studies have been conducted to evaluate bioavailability through non-oral routes. In one study of evaluating pulmonary bioavailability, beagle dogs were administered a solution aerosol formulation of LA at dose levels of 0, 0.5, 1, and 2 mg/day for 14 consecutive days (Adjei et al., 1992).

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doi:10.1016/j.ijpharm.2006.07.051

A dose-dependent increase in the area under the plasma-time course curve was observed. The bioavailability of LA on day 1 was approximately double than the day 14 and the data suggested up to 40% absorption of the drug from the lung following administration as an inhalation aerosol. Thus, pulmonary administration did not maintain a constant plasma level of LA critical for its intended use in prostate cancer.

Microspheres of LA have been prepared successfully with PLA using a dispersion/solvent evaporation-extraction method (Persad, 2002; Sohier et al., 2003). However, microspheres showed high burst release and lower encapsulation efficiency (Cleland, 2001). The process is costly due to multi-steps involved in the preparation of microspheres. A novel liposomal formulation of LA termed 'liposomes in liposomes' has been reported to exhibit better encapsulation efficiency and stability than microspheres but 50% of encapsulated LA released within 53 h and constant release was observed only from 15 to 45 h (Saroglou et al., 2006). Thus, the liposomal formulations are not suitable for long-term delivery of LA.

Viadur™ (LA implant) is a sterile, non-biodegradable, osmotically driven miniaturized implant designed to deliver LA for 12 months at a controlled rate (Marks, 2003). Lupron Depot-4 Month containing 30 mg LA is also available (Physicians' Desk Reference, 2002). The control release profiles from Viadur and Lupron were well defined and could be manipulated accurately to obtain desired rate of release but they were made of non-biodegradable polymers and therefore required to be removed surgically upon complete release of the incorporated LA.

In situ gel forming and injectable phase-sensitive polymer systems are promising for controlled delivery of drugs (Hatefi and Amsden, 2002). The phase sensitive of polymer systems utilize a solvent(s) system and a polymeric matrix (Okumu et al., 2001). The solvent system makes use of both hydrophilic and hydrophobic organic solvents. The hydrophilic solvents used by various researchers are benzyl alcohol, *N*-methyl pyrrolidone, and ethanol, and the hydrophobic solvents used are benzyl benzoate, miglyol, and triacetin (Cleland, 2001). The hydrophilic solvent leaves the gel upon injection, causing the formation of a shell around the exterior of the depot, while the hydrophobic solvent remains behind, slowing aqueous influx, decreasing the rate of polymer hydrolysis, prolonging the release of incorporated agent and potentially increasing protein/peptide stability (Brodbeck et al., 1999; Chandrashekhar et al., 2001; Ravivarapu et al., 2000).

In this study, we investigated the in vitro release profiles of LA from phase-sensitive polymer-based injectable delivery systems and their biocompatibility. Furthermore, in vivo absorption of LA from the delivery systems and testosterone levels in rabbits was also investigated.

2. Materials and methods

2.1. Materials

DL-Polylactic acid (PLA) ($M_w \sim 16{,}000$ Da, intrinsic viscosity 0.19 dl/g) was purchased from Boehringer Ingelheim, Petersburg, VA. LA was a gift sample from TAP Pharmaceuticals, Inc. (Deerfield, IL). LA enzyme immunoassay (EIA) and testosterone radio immunoassay (RIA) kits were purchased from Peninsula Laboratories, Inc. (San Carlos, CA) and Cayman Chemicals (Ann Arbor, MI), respectively. All other chemicals used were of analytical grade. Rabbits (New Zealand, white, male, 10 weeks old, and approximately 1.5 kg body weight) were purchased from Harlan, Indianapolis, IN, USA.

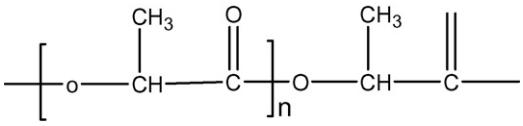

Fig. 1. The chemical structure of polylactic acid (PLA).

2.2. Preparation of leuprolide acetate formulation

PLA (Fig. 1) was dissolved into solvents mixture [benzyl benzoate (BB) and benzyl alcohol (BA)] by keeping the vial containing PLA and solvents mixture into a shaker water bath at 37 °C for 24 h. LA was incorporated into the polymer solution by homogenizing at 8000 rpm for 2 min to get homogeneous dispersion of the drug. Four formulations were prepared differing in proportion of BB and BA (Table 1). Injectability of the formulations was observed through a 25-gauge needle.

2.3. In vitro release of leuprolide acetate

Five hundred microliter of the formulation containing LA was injected into 15 ml of isotonic phosphate buffer (pH 7.4) contained in a glass vial. The formulation immediately changed into gel depot. The vials, containing in situ formed gel depot, were kept in a reciprocal shaking water bath (Precision Scientific, Winchester, VA) at 37 °C and 35 rpm. Five milliliter of aliquot was withdrawn at specified time points. The volume withdrawn was replaced with fresh isotonic phosphate buffer (pH 7.4).

The amount of LA in the released samples was determined by stability indicating high performance liquid chromatography (HP 1050 series) using a 220 nm UV detection, C_{18} MICRSORB-MV™ column (4.6 mm × 15 cm), mobile phase (0.03 m, 70% diabasic ammonium phosphate buffer:30% acetonitrile), and 2.0 ml/min flow rate (Singh et al., 2000).

Table 1
Compositions of phase-sensitive polymer-based leuprolide acetate delivery systems

Formulations	DL-PLA (%, w/v)	BB (%, v/v)	BA (%, v/v)	Leuprolide acetate (%, w/v)
1	15	100	–	3
2	15	95	5	3
3	15	90	10	3
4	15	85	15	3

DL-PLA, DL-poly(lactide); BB, benzyl benzoate; and BA, benzyl alcohol.

2.4. Incorporation efficiency

Incorporation efficiency was calculated by using the following formula:

$$\%\text{Incorporation efficiency} = \frac{\text{initial amount of drug} - \text{burst release}}{\text{initial amount of drug}} \times 100$$

2.5. In vitro biocompatibility of delivery systems

The smart polymer-based delivery systems (without drug) were extracted in PBS (pH 7.4) at 70 °C for 10 days. The polymers degrade faster at elevated temperatures, which simulate the long-term effects of in situ gel formed implant (artificial ageing extract). After 10 days of incubation, the pH of the extract was measured and adjusted to 7.4 by adding 1 M NaOH. The extract was filtered through a 0.2 μm filter and handled aseptically.

The extracts were diluted with double concentrated growth medium to a ratio of 1:1–1:16. Two hundred microliter of each dilution was directly added to Crandell Feline Kidney (CRFK) cell cultures. Medium produced in the same way without polymer extract was used as a negative control (PBS, pH 7.4) while 2% dimethyl sulfoxide (DMSO) in growth medium served as a positive control.

2.5.1. MTT assays

This assay is based on the ability of living cells to reduce a water-soluble yellow dye, MTT, to a water-insoluble purple formazan product by mitochondrial succinate dehydrogenase. Cells (8×10^3 per well) were plated into 96-well microtiter plates. After 5 h plating, the growth medium was carefully decanted and replaced by 200 μl per well of a freshly prepared dilution series of the extracts. At least eight replicates were prepared for each dilution and for the positive and negative controls. The extracts were exposed to the cells for 24, 48, and 72 h at 37 °C in a humidified 5% CO_2 atmosphere. Ten microliter per well of MTT solution (5 mg/ml PBS, pH 7.4) was added and incubated for 4 h. The formazan crystals formed were dissolved by adding 100 μl per well of a freshly prepared acidified isopropanol. The colorimetric staining of the plates was evaluated on a multi-well plate reader at 570 nm. To ensure that the polymers themselves do not contribute to the reduction of the dye, polymer solutions alone were assayed in a microtiter plate by MTT assays (Ignatius and Claes, 1996). Absorbance readings obtained were used to determine the viability of cells.

2.6. In vivo measurement of leuprolide acetate and testosterone

Polymer formulation containing LA was injected subcutaneously on the upper shaved area of the neck of the rabbits, and 1 ml of blood sample was collected from a marginal ear vein at specific time points. The blood sample was centrifuged to obtain serum and was analyzed for LA and testosterone.

2.6.1. Determination of serum LA level

Serum LA concentration was determined following the protocol provided with the leuprolide EIA kit (Peninsula Laboratories, Inc., San Carlos, CA). Briefly, leuprolide was extracted by the C18 Sep-Column extraction method. Chromatography solvents consisted of Buffer A and Buffer B. Buffer A was prepared by dissolving 1% trifluoroacetic acid (TFA, HPLC Grade) in 90% distilled water. Buffer B was a mixture consisting of 60% acetonitrile (HPLC Grade), 1% TFA and 39% distilled water. Two hundred microliter of serum was taken in a centrifuge tube, acidified with 200 μl of Buffer A, and centrifuged at 10,000 × *g* for 20 min at 4 °C. Supernatant was loaded to a C18 Sep-column which was pre-equilibrated by washing with 100% acetonitrile (1 ml, once) followed by Buffer A (3 ml, three times). The column was slowly washed with Buffer A (3 ml, three times), and the wash was discarded. LA was eluted with Buffer B (3 ml, once), and eluent was collected in a propylene tube. Eluent was evaporated to dryness by a centrifugal concentrator. The dry residue consisting LA was reconstituted in 200 μl of assay buffer provided with the kit. Extraction efficiency was determined to be 92%.

Six standards were prepared in the range of 0–10 ng/ml by diluting the standard with assay buffer provided with the kit. Fifty microliter of standard/sample, 25 μl of primary antisera, and 25 μl of biotinylated peptide solution were added into each well (except blank well) and incubated for 2 h. Thereafter, contents of the well were discarded; the plate was washed five times with assay buffer and blotted dry. One hundred microliter of streptavidin–HRP solution was added to each well (except blank well) and incubated for 1 h. Thereafter, the plate was washed five times. One hundred microliter of the 3,3′,5,5′ tetramethyl benzidine dihydrochloride (TMB) solution was added to each well and kept for incubation. After 1 h, 100 μl of 2N HCl was added to each well, and absorbance was read at 450 nm by a plate reader. This assay is reported to have sensitivity of 0.04 ng/ml.

2.6.2. Determination of serum testosterone level

Serum testosterone concentrations were determined by following the protocol provided with the testosterone RIA kit (Cayman Chemical, Ann Arbor, MI). Briefly, testosterone was extracted from serum by taking 200 μl of serum in a glass test tube containing 2 ml of anhydrous ether and vortexing for 5 min. The test tube was kept in a −20 °C freezer to freeze the lower aqueous layer. The ether layer was separated by decanting and evaporated to dryness. Two hundred microliter of EIA buffer was added to dry the extract. Extraction efficiency was found equal to 95%. Eight testosterone standard solutions were prepared in the range of 0.5–0.004 ng/ml. One hundred microliter of EIA buffer was added to each of the Non-Specific Binding (NSB) wells. Fifty microliter of EIA buffer was added to each of the Maximum binding (Bo) wells. Fifty microliter of standard/sample, 50 μl of testosterone AchE tracer, and 50 μl of testosterone antiserum were added to each well. Nothing was added to blank wells; EIA buffer and tracer were added to the NSB well; EIA buffer, tracer, and antibody were added to Bo well; and only tracer was added to the total activity well during the development step. The plate was incubated for 1 h. Thereafter, wells were emptied and rinsed

five times with wash buffer. Two hundred microliter of Ellman's reagent was added to each well except the TA well where only 5 μl was added. The plate was kept in an orbital shaker under dark for 90 min, and absorbance was read at 405 nm by a plate reader. This assay is reported to have a lower limit of sensitivity of 6 pg/ml.

2.7. Data analysis

Statistical comparisons were made using Student's *t*-test and analysis of variance (ANOVA). The level of significance was used as $p < 0.05$. The amount of LA was calculated by plotting the known concentration of leuprolide on a log scale and the corresponding absorbances on the *Y*-axis on a linear scale.

The amount of testosterone was calculated by following these steps:

i. Absorbance readings from NSB and Bo wells were averaged.
ii. Corrected maximum binding (corrected Bo) was obtained by subtracting NSB average absorbance from Bo average absorbance.
iii. Calculated the %B/Bo by dividing sample reading by corrected Bo and multiplied by 100.
iv. Standard curve was plotted by taking %B/Bo for standards on the *Y*-axis on a linear scale and testosterone concentration on the *X*-axis on a log scale.

The sigmoidal standard curves of LA and testosterone were fitted by simulating logistic non-linear fit model until χ^2 were reducing. The sigmoidal curve fit was represented by the following equation:

$$Y = \frac{A_1 - A_2}{1 + (X/X_o)^P} + A_2,$$

where Y are the values represented on Y-axis; X the values represented on X-axis; A_1 is 1.20951 (for LA), 106.13839 (for testosterone); A_2 is 0.02267 (for LA), 0.43684 (for testosterone); $X_o = 0.32694$ (for LA); 0.0438 (for testosterone); $P = 1.26102$ (for LA, 4 d.f.); 0.75261 (for testosterone, 7 d.f.); $\chi^2 = 1.05496E-4$ (for LA, 4 d.f.); 14.42306 (for testosterone, 7 d.f.).

3. Results and discussion

3.1. In vitro release of leuprolide acetate

Four formulations of LA, using DL-PLA and a solvent mixture of different ratios of BB and BA, were prepared (Table 1). Fig. 2 shows the in vitro release of LA from formulations 1–4. We found 3.04 ± 0.35, 4.63 ± 0.05, 7.56 ± 0.47, and 9.03 ± 0.13% burst release and 120, 150, 155, 175 μg/day mean release rate of LA from formulations 1, 2, 3, and 4, respectively. A greater release rate was found with formulations containing a greater proportion of BA (hydrophilic fraction) which may be due to faster gelation and/or polymer degradation caused by the presence of a greater amount of hydrophilic fraction of the solvent mixture (Eliaz and Kost, 2000).

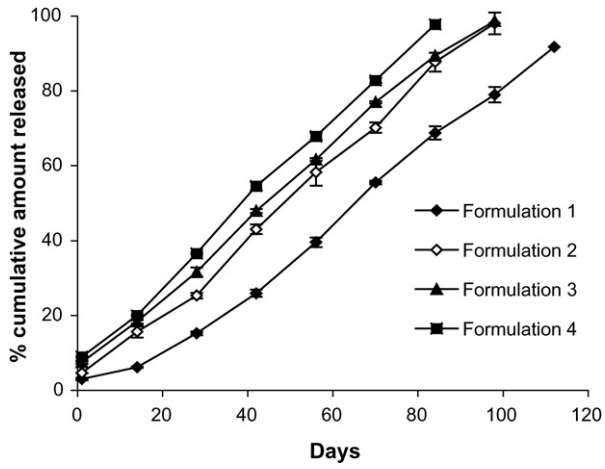

Fig. 2. In vitro release of leuprolide acetate from phase-sensitive polymer formulations.

The burst release of LA from the delivery systems was lower than microspheres because proteins/peptides may get adsorbed on the surface due its particulate nature and involvement of interfaces during manufacturing (Sohier et al., 2003). We observed therapeutically useful sustained elevated levels of 120–175 μg/day for 3 months in contrast to microspheres (e.g. Lupron®) which generally shows triphasic release profiles. Although Viadur® exhibits sustained levels, it is composed of non-biodegradable materials and requires implantation and removal by surgery which is inconvenient to patients. An oral microemulsion of LA is reported which is easy to administer but it releases all of the LA instantaneously and hence the recommended dosing frequency is daily (Zheng and Fulu, 2006). Dry powder inhaler of LA although easy to use but the bioavailability was ~50% in comparison to that of subcutaneous administration (Shahiwala and Misra, 2005).

We used different proportion of BA and BB in formulations 1–4 to obtain an optimal hydrophobicity and hydrophilicity as BA is comparatively hydrophilic and BB is hydrophobic. The in vitro release method used in this study was validated and widely used by other researchers (Okumu et al., 2001; Duenas et al., 2001). In addition, we determined the amount of LA in the released samples by a stability indicating HPLC method (Singh et al., 2000). The LA was stable in the formulation. We did not observe any additional peak in the HPLC chromatograms from the drug recovered from the formulations.

3.2. Incorporation efficiency

We calculated the efficiency of the formulations for incorporating LA as percentage of LA remaining after 24 h divided by its initial amount used while preparing the formulation. We found 96.96 ± 0.35, 95.37 ± 0.05, 92.44 ± 0.47, and 90.97 ± 0.13% incorporation efficiency of LA from formulations 1–4, respectively. Incorporation efficiency decreased with increasing the hydrophilic component in the solvent mixture.

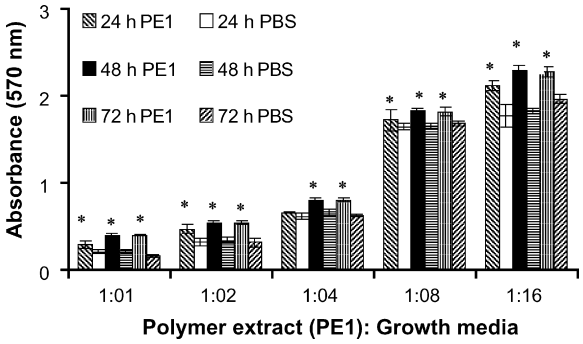

Fig. 3. In vitro biocompatibility of control polymer extract and polymer extract PE1 by MTT cell viability assay. [PBS = phosphate-buffered saline, PE1 = polymer extract made with benzyl benzoate, * significantly greater (paired *t*-test, $p < 0.05$) cell viability in comparison to control which increased significantly (ANOVA, $p < 0.05$) with increasing ratio of media except at 1:04 ratio for 24 h].

3.3. In vitro biocompatibility

The long-term biocompatibility of the delivery system was evaluated by MTT assay, using the extracts of the polymer solution in PBS (pH 7.4). In this assay, living cells reduce a water-soluble yellow dye, MTT, to a water-insoluble purple formazan product. The formazan product was quantified spectrophotometrically by measuring absorbance at 570 nm. Absorbance readings are related with the viability of cells for mitochondrial activity; higher the absorbance readings, greater the cell viability (Ignatius and Claes, 1996).

Figs. 3 and 4 show absorbance measured by MTT assay of CRFK cells in the growth media diluted with control extract or polymer extracts (PE1 and PE2). We found significantly higher ($p < 0.05$) cell viabilities in growth media diluted with polymer extracts than diluted with control for all dilutions and exposure periods.

The pH of the polymer extract was adjusted to 7.4 to exclude the effect of the acidic medium on surrounding tissues. We added 8×10^3 cells per well to prevent exhaustion of growth media by an excessive number of cells. We observed significantly ($p < 0.05$) higher viability in growth media diluted with polymer extract than the control. Polymer extracts generate lactic acid out of polymer degradation, which can stimulate mitochondrial activity by acting as a metabolic substrate. This increased mitochondrial activity may be the reason for significantly ($p < 0.05$) greater absorbance of cells containing media diluted with polymer extract than control.

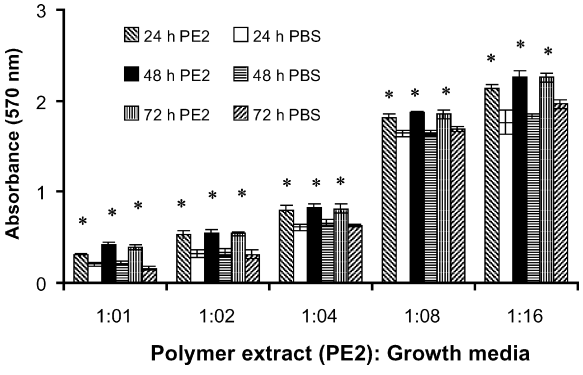

Fig. 4. In vitro biocompatibility of control polymer extract and polymer extract PE2 by MTT cell viability assay. [PBS = phosphate-buffered saline, PE2 = polymer extract made with benzyl benzoate:benzyl alcohol (95:5), * significantly greater (paired *t*-test, $p < 0.05$) cell viability in comparison to control which increased significantly (ANOVA, $p < 0.05$) with increasing ratio of media].

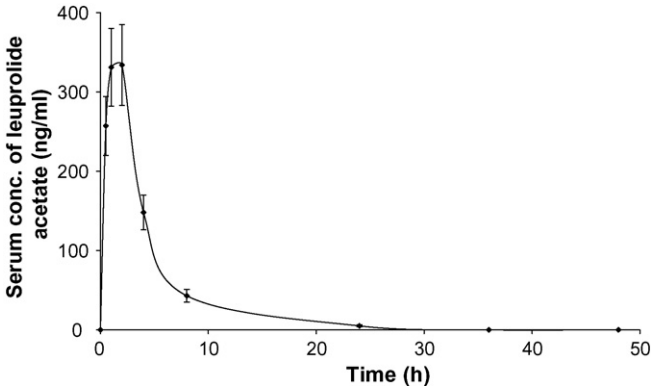

Fig. 5. In vivo absorption of leuprolide acetate following subcutaneous injection of leuprolide acetate solution.

3.4. In vivo absorption of leuprolide acetate

We prepared two formulations named formulations 1v and formulation 2v each containing LA equivalent to 10 mg/kg of body weight. Fig. 5 shows the serum concentration of LA following subcutaneous administration of LA solution (PBS, pH 7.4). The mean peak serum concentration (C_{max}) of 357 ± 37 ng/ml was achieved within 30 min. The serum concentration of LA decreased rapidly after 2 h and reached the baseline level after 24 h. We could not detect any LA in 36 and 48 h serum samples.

Fig. 6 shows LA serum concentrations following subcutaneous administration of polymer-based formulations. We found a controlled absorption of LA from both the polymeric formulations in comparison to LA solution. The peak serum concentra-

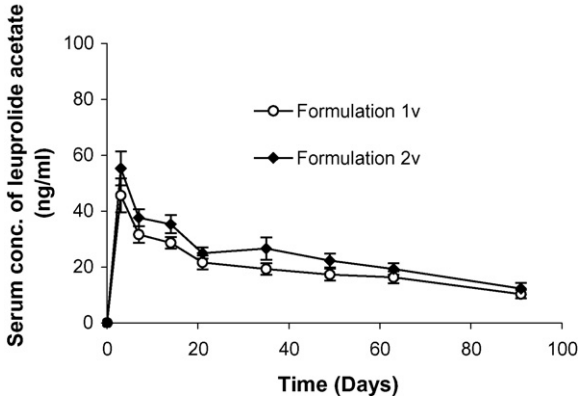

Fig. 6. In vivo absorption of leuprolide acetate following subcutaneous injection of polymeric formulations. [Formulation 1v = DL-PLA (15%, w/v) + BB (100%, v/v) + LA; formulation 2v = DL-PLA (15%, w/v) + BB (95%, v/v) + BA (5%, v/v) + LA.]

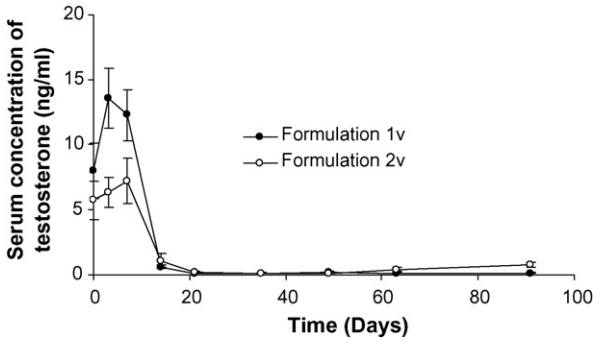

Fig. 7. Serum testosterone levels following subcutaneous injection of polymer-based leuprolide acetate formulations [formulation 1v = DL-PLA (15%, w/v) + BB (100%, v/v) + LA (15%, w/v); formulation 2v = DL-PLA + BB (95%, v/v) + BA (5%, v/v) + LA.]

tion of LA (40–66 ng/ml serum) was observed in 3 days' samples from both the polymer formulations. We observed a constant serum LA levels in the range of 10–20 ng/ml and 10–25 ng/ml after 2nd week from formulation 1v and 2v, respectively. Serum levels of LA from formulation 1v were significantly lower ($p < 0.05$) in 14, 35, and 49 days samples in comparison to those from formulation 2v which might be due to a greater degradation rate assisted by the presence of the hydrophilic fraction (BA) in the formulation 2v.

3.5. *In vivo pharmacodynamic effect of leuprolide acetate*

Fig. 7 shows the testosterone serum concentrations following subcutaneous administration of two polymer-based formulations of LA. Serum testosterone concentration initially increased from 4–8 ng/ml to 8–13 ng/ml from both the formulations, but began to decline and reached the castration level (0.5 ng/ml) after 14 days, which was maintained until the end of study for formulation 1v (91 days) and formulation 2v (63 days).

LA delivered in biologically active form from the polymer formulations was supported by its pharmacodynamic effect. The pharmacodynamic effect was due to controlled release of LA and protection provided by the formulation components against various peptide degradation mechanisms.

4. Conclusions

Phase-sensitive polymer delivery systems controlled the in vitro release of incorporated LA. The release rate of LA from the delivery system was influenced by the hydrophilic fraction of the solvent system. The polymer system was found biocompatible. In vivo in rabbits study showed that the phase-sensitive polymer formulations could be used for delivering LA at a controlled rate for 3 months for the purpose of reducing testosterone to castration level. Future study should use increased polymer concentration as well as larger molecular weights of PLA in order to extend further the release of LA from 3 to 6 months. Furthermore, we would extend the biocompatibility studies from in vitro to in vivo in rabbits.

Acknowledgement

Financial support from NIH grant # HD 41372 is acknowledged.

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