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Evaluation of Orntide Microspheres in a Rat Animal Model and Correlation to In Vitro Release Profiles

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ABSTRACT Orntide acetate, a novel luteinizing hormone-releasing hormone (LHRH) antagonist, was prepared and evaluated in vivo in 30-day and 120-day sustained delivery formulations using a rat animal model. Orntide poly(d,l- lactide-co-glycolide) (PLGA) and poly(d,l- lactide) (PLA) microspheres were prepared by a dispersion method and administered subcutaneously in a liquid vehicle to rats at 2.2 mg Orntide/kg of body weight (30-day forms) or 8.8 mg Orntide/kg (120-day forms). Serum levels of Orntide and testosterone were monitored by radioimmunoassays, and a dose-response study at 4 doses (3, 2.25, 1.5, and 1.75 mg Orntide/kg) was conducted to determine the effective dose of Orntide. Microspheres with diameters between 3.9 and 14 μ were prepared. The onset and duration of testosterone suppression varied for different microsphere formulations and were influenced both by polymer properties and by microsphere characteristics. Microspheres prepared with 50:50 and 75:25 copolymers effectively sustained peptide release for 14 to 28 days, whereas an 85:15 copolymer and the PLA microspheres extended the pharmacological response for more than 120 days. Increase in drug load generally accelerated peptide release from the microspheres, resulting in higher initial serum levels of Orntide and shorter duration of the release. In general, apparent release was faster in vivo than under in vitro conditions. Orntide microspheres effectively suppressed testosterone in rats, providing rapid onset of release and extended periods of chemical castration. Testosterone suppression occurred immediately after microsphere administration without the initial elevation seen with LHRH superagonists.

Key Words: LHRH antagonist; Orntide acetate; Peptide controlled delivery; PLGA microspheres; Prostate cancer.

INTRODUCTION

Since the discovery and structural elucidation of luteinizing hormone–releasing hormone (LHRH), numerous analogues have been synthesized and studied for potential application in gynecology and oncology as antigonadotropic agents for ovarian and testicular suppression [1-4]. Leuprolide acetate, developed and marketed by TAP Pharmaceuticals, Inc. (Deerfield, Ill.) as Lupron Depot®, is one of the most widely used LHRH superagonists in the treatment of sex hormone-dependent tumors, such as prostatic carcinoma in men [5-7]. During continuous administration, Leuprolide initially stimulates production and release of testosterone, but the prolonged exposure to an LHRH superagonist eventually causes down-regulation of LHRH receptors and inhibition of luteinizing hormone (LH) release, which finally leads to chemical castration [8,9]. Due to this initial testosterone elevation, which may last in humans from 5 to 8 days, approximately 11% of all treated patients experience painful and potentially dangerous flare-ups of disease [10-14].

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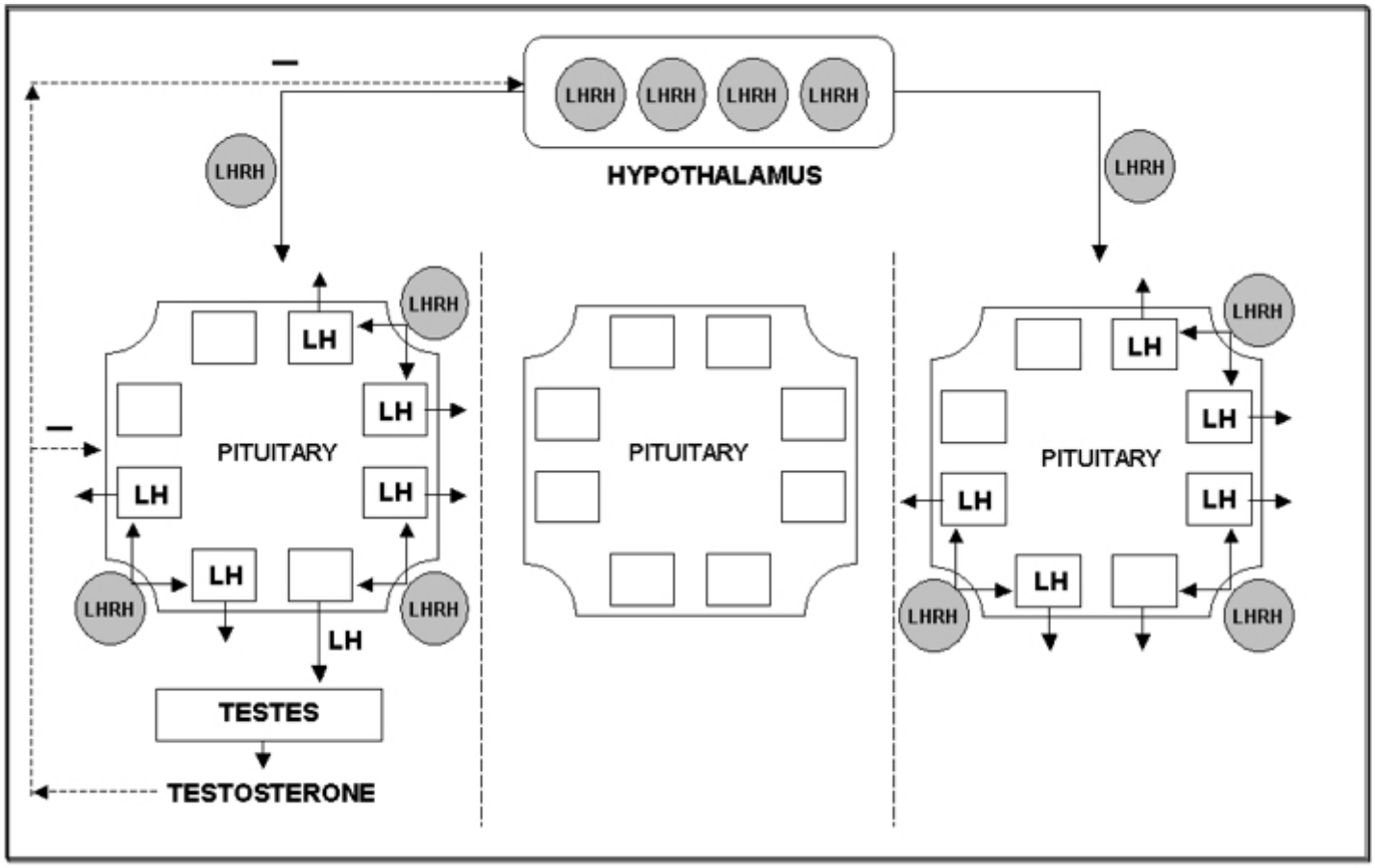
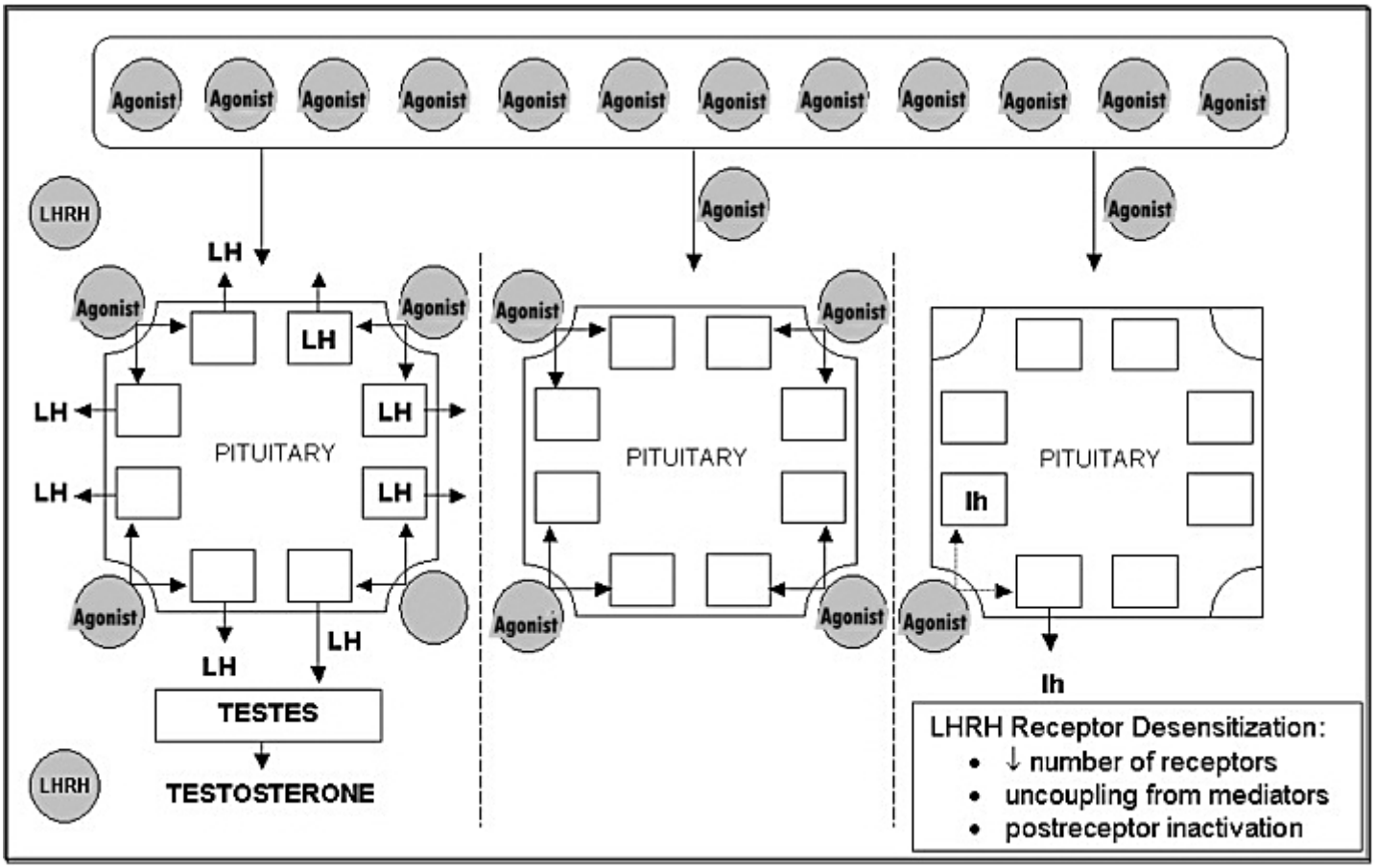


Scheme 1. Normal physiology of the pituitary-gonadal axis.

Scheme 1 illustrates normal physiology of the hypothalamo-pituitary-gonadal axis. LHRH, a peptide hormone synthesized in the arcuate nucleus of the hypothalamus, is released in response to low testosterone levels and is transported to the pituitary gland, via capillaries known as the hypothalamic-pituitary portal system, where it binds to the surface receptors of gonadotropin-producing cells, or gonadotropes. Once the LHRH receptors are stimulated, the gonadotropes synthesize and secrete gonadotropins by exocytosis of storage granules containing LH and follicle-stimulating hormone (FSH). Both hormones are glycoproteins that consist of €α and ß subunits. FSH stimulates the development of the seminiferous tubules and maintains spermatogenesis, and LH stimulates the Leydig cells in the testes to produce and secrete testosterone. Under normal physiologic conditions, LHRH is rapidly removed from the site by enzymes that cleave the glycine-leucine linkage between positions 6 and 7. In this way the gonadotropins are released in a regular pulsatile fashion, which is necessary to maintain normal functions of the pituitary gonadotropes and gonads [2].

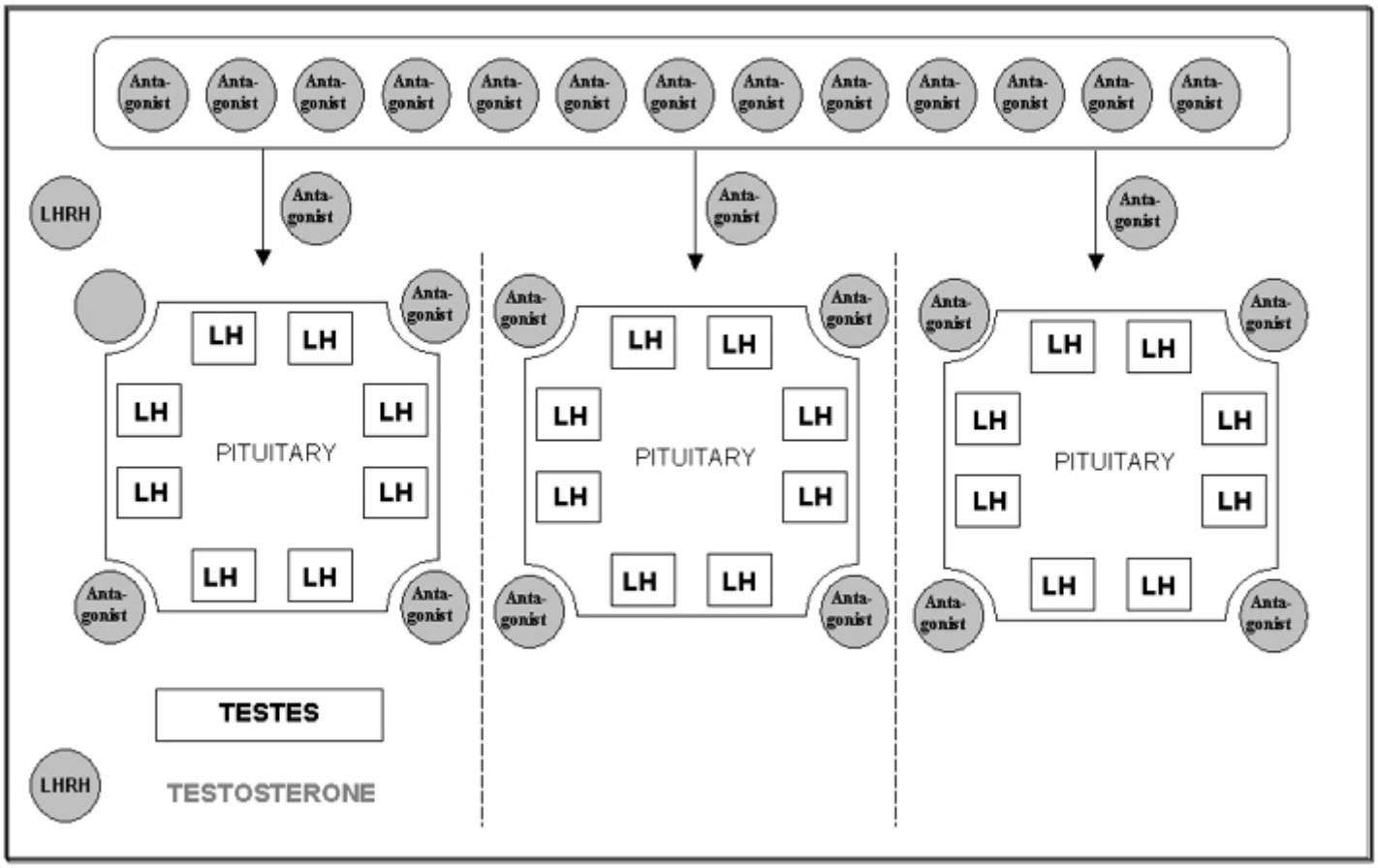
Scheme 2 shows the effect of continuous administration of an LHRH superagonist on the pituitary-testicular axis. Initially, a transient phase of testosterone elevation caused by stimulation of the pituitary LHRH receptors and increased release of LH is observed. Soon after, however, the gonadotrophic receptors become desensitized and unresponsive to further stimulation. The receptor down-regulation process includes reduction in the number of surface receptors, uncoupling of the receptors from intracellular mediators, and postreceptor inactivation [2,15-17]. Finally, instead of producing active LH, gonadotrophs start to produce and release an inactive form of LH (shown in **Scheme 2** as "lh") that is unable to stimulate production of testosterone in testes.



Scheme 2. The effect of continuous exposure to a LHRH superagonist on the pituitary-gonadal axis physiology.

It has been reported that immediate and complete testosterone suppression may be achieved with LHRH antagonists [2]. These compounds show a much higher affinity to the pituitary LHRH binding sites than does native LHRH; they also compete with endogenous LHRH at the LHRH receptor sites. Blockade of the pituitary LHRH receptors prevents stimulatory effects of native LHRH and results in rapid inhibition of the pituitary- gonadal axis; thus, testosterone suppression is immediate without the characteristic stimulatory phase for LHRH superagonists (**Scheme 3**). In addition, it has been reported that LHRH antagonists directly inhibit growth of numerous tumors, including mammary, pancreatic, and prostatic tumors, by binding to receptors localized on tumor cells [18-21].

More than 3000 LHRH analogues have been synthesized and studied [2]. Whereas the LHRH agonists have already found clinical application in the therapy of gonadal steroid–associated diseases, the LHRH antagonists have not reached this stage. Animal studies with early LHRH antagonists revealed that, due to high histamine release from mast cells, these compounds caused severe adverse effects, including anaphylactoid reactions, hypotension, skin lesions, and formation of edema [22,23]. During the past 2 decades, developers of safer LHRH antagonists focused on increasing antigonadotropic potency and minimizing histamine-releasing properties. As a result of enormous efforts by several research groups, a series of modern, safer, and more potent LHRH antagonists has been synthesized [24,25]. Orntide acetate, synthesized by Cyril Y. Bowers, Karl Folkers, and Anna Janecka at Tulane School of Medicine, New Orleans, LA, is one of the most promising LHRH antagonists developed to date (**Table 1**).



Scheme 3. The effect of continuous exposure to a LHRH antagonist on the pituitary-gonadal axis physiology.

Table 1. Leading LHRH antagonists (Modified from: Cyril Y. Bowers, Karl Folkers, and Anna Janecka, United States Patent #US5480969, 1996)

LHRH Antagonist	Antiovulatory Activity (%/μg)					Histamine Release ED_{50} (μg/mL)
	0.125	0.25	0.5	1.0	1.5	
Nal-Arg (Rivier et al., 1984)			50			0.17
Nal-Glu (Rivier et al., 1986)			50		100	1.6
Antide (Ljungqvist et al., 1987)			36	100		>300
Argtide (Janecka et al., 1991)	63	89				31
SB-75 (Bajusz et al., 1988)					75	1.5
RS-26306 (Nestor et al., 1985)		50				13
Ornitide (Bowers et al., 1996)		25	100			100

Note: The most preferred LHRH analogues of the table have high antiovulatory activity and also a high effective dose level for histamine (ED_{50} = dose releasing 50% of total histamine from mast cells; for native LHRH ED_{50}=170 μg/mL).

It combines both high antigonadotropic potency and safety. At present, this analogue is undergoing preclinical evaluation in animals and soon will enter the first phase of clinical trials. Therefore, sustained-release formulations of Ornitide acetate are urgently needed to make the clinical use more feasible. Such preparations may, in addition, help to reduce the therapeutic dose required for continuous suppression of pituitary and gonadal functions and may cause fewer adverse effects.

To enable further studies and clinical trials with Ornitide, this analogue must be formulated as a therapeutic system that provides the highest efficiency and safety of the treatment and preferably good patient compliance. Therefore, the purpose of this work was to prepare controlled-release microparticulate Ornitide formulations using biodegradable low-molecular-weight polylactides, poly(d,l- lactide) (PLA) and poly(d,l- lactide-co-glycolide) (PLGA), and to evaluate these formulations for their efficacy in suppressing testosterone in a rat animal model.

MATERIALS AND METHODS

Materials

Orntide acetate ([NacDNal1 DpClPhe 2 D3Pal 3 PicLys 5 D(6Anic)Orn 6 Ilys 8 DAla 10] - LHRH) was supplied by California Peptide Research, Inc (Napa, CA). Orntide 30-day and 120-day PLGA and PLA microspheres were developed in collaboration with Oakwood Laboratories, LLC (Oakwood, OH) [19]. Three 50:50 PLGA copolymers (MW 10 777–31 281) with different degrees of hydrophilicity, 75:25 PLGA (MW 11 161), and a PLA homopolymer (MW 9489) were obtained from Boehringer Ingelheim, Inc (Ingelheim, Germany), and 85:15 PLGA (MW 17 903) was obtained from Birmingham Polymers, Inc (Birmingham, AL). The hydrophilic resomers (H) had free carboxyl end groups, whereas the hydrophobic ones were capped with long-chain alkyl alcohols. Polyvinyl alcohol (PVA) (average MW, 30 000-70 000) was obtained from Sigma Chemical Co (St Louis, MO). The solvents and other excipients were analytical grade and were purchased from commercial sources; dialysis tubes (Tube-O-Dialyzer®) were from Research Products International Corp (Mount Prospect, IL), and a Spectra/Por® CE dialysis membrane (molecular weight cut-off, or MWCO, 300 000 Da) was from Spectrum Medical Industries, Inc (Houston, TX). Injections of free Orntide acetate were prepared by dissolving the peptide in water to reach the desired concentration. Male Sprague Dawley rats weighing approximately 300 g were purchased from Harlan (Indianapolis, IN). The studies were conducted at the University of Kentucky College of Pharmacy Animal Research Facility.

Methods

High-Performance Liquid Chromatography (HPLC) Method for Peptide Assay

The peptide was analyzed by reverse-phase HPLC (Bondclone 10 C18 column, 150 x 3.90 mm) using an elution phase of 34% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid in water. Ultraviolet detection was at 215 nm [26].

Preparation of Orntide Microspheres

Orntide microsphere batches (0.9-2.7 g) were prepared by a dispersion method, followed by solvent extraction and evaporation [26]. A solution of peptide in methanol was combined with a solution of PLGA or PLA in methylene chloride and stirred until clear. The solution was then slowly injected into a 1 L reactor with baffles (Ace Glass Inc, Vineland, NJ) containing the continuous phase (CP; 0.35% [wt/vol] solution of PVA; pH, 7.2) and stirred at 5500 rpm with a Silverson™

L4R homogenizer (Silverson Machines Ltd, Waterside, England). The temperature of the reactor was maintained initially at 25ºC for 30 minutes and then at 40ºC for 60 minutes. Once microspheres were formed and hardened, the contents of the reactor were transferred to a filtration apparatus equipped with a 0.8 µ membrane filter (Gelman Sciences, Ann Arbor, MI) and the recovered product was washed with water and dried under reduced pressure for 48 hours at room temperature.

Microsphere Characterization

Peptide content in microspheres was determined by HPLC after dissolving the microspheres in chloroform and extracting the peptide with a 0.1 mol/L acetate buffer (AB) with a pH of 4.0. Total product yield was assessed gravimetrically on the basis of polymer/drug recovery. Microsphere bulk density was determined with the tapping method using approximately 100 taps and a 10 mL graduated glass test tube. Particle size distribution was determined using a laser diffraction technique (Malvern 2600c Particle Sizer, Malvern, UK).

In Vitro Release Study

A previously described dialysis technique was used to determine the in vitro release from Orntide microspheres [27]. Approximately 20 mg of the microspheres were quantitatively transferred to a 7 mL dialysis tube (Tube-O-Dilalyzer; MWCO, 300 000 Da) containing 5.0 mL of AB, which in turn was placed in a 50 mL tube containing 40 mL of the same release medium. The contents of the larger tube were continuously stirred with a magnetic stirrer. At various times, 1.0 mL of the supernatant was removed from the

50 mL tube and 1.0 mL of fresh buffer was added. The sample was analyzed for peptide concentration using the HPLC method.

Animal Study

Male Sprague Dawley rats (n = 6) weighing approximately 300 g were used to study testosterone suppression during treatment with liquid Orntide acetate and Orntide microspheres. The formulations were injected subcutaneously at the lower back section of the neck after reconstitution in a liquid vehicle (1% [wt/vol] carboxymethylcellulose and 2% [wt/vol] mannitol). Blood samples were collected from the tail vein at specific times. The samples were centrifuged in Microtainer®

€tubes (Becton Dickinson, Franklin Lakes, NJ), and serum was collected. Serum samples were frozen and stored at -20ºC until analysis.

Serum Orntide levels in rats were assessed using a radioimmunoassay developed at Tulane School of Medicine, New Orleans, LA. Tyr1-Orntide was radioiodinated by the lactoperoxidase method, and the labeled ligand was purified by HPLC. Orntide was conjugated by the carbodiimide method, and an antibody to Orntide was produced in rabbits. The lower detection limit of the assay was 0.008 ng/mL. The intra- and interassay coefficients of variation were 6% and 9%, respectively.

Serum testosterone levels were assayed using Active™ Testosterone RIA DSL-4000 kits (Diagnostic Systems, Inc, Webster, TX). The lower limit of detection for this assay was 0.08 ng/mL, and the intra- and interassay coefficients of variation were 10% and 9%, respectively. The cross-reactivity of the testosterone antiserum was less than 6%.

Statistical Evaluation of Data

Data are presented as means ±standard deviation. For values below the assay detection limit, the limit was used for calculations. To evaluate the effectiveness of continuous treatment with Orntide microspheres to suppress testosterone level in rats, the areas under serum Orntide and serum testosterone curves were calculated using GraphPad Prism (GraphPad Software, Inc, San Diego, CA). The results were analyzed by one-way analysis of variance at α-level of 0.05.

RESULTS AND DISCUSSION

Dried microspheres formed a free- flowing powder with the average particle size varying from 3.9 to 19 µ (Table 2). Average peptide incorporation efficiency was 84%, and drug load varied from 6.63% to 17.2%. As shown in Figure 1, a single administration of liquid Orntide to rats at 0.1 mg/kg resulted in serum Orntide peak 6 hours later (observed C_{max} 35 ng/mL). At the same time mean serum testosterone level decreased from the initial 3.4 ng/mL to below 0.1 ng/mL.

Table 2. Physical characteristics of Orntide microspheres

Polymer		Molecular Weight (Da)*	Drug Load (wt %)	In cor Eff (wt %)	Yie (w %)	Bulk Density (g/cc)	Particle Size (µ) <90%	<50%	< 10
Code	Type								
502H	PLGA 50:50	10 777	6.63	61	68	0.32	29	10	2.3
503H (1)	PLGA 50:50	28 022	11.2	86	77	0.48	36	14	2.0
503H (2)	PLGA 50:50	28 022	14.6	81	78	0.49	19	5.9	1.2
503 (1)	PLGA 50:50	31 281	8.91	81	78	0.66	27	11	1.7
503 (2)	PLGA 50:50	31 281	13.6	91	70	0.63	28	10	1.6
7525H	PLGA 75:25	11 161	10.8	83	70	0.51	29	11	2.3
8515H	PLGA 85:15	17 903	14.2	89	74	--	26	6.6	1.7
202H (1)	PLA	9489	9.80	88	80	--	29	9.8	3.4
202H (2)	PLA	9489	15.1	94	86	--	23	3.9	1.5
202H (3)	PLA	9489	17.2	82	71	--	46	14	3.3

*) Average molecular weight.

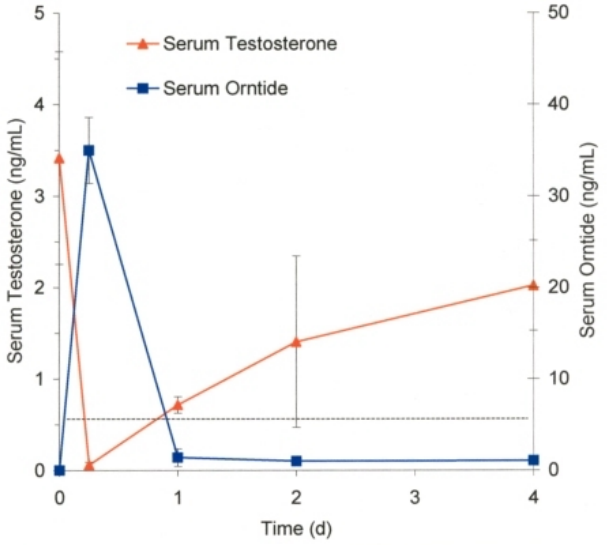


Figure 1. Serum Orntide and testosterone levels in rats after a single administration of liquid Orntide (0.1 mg/kg). Dotted line at 0.5 ng/mL indicates castration level of testosterone.

These results indicate that Ornitide acetate is an effective antigonadotropic agent with a rapid onset of action. The testosterone suppression was reversed 24 hours after Ornitide administration, suggesting that Ornitide acetate was rapidly degraded in animals probably as a result of poor resis tance to peptidases and physical instability at physiological pH [28,29]. Short biological half- lives of LHRH analogues have been one of the reasons for frequent administrations and poor patient compliance during chronic treatment [11].

Figure 2 shows serum Ornitide levels in rats after administration of 30-day Ornitide microspheres (2.2 mg Ornitide/kg) prepared with different resomers of PLGA. The 502H microspheres resulted in a high initial serum Ornitide level (observed C_{max} of 69 ng/mL at 6 hours) but did not sustain elevated serum Ornitide levels beyond 15 days. The 7525H microspheres had the highest initial release (C_{max} of 147 ng/mL at 6 hours) and maintained serum Ornitide levels above 1 ng/mL for more than 22 days.

The 503H (1) microspheres produced a sharp Ornitide peak at 6 hours (27 ng/mL), and C_{max} of 29 ng/mL was observed on day 8; the mean serum level of Ornitide remained above 1 ng/mL for approximately 20 days. The 503H (2) microspheres with a higher drug load (14.6%) showed the highest serum Ornitide concentration at 6 hours ($C_{max}$of 25 ng/mL), and a second smaller peak occurred on day 4; the mean serum Ornitide level remained above 1 ng/mL for 36 days. These serum Ornitide profiles indicate that drug load had an effect on the in vivo release rate from the 503H microspheres; increase in drug load shifted the maximum release from the microspheres from 8 to 4 days.

Serum testosterone levels in rats treated with 30-day Ornitide PLGA microspheres and in a control group are shown in **Figure 3**. The 503 (1) and (2) hydrophobic microspheres did not suppress testosterone in rats with the only exception of the 6-hour data point when the levels decreased to below 0.5 ng/mL (castration level).

The lack of testosterone suppression for the 503 microspheres was expected because very little release from these microspheres was detected in vitro [26].

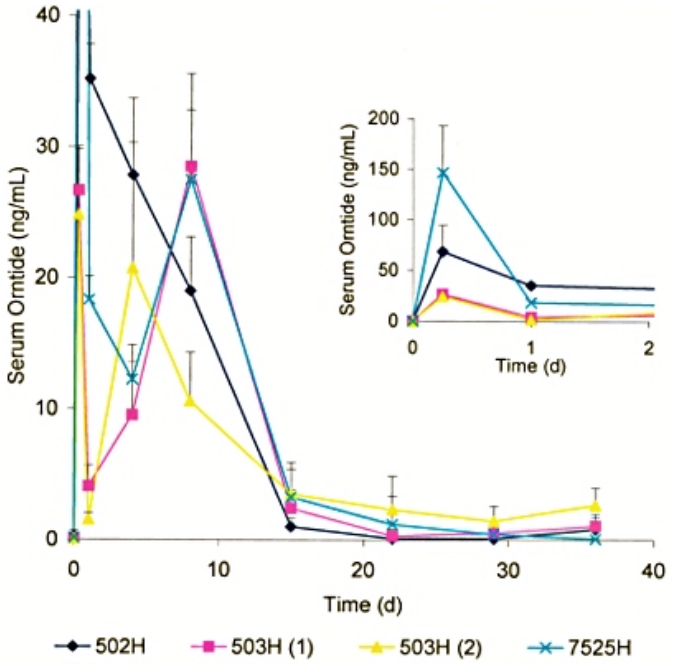


Figure 2. Serum Ornitide levels in rats after administration of 30-day Ornitide PLGA microspheres (2.2 mg Ornitide/kg).

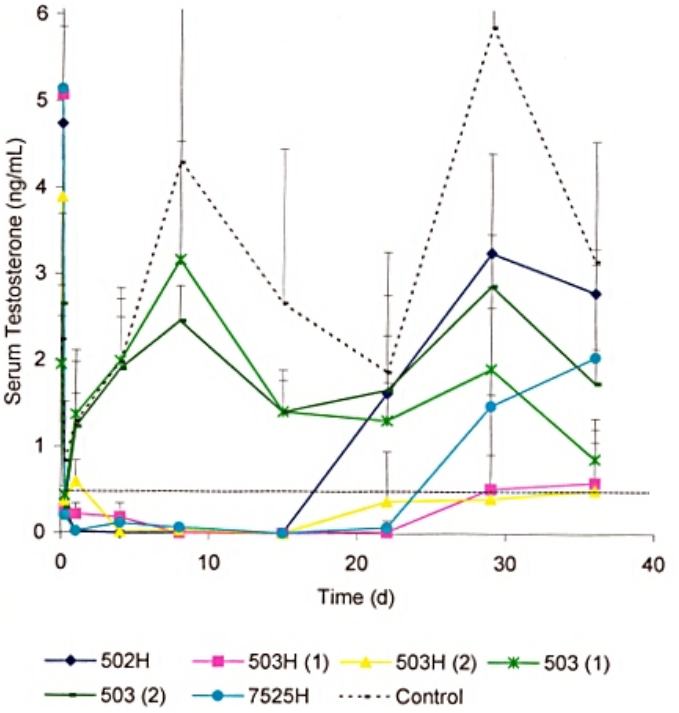


Figure 3. Testosterone suppression in rats after administration of 30-day Ornitide microspheres (2.2 mg Ornitide/kg). Dotted line at 0.5 ng/mL indicates castration level of testosterone.

The initial suppression of testosterone in these two groups could be ascribed to the release of free Orntide adsorbed to the microspheres' surface or ascribed to stress during administration; stress also affected serum levels in the animals in the control group. Rapid onsets.of testosterone suppression were seen in all animal groups treated with hydrophilic Orntide microspheres, but only the 503H microspheres maintained castration levels of testosterone for 4 weeks. The 503H (1) microspheres showed a better initial testosterone suppression than the 503H (2) microspheres and maintained testosterone level below 0.1 ng/mL for more than 22 days (**Figure 3**). The difference in suppression duration between the two 503H microsphere batches illustrates the effect of drug load on the release rate from PLGA delivery systems. Incorporation of hydrophilic drugs into polymeric matrices increases water uptake by the microspheres, thereby accelerating hydrolytic degradation of the polymer; the opposite is also true, and a high load of a hydrophobic drug may delay polymer degradation [28].

Dose-response study with the 503H (1) microspheres showed that testosterone suppression in rats was dose-dependent and could be enhanced by increasing the initial dose of Orntide microspheres (**Figure 4**). Increase of the Orntide dose to 3 mg/kg extended testosterone suppression by an additional week and provided a sustained-release Orntide formulation effective in vivo for 30 days.

Serum Orntide levels in rats after administration of 120-day Orntide microspheres (8.8 mg Orntide/kg) are shown in **Figure 5**. Initially, all microspheres produced high levels of Orntide in serum ranging from 52 ng/mL (8515H microspheres) to 212 ng/mL (202H [2] microspheres) at 6 hours. The 202H (1), with a low drug load, resulted in lower Orntide levels, and after the initial peak at 6 hours, the mean Orntide concentration decreased to 6.5 ng/mL on day 1 and remained below that for the remainder of the study. This serum Orntide level was not sufficient to suppress testosterone and maintain castration in rats for an extended period of time (**Figure 6**).

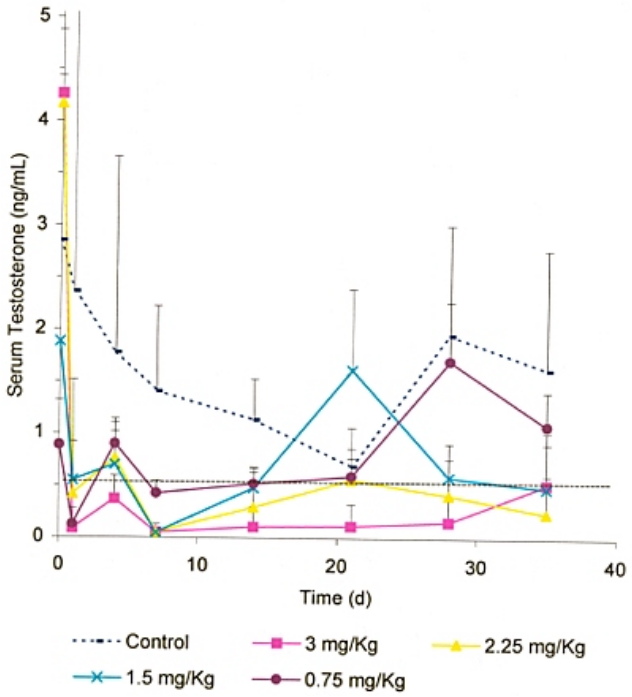


Figure 4. Dose-response study in rats with 30-day Orntide PLGA503H microspheres. Dotted line at 0.5 ng/mL indicates castration level of testosterone.

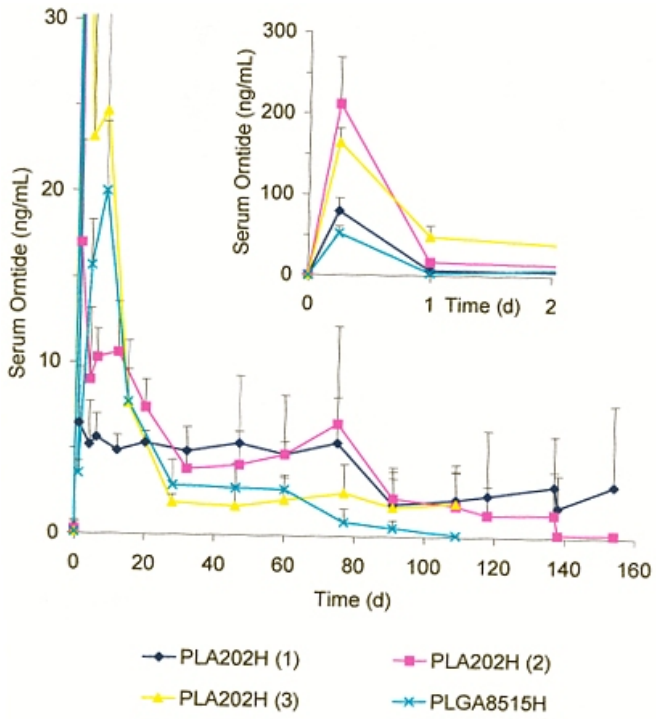


Figure 5. Serum Orntide levels in rats after administration of Orntide 120-day microspheres.

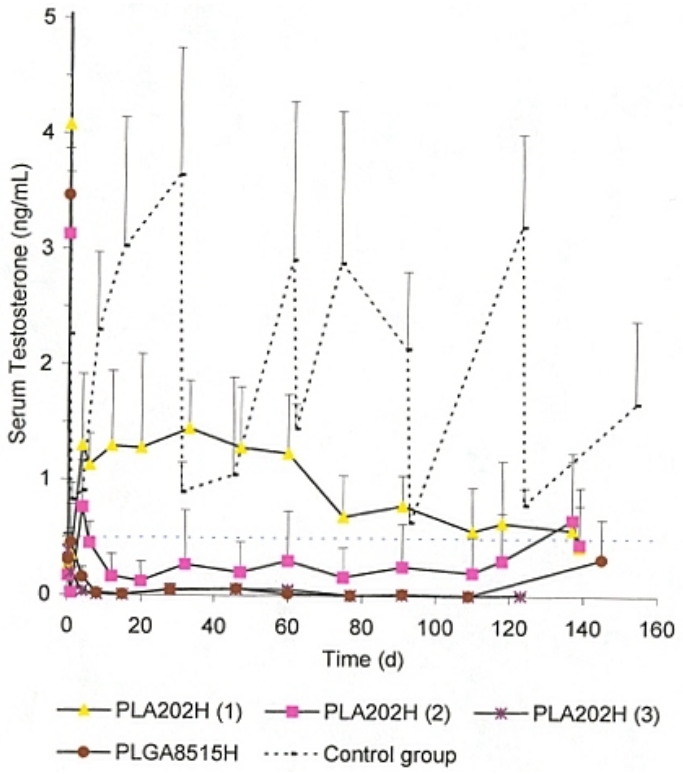


Figure 6. Testosterone suppression in rats after administration of 120-day Orntide microspheres. Dotted line at 0.5 ng/mL indicates castration level of testosterone.

In contrast, the 202H (2) microspheres maintained a serum Orntide level that was twice as high as that produced by the previous batch during the first 2 weeks and suppressed testosterone in rats to below 0.5 ng/mL for approximately 130 days, with a sma ll elevation to 0.8 ng/mL on day 4. Even more rapid castration was seen in rats treated with the 202H (3) and 8515H microspheres: mean testosterone levels decreased to below 0.5 ng/mL within 6 hours and remained below 0.1 ng/mL for 4 months (**Figure 6**). Interestingly, these 2 batches of microspheres maintained higher serum Orntide levels than did the 2 previous batches (202H [1] and [2]) only during the first 10 days ; for most of the study, the Orntide levels were below 3 ng/mL. This indicates that to achieve a rapid and prolonged castration in rats, it is crucial to maintain higher serum Orntide levels initially (above 10 ng/mL for the first 10-14 days), and it seems that the subsequent levels as low as 3 ng/mL are sufficient to suppress testosterone for several months. This may be an advantage because the biodegradable PLGA and PLA microspheres cause

Table 3. Response areas under serum Orntide and testosterone curves in rats treated with Orntide microspheres

Formulation (Orntide mg/kg)	< mL) d (ng curve Orntide serum under area Mean>	Mean area under testosterone curve (ng d/mL)
30-Day Control (0.0)		118± 33
502H microspheres (2.2)	314± 36	41±15
503H (1) microspheres (2.2)	237± 54	5.5 ±4.5
503H (2) microspheres (2.2)	204 ± 59	4.5 ± 3.1
503 (1) microspheres (2.2)	ND	62 ± 14
503 (2) microspheres (2.2)	ND	71 ± 13
7525H microspheres (2.2)	336 ± 49	20 ± 12
120-Day Control (0.0)	–	299 ± 34
202H (1) microspheres (8.8)	631± 134	133± 25
202H (2) microspheres (8.8)	705 ± 128	7.2 ± 4.3
202H (3) microspheres (8.8)	559± 125	4.4 ± 0.8
8515H microspheres (8.8)	427 ± 44	10 ± 5.8

a "burst release" followed by an extended phase of steady release [29].

Table 3 shows mean areas under serum Orntide and testosterone curves in rats after administration of various Orntide formulations. All microsphere batches resulted in significant decrease of testosterone levels compared with control groups. Statistically, the 503H (2) and 503H (1) microspheres were most effective among the 30-day formulations (area under the curve [AUC] T_{036d} 4.5 ± 3.1 and 5.5 ± 4.5 ng d/mL, respectively), whereas, the 202H(3) microspheres showed the best testosterone suppression over a 4-month period (AUC $T_{0\text{-}115\ d}$ 4.4 ± 0.8 ng d/mL). On average, the 30-day formulations produced an area under serum Orntide curve (Orntide AUC) of 273 ng d.mL, and the 4-month Orntide microspheres resulted in a mean AUC of 581 ng d/mL.

Cumulative Orntide AUCs were compared with in vitro release profiles previously obtained with a dialysis method [26]. Assuming that Orntide acetate has a very short biological half- life and that the peptide does not stimulate an antibody reaction, then the cumulative Orntide.AUC should reflect the in vivo release from sustained-release Orntide formulations. **Figure 7A** shows the Orntide AUC curve and in vitro release profile obtained with a dialysis method in 0.1 mol/L acetate buffer, pH 4.0 at 37° C, for the 50:50 PLGA (503H [2]) microspheres.

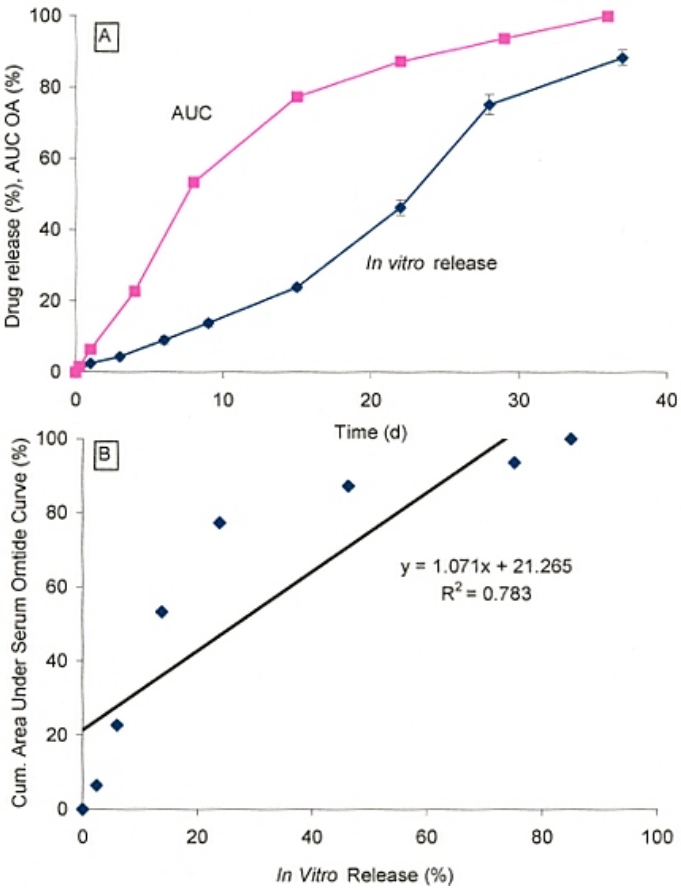


Figure 7. 50:50 PLGA microspheres [503H(2)]: A) Mean area under serum Orntide curve vs. in vitro release profile, and B) in vivo-in vitro correlation.

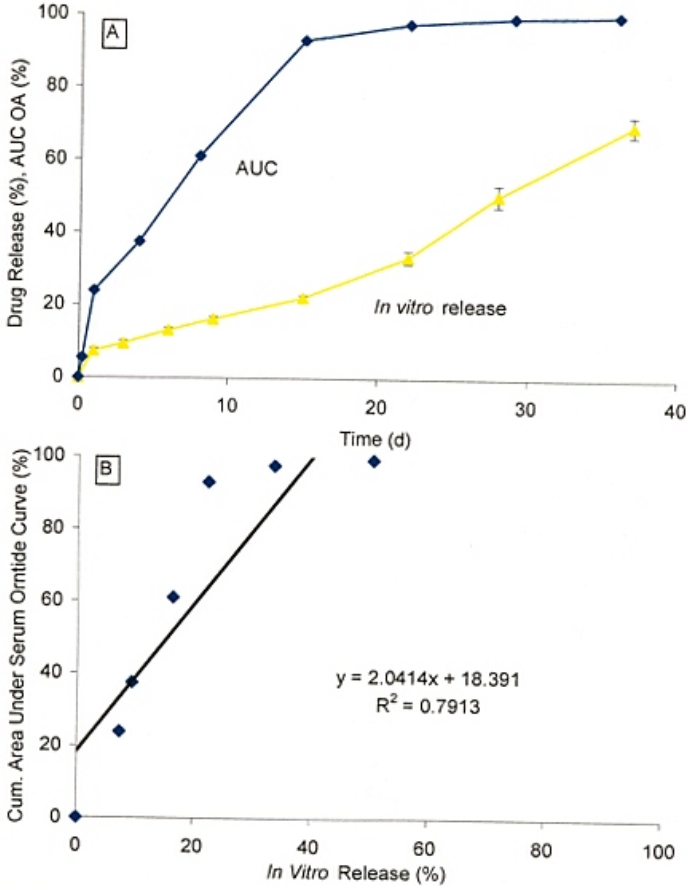


Figure 8. 75:25 PLGA microspheres (7525H): A) Mean area under serum Orntide curve vs. in vitro release profile, and B) in vivo-in vitro correlation.

The Orntide AUC curve shows that the in vivo release started immediately after administration and was rapid during the first 15 days (77% release). During the same period, only 24% of the initial drug load was released from the microspheres in the in vitro study; 50% release was reached after T_{50} of 23 days (in vivo T_{50} was 7 days). After the initial 25% release, the in vivo and in vitro release rates became similar, although the in vitro release curve remained delayed by approximately 2 weeks for the entire study. Even faster in vivo release was seen for the 7525H Orntide microspheres, where the initial release reached 24% after 1 day and was over 93% after 15 days (**Figure 8A**).

In contrast, only 7.3% of the drug was released within 1 day during the in vitro study, and the microspheres continued to release with a steady rate of 1.7% per day, reaching 50% after 28 days. Similarly, higher initial in vivo release was seen for the 120-day forms. **Figure 9A** shows a typical S-shaped in vitro release profile and cumulative Orntide AUC for the 8515H microspheres.

The release rate was much slower under in vitro conditions during the first month (14%) than in vivo, where a similar release occurred within 4 days. The PLA (202H [3]) microspheres released in vivo more than 60% of the drug within 2 weeks and continued to release 0.37% per day (**Figure 10A**). In contrast, only 16% release occurred in vitro in 14 days, but subsequently the release rate stabilized at 0.38% per day.

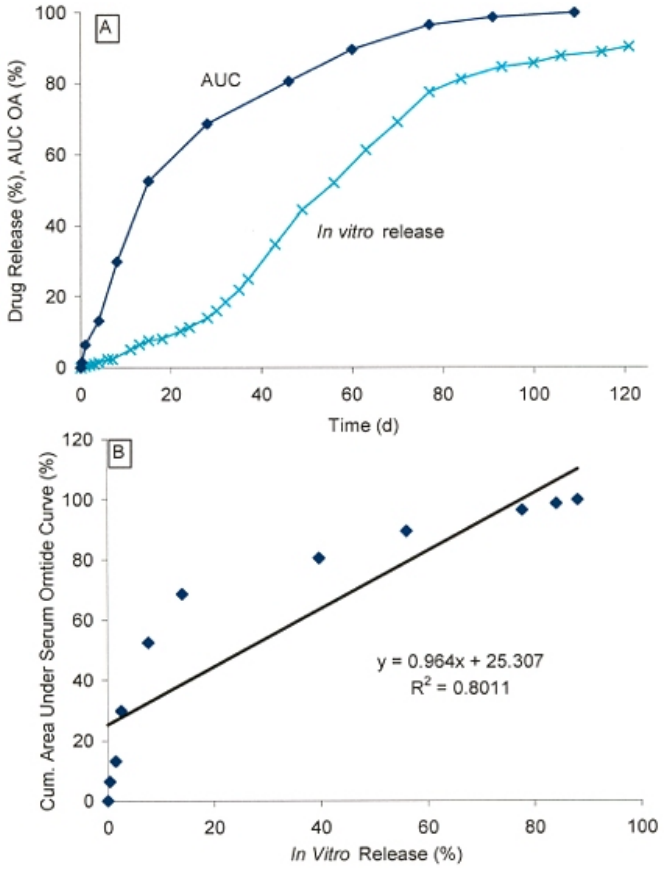


Figure 9. 85:15 PLGA microspheres (8515H): A) Mean area under serum Orntide curve vs. in vitro release profile, and B) in vivo-in vitro correlation.

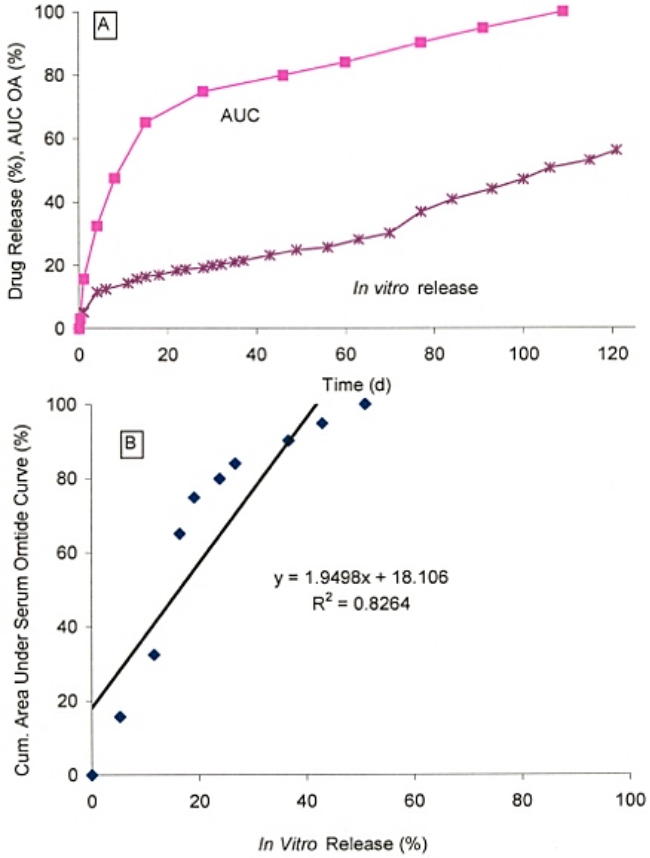


Figure 10. PLA microspheres [202H(3)]: A) Mean area under serum Orntide curve vs. in vitro release profile, and B) in vivo- in vitro correlation.

CONCLUSIONS

The comparison between in vitro and in vivo release profiles indicates that the initial in vivo release is several times higher than under in vitro conditions. The biggest difference in release rates was seen during the first 14 days for the 30-day forms and during the first month for the 120-day microspheres. The higher in vivo release rate resulting in poor overall in vivo–in vitro correlation (**Figures 7B** and **10B**) could be ascribed to several factors, including 1) higher peptide solubility in serum than in a buffer system, 2) presence of hydrolytic enzymes, 3) formation of acidic microenvironment within degrading microspheres, and 4) effect of plasma proteins on degradation of polyesters [30-35].

A recently published study showed that solubility of vapreotide pamoate (somatostatin analogue) was greatly enhanced in protein-containing media, such as aqueous bovine serum albumin (BSA) solution and human serum, and was dependent on protein concentration [30]. The increased peptide solubility in serum could affect the release rate from biodegradable microspheres because of a higher dissolution rate of a free fraction of the peptide trapped inside the matrix pores or adsorbed to the microspheres' surface.However, it seems unlikely that the higher solubility in serum could play a major role in release over a period of several months because the rate of peptide release from biodegradable microspheres is in most cases limited by the rate of hydrolytic degradation of the polymeric matrix (erosion-controlled release) [36]. The hydrolytic activity of esterolytic enzymes

could also accelerate the in vivo release from biodegradable systems, but it seems more likely that the catalytic effect of these enzymes would be more pronounced during the latter part of treatment, when the highly hydrophobic polymer is more accessible to the enzymes because of a greater contact area and the formation of a large number of hydrophilic oligomers [31].

A recent study performed with the application of electron paramagnetic resonance (EPR) showed the rapid formation of a highly acidic microenvironment within biodegradable microspheres [33]. Decrease in pH within polyester microspheres to values below 3 could result in accelerated polymer erosion caused by the catalytic effect of lactic and glycolic acids on ester hydrolysis and hence could increase the release rate. The microenvironment effect may play a more important role in vivo than under in vitro conditions because of the presence of plasma proteins and their.effect on polymer degradation properties [34,35]. Serum albumin, γ-globulins, and fibrinogen were shown to form adsorption layers on the surface of PLA microspheres through hydrophobic interactions in such a way that the outer part of the adsorbed protein layer is rich in positively charged groups and the inner part is rich in negatively charged groups, which causes a variation in the zeta potential of plasma protein-coated microspheres. The potential increase on the polymer-protein interface causes an increase in H^+ concentration at that interface, which may result in an acceleration of the hydrolytic degradation of the microspheres. Also, the presence of plasma proteins can increase the solubility of PLA, causing the polylactide to exist in an expanded form and hence increase its availability for hydrolytic and enzymatic processes. Therefore, the formation of a highly acidic microenvironment inside biodegradable systems resulting from an accumulation of polymer degradation products and increase in H^+concentration on the polymer-plasma protein interface may indeed be responsible for higher initial in vivo releases than under in vitro conditions.

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