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A Novel *In Vitro* Release Technique for Peptide-Containing Biodegradable Microspheres

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ABSTRACT The purpose of this study was to develop and evaluate a dialysis *in vitro* release technique for peptide-containing poly(d, l-lactide-co-glycolide) (PLGA) microspheres (ms) that would correlate with *in vivo* data. Using a luteinizing hormone– releasing hormone analogue (LHRH), Orntide acetate, solubility and stability were determined in 0.1 M phosphate buffer (PB), pH 7.4, and in 0.1 M acetate buffer (AB), pH 4.0, with high-performance liquid chromotography (HPLC), and peptide permeability through a dialysis membrane (molecular weight cut-off 300,000) was determined. Orntide ms were prepared by a dispersion/solvent extraction/evaporation method and characterized for drug content (HPLC), particle size distribution (laser diffraction method), and surface morphology (scanning electron microscopy). *In vitro* release was studied in PB using a conventional extraction method and with a new dialysis method in AB. Gravimetric analyses of polymer mass loss and matrix hydration, and peptide adsorption to blank PLGA ms (50:50, M_w 28 022) were carried out in PB and AB upon incubation at 37° C. Serum Orntide and testosterone levels in rats after administration of Orntide ms were determined by radioimmunoassay. Orntide acetate solubility was influenced by pH; approximately 2.3 mg/mL dissolved in PB and > 18 mg/mL in AB. Stability was pH- and temperature-dependent. The peptide was very stable at pH 4.0, 4° C, but degraded rapidly at pH 7.4, 37° C. Peptide permeability through the dialysis membrane was accelerated by agitation and >95% equilibrium was reached within 48 hours. The overall release rate was higher with the dialysis method. Mass loss of the Orntide ms was faster in AB (50% loss in 3 weeks; 95% in 35 days) than in PB (65% in 35 days). In contrast, hydration after 35 days was 4-fold higher in PB. The nonspecific adsorption to blank ms was greater in PB (128 μ g Orntide/10 mg PLGA) compared with AB (< 5 μ g Orntide/ 10 mg PLGA). Administration of 30-day Orntide PLGA ms to rats resulted in an initial serum Orntide level of 21 ng/mL after 6 hours and a C_{max} of 87 ng/mL after 6 days. Testosterone levels were suppressed immediately after ms administration (3 mg Orntide /Kg) from 5.2 ng/mL to 0.3 ng/mL (after 24 hours) and remained suppressed for 38 days. Orntide acetate solubility and degradation kinetics were markedly influenced by pH of the buffer systems and mass loss; matrix hydration, as well as the nonspecific adsorption to blank ms, was pH-dependent. The *in vitro* release profile obtained with the dialysis method in AB correlated well with the *in vivo* data, thereby providing a more reliable prediction of *in vivo* performance.

KEYWORDS: Orntide Microspheres, Solubility, Stability, *In vitro* release, Dialysis, PLGA

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INTRODUCTION

Biodegradable polymeric drug delivery systems (DDS), such as poly(d,l-lactide) (PLA) and poly(d,l-lactide-co-glycolide) (PLGA) microspheres (ms), have been studied extensively during the past 3 decades as a formulation approach to protect encapsulated drugs from degradation, enhance bioavailability, and sustain drug release [1, 2]. Although biodegradable DDS have been

used to deliver many therapeutic agents, such as traditional small-molecule drugs (eg, cisplatin , prostaglandin E_2, progesterone, mitomycin C) [3-6], proteins (eg, human bone morphogenetic protein, beta-lactoglobulin, nerve growth factor) [7-9] and, recently, plasmid DNA [10-12], these systems seem to be particularly suited for delivery of therapeutic peptides [1]. The following factors can be cited for incorporating peptide drugs into polymeric matrices: 1) high potency of peptide drugs and relatively small therapeutic doses; 2) very poor absorption and bioavailability after oral administration due to high molecular weight and rapid degradation in the GI environment; 3) short biological half-life due to chemical and enzymatic degradation (peptidases) in body fluids; 4) specific mechanisms of therapeutic action requiring continuous delivery of a small amount of the drug over an extended time; and 5) good resistance to physical processes during microencapsulation (mechanical stress, exposure to organic solvents, temperature fluctuations) [1].

While the literature describes peptide and polymer characterization methods, various techniques of microencapsulation, and *in vivo* evaluation of these formulations in different animal models, information on *in vitro* release techniques and conditions under which these tests should be conducted is sparse. Several microencapsulated drugs are currently on the market (Lupron Depot® , Enantone Depot® , Decapeptyl® , Parlodel LA®). However, there is no standardized and validated *in vitro* release technique that would enable a good prediction of the *in vivo* release onset, overall *in vivo* release rate, and release duration. The most commonly used *in vitro* release method involves incubation of the ms at 37° C in a certain volume of release medium, generally pH 7.4 phosphate buffer (PB) containing a preservative and optionally a surfactant and the subsequent extraction of the ms for residual peptide. In most cases the *in vitro* release profile obtained with this method does not correlate with the *in vivo* results, and the *in vitro* release is often incomplete [13].

In vitro release from biodegradable polymeric DDS has been shown to be influenced by many factors, such as 1) release medium composition (buffer system, ionic strength, pH, surfactants); 2) test conditions (temperature, agitation, frequent supernatant removal); 3) drug stability, solubility and adsorption to degrading matrix; and 4) formation of internal acidic microenvironment [13-18]. Taking all these factors into consideration makes it clear that *in vitro* release testing is a complex problem requiring serious consideration.

The goal of this work was to develop and evaluate a dialysis *in vitro* release technique for peptide-loaded biodegradable ms that would enable a good correlation between *in vivo* and *in vitro* data and could be used to predict *in vivo* performance of experimental microparticulate formulations. Ornitide acetate, a decapeptide, was selected as the peptide and PLGA with free carboxylic acid end groups was selected as the polymer.

MATERIALS AND METHODS

Materials

Orntide acetate

([NacDNal^{1}DpClPhe^{2}D3Pal^{3}PicLys^{5}D(6Anic)Orn^{6}Ilys^{8}Dala^{10}]-LHRH) was supplied by California Peptide Research, Inc. (Napa, CA). PLGA (50:50, M_w 28,022) with free carboxyl end groups was obtained from Boehringer Ingelheim, Inc. (Ingelheim, Germany) and used for ms preparation. The solvents and other excipients used were analytical grade and were purchased from commercial sources. Dialysis tubes (Tube-O-Dialyzer®) were purchased from Research Products International Corp. (Mount Prospect, IL), and a Spectra/Por® CE dialysis membrane (MWCO 300,000 Da) was supplied by Spectrum Medical Industries, Inc. (Houston, TX).

Methods

HPLC method for peptide assay

The peptide was analyzed by reverse phase high-performance liquid chromatography HPLC (Bondclone 10 C18 column, 150 mm × 3.90 mm). The elution phase consisted of 34% (v/v) acetonitrile and 0.1 % (v/v) trifluoroacetic acid in water. UV detection was at λ_{max} 215 nm.

Stock solutions.

Stock solutions of Orntide acetate were prepared by dissolving 20 mg of the peptide in 200 mL of 0.1 M PB, pH 7.4, or 0.1 M acetate buffer (AB), pH 4.0, containing 0.02% (w/v)] of sodium azide. The solutions were then filtered through a 0.22 μ filter (Millipore Co., Bedford, MA) into sterile containers and used immediately or stored at -20° C for later experiments.

Peptide solubility

The solubility of Orntide acetate was studied in two media: 1) 0.1 M PB, pH 7.4, and 2) 0.1 M AB, pH 4.0. The solubility was determined by incubating an excess of peptide in 0.5 mL of test medium at room temperature in tightly sealed glass vials. The samples were continuously agitated using a LabQuake® shaker (Barnstead-Thermolyne, Dubuque, IA) . At each time point two samples were filtered using 0.45 μ Millex-HV_4 syringe filter (Nihon Millipore Kogyo K.K., Yonezawa, Japan), and the solubilized peptide was analyzed by HPLC.

Peptide stability

Peptide stability was determined in 1) 0.1 M PB, pH 7.4, and 2) 0.1 M AB, pH 4.0, upon incubation at 4° C, 25° C, and 37° C. Approximately 5.0 mL of fresh stock solutions were placed in 7-mL scintillation glass vials. The vials were sealed and incubated under continuous orbital rotation (LabQuake® shaker). Periodically, aliquots were taken from each vial and the amount of intact peptide was assayed by HPLC.

Peptide permeability through dialysis membrane

5.0 mL of Orntide stock solution in AB were quantitatively transferred to a 7-mL dialysis tube closed at one end with a dialysis membrane (MWCO 300,000 Da). Each dialysis tube containing Orntide solution was then placed in a 50-mL tube filled with 40 mL of the same buffer. The contents of the larger tube were either stirred continuously with a magnetic stirrer or were not agitated at all. All tubes were incubated at 37° C and at each time point a 1.0-mL sample was withdrawn from the larger tube and analyzed for peptide concentration. The removed volume of test medium was replaced with fresh buffer.

Microsphere preparation

PLGA ms containing Orntide acetate were prepared by a dispersion method followed by solvent extraction/evaporation [19]. Briefly, a solution of peptide in methanol was combined with a solution of PLGA in methylene chloride and stirred using a magnetic stirrer for approximately 10 minutes. The clear solution was then slowly injected into a reactor containing the continuous phase (0.35% [w/v] solution of polyvinyl alcohol) and stirred at 5500 rpm with a Silverson L4R homogenizer (Silverson Machines, Ltd, Waterside, Chesham, Buckinghamshire, UK). The temperature of the reactor was maintained initially at 25° C for 30 minutes and after that at 40° C for 60 minutes using a circulating water bath. Once ms formed and hardened, the contents of the reactor were passed through a 0.8-μ membrane filter (Gelman Sciences, Ann Arbor, MI), and the recovered ms were washed with water and dried under reduced pressure for 48 hours at room temperature.

Microsphere characterization

Peptide content in ms was determined by HPLC after dissolving the ms in chloroform and extracting the peptide with AB. Particle size distribution was determined using a laser diffraction technique (Malvern 2600c Particle Sizer, Malvern, UK). Surface morphology was analyzed by scanning electron microscopy.

In vitro studies

Release in PB (extraction method). A known amount of Orntide ms was placed in a 15-mL polypropylene tube filled with 10.0 mL of 0.1 M PB, pH 7.4 and incubated at 37° C. At each time point, remaining ms were recovered by centrifugation and assayed for drug content, as described above.

Release in AB (dialysis method). Orntide ms were accurately weighed and placed in a 7-mL dialysis tube (Tube-O-Dilalyzer® , MWCO 300,000 Da) filled with 5.0 mL of 0.1 M AB, pH 4.0, 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 (Figure 1). At each time point 1.0 mL was removed from the 50-mL tube and 1.0 mL of fresh buffer was added. Peptide content was determined by HPLC.

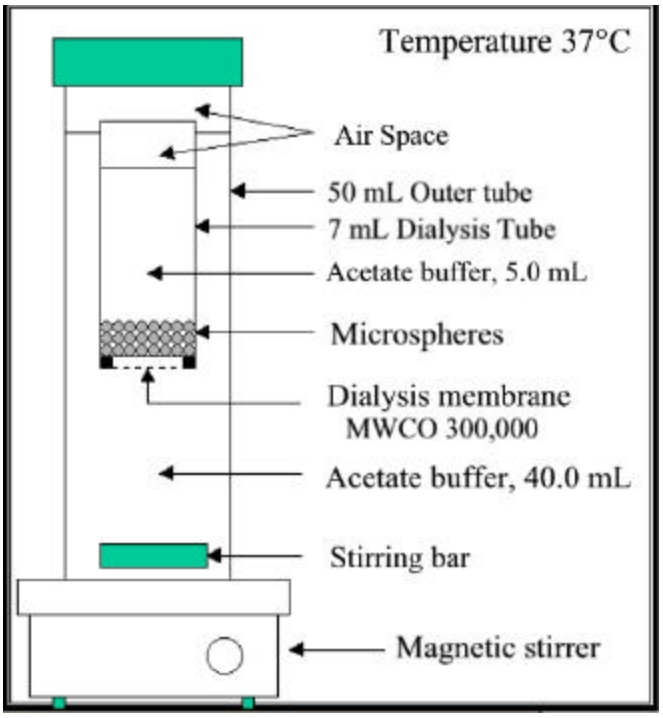

Figure 1. Dialysis setup for *in vitro* release testing. The enclosure represents an incubator at 37 C.

Mass loss and hydration of PLGA microspheres. A previously reported technique to determine mass loss of the polymeric ms was used [20]. A known amount of ms (20 mg - 40 mg) was accurately weighed into 15-mL glass vials (initial weight, W_o). To each vial, 10.0 ml of 0.1 M PB, pH 7.4, or 0.1 M AB, pH 4.0, were added and the vials were placed in a 37° C incubator. Samples were withdrawn at each time point and collected by filtration. The wet ms were weighed accurately (wet weight, W_w), dried for 48 hours under reduced pressure at room temperature, and weighed again (dry weight, W_d). The mass remaining (MR) and the degree of hydration (DH) were calculated as follows:

$$MR\ (\%) = (W_d / W_o) \cdot 100 \qquad (1)$$

$$DH = (W_w - W_d) / W_d \qquad (2)$$

Peptide adsorption to PLGA microspheres

Fifty milliliters of Orntide stock solutions in PB and AB were incubated at 37° C with 10 mg of blank ms under continuous orbital agitation (LabQuake® shaker). At each time point 2 samples of each suspension were removed from the incubator and filtered through 0.45 µ syringe filter; the amount of peptide in solution was determined by HPLC.

In vivo studies

Two groups of male Sprague Dawley rats (n = 6) weighing approximately 300 g were used to evaluate *in vivo* performance of Orntide ms. The ms were injected subcutaneously at the back of the neck after reconstitution in a suitable vehicle (1% carboxymethylcellulose and 2% mannitol [w/v]). Blood samples were collected from the tail vein at specific time points. The samples were centrifuged in Microtainer® tubes (Becton Dickinson & Co., Franklin Lakes, NJ) and serum was collected. Serum samples were frozen and stored at -20° C until analysis.

Serum Orntide levels in rats were assessed using a radioimmunoassay (RIA) method 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 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 with the exception of the *in vitro* release profiles,

where single data points are presented. For values below the assay limit the detection limit was used for calculations. To analyze the *in vivo-in vitro* correlation, cumulative area under serum Orntide curve was calculated for 0 to 38 days using the trapezoid rule with application of GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

RESULTS AND DISCUSSION

Peptide solubility

Figure 2 shows that the solubility of Orntide acetate in 0.1 M PB, pH 7.4, increased with time and reached an apparent equilibrium at 2.3 mg/mL after 4 hours at room temperature. Orntide acetate was substantially more soluble at lower pH (>18 mg/mL), but the equilibrium concentration of the solubilized peptide in 0.1 M AB, pH 4.0, could not be determined due to gelation of the highly concentrated and viscous solution. Gelation of another luteinizing hormone-releasing hormone LHRH analogue, leuprolide acetate, was reported previously in the literature and it was induced by increased peptide concentration, introduction of salts, or agitation [21]. The higher solubility of Orntide acetate in AB could be a result of increased dissociation of the peptide salt at lower pH.

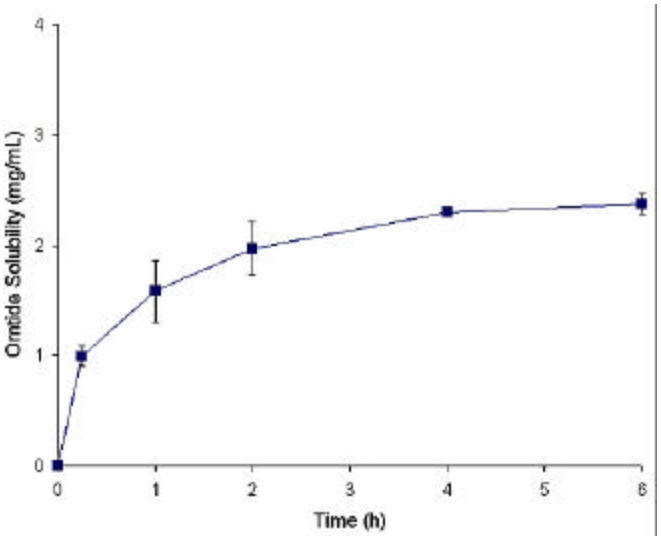

Figure 2. Solubility of Orntide acetate in 0.1 M phosphate buffer pH 7.4 at room temperature (n = 2).

Peptide stability

Figure 3 shows the effects of pH and temperature on stability of Orntide acetate in solution. The peptide was substantially more stable in AB pH 4.0 than in PB pH 7.4, and there was no detectable decrease in peptide concentration in AB for the first 3 days. After that the peptide concentration in AB decreased slightly (loss after 7 days was 5%, 6%, and 8% at 4° C, 25° C, and 37° C, respectively). In PB pH 7.4 at 37° C, 17% of the peptide degraded within 24 hours and almost 50% degraded after 7 days. Peptide degradation in PB was slower at lower temperatures and as much as 77% of the initial amount remained after 1 week at 4° C and 25° C.

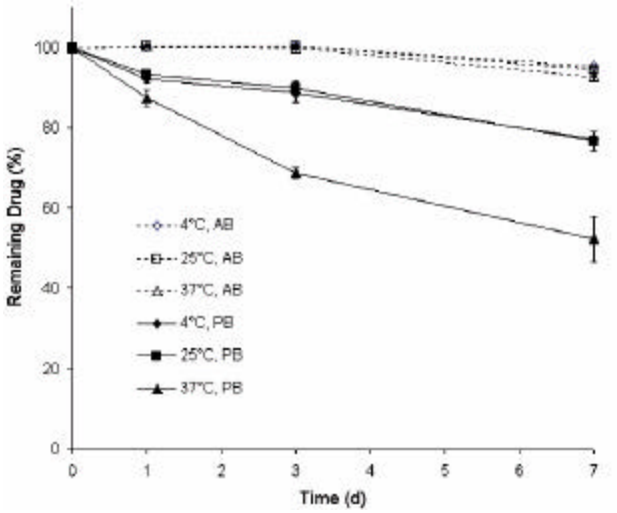

Figure 3. Orntide acetate stability in 0.1 M phosphate buffer (pH 7.4) and 0.1 M acetate buffer (pH 4.0) at various temperatures (n = 3).

Peptide permeability through dialysis membrane

Under continuous agitation, Orntide acetate penetrated the dialysis membrane at a moderate rate, reaching 95% of the theoretical equilibrium concentration within 48 hours (Figure 4). A slower equilibration was observed without agitation (<80% after 48 hours). This finding suggests that application of a dialysis technique to *in vitro* release testing requires constant agitation of the contents of the outer tube in order to achieve a faster equilibrium between the two tubes.

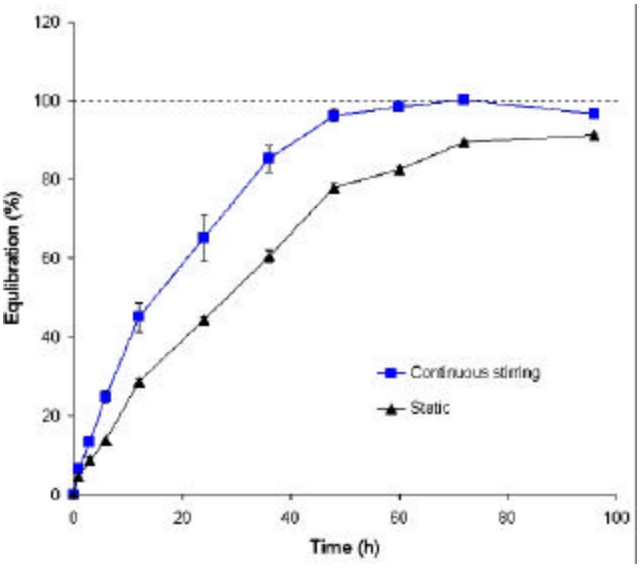

Figure 4. Peptide permeability through dialysis membrane (MWCO 300 000) at 37 C (n = 2).

Microsphere characterization

The ms were spherical with a seemingly nonporous surface and formed a free-flowing powder. The mean particle size was 9.60 ± 2.07 µ and 90% of the particles were below 12.1 µ (Figure 5). Drug incorporation efficiency was 81%, and the final drug content was 9.0%. Most of the onincorporated remaining peptide was detected in the continuous phase, and the final mass balance in respect to the peptide was 91%.

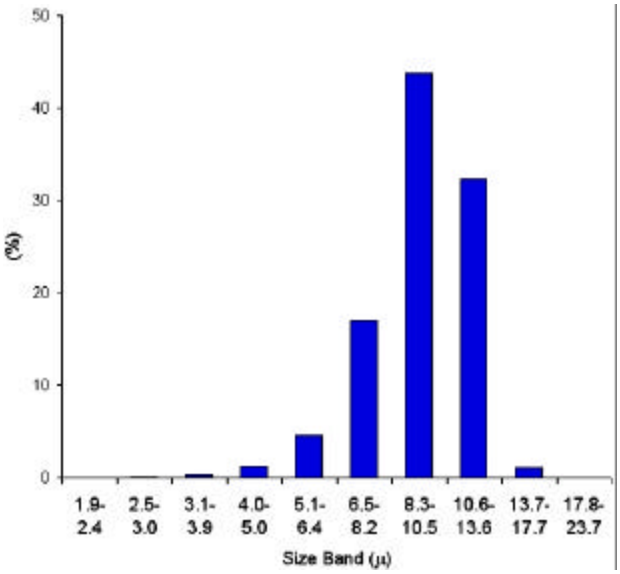

Figure 5. Particle Size Distribution

In vitro release

The *in vitro* release profiles from Orntide ms in 0.1 M PB, pH 7.4, obtained with the extraction method and in 0.1 M AB, pH 4.0, generated with the dialysis method are shown in Figure 6. Initially, approximately 6% of the drug released within 24 hours in PB, but the release rate decreased markedly and only an additional 12% released over the next 34 days (overall release rate was ~ 0.5% per day). In contrast, the *in vitro* release from Orntide ms in AB pH 4.0 (dialysis method) was initially slower: only 0.5% released in 24 hours and another 5% in the following 6 days. Subsequently, the release rate increased in a nearly linear manner to 3.5% per day, and release was essentially complete in 35 days (overall release rate was 2.9% per day).

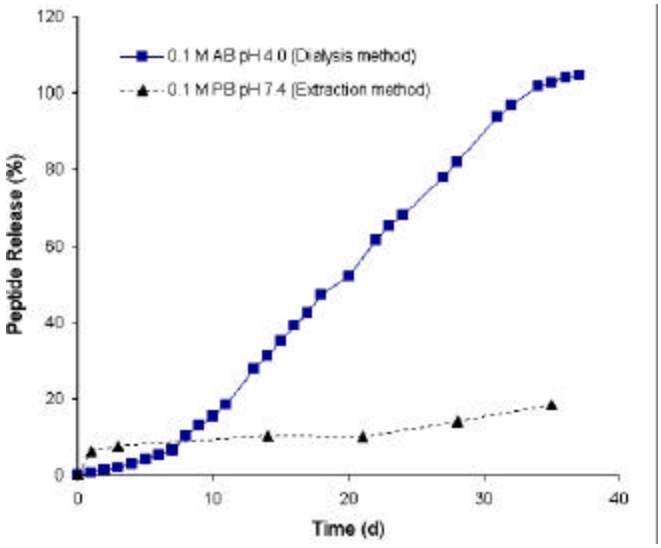

Figure 6 *In vitro* release from Orntide microspheres obtained with the extraction and dialysis methods.

The release method and the release medium influenced the *in vitro* release kinetics from the Orntide ms. The 6-fold difference in the apparent release rates between the two methods could be a result of 1) higher solubility of the peptide and faster degradation of the polyester drug matrix at pH 4.0, and/ or 2) higher adsorption of the peptide to the degrading microspheres at PB pH 7.4.

Mass loss and hydration of Orntide microspheres

Figure 7 shows the influence of buffer type and pH on the *in vitro* degradation of Orntide PLGA ms.

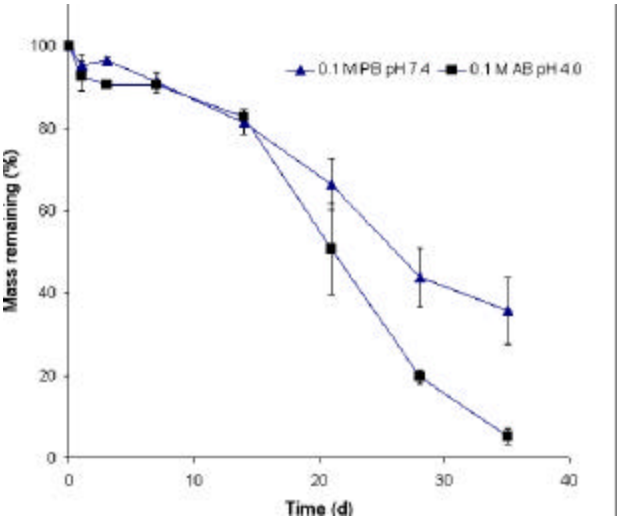

Figure 7. *In vitro* mass loss of Orntide PLGA microspheres in phosphate and acetate buffers at 37 C (n = 2).

Initially, the ms degraded with similar rates in PB and AB, and during 14 days the initial ms weight decreased by 19% and 17%, respectively. After that the polymer degradation was markedly faster in AB, resulting in 49% mass loss after 21 days and 95% after 35 days. In contrast, only 34% of the initial ms weight was degraded in PB within 21 days and 64% after 35 days. Interestingly, the hydration of Orntide ms was 4-fold higher in PB than in AB (Figure 8). This difference in water absorption by the ms in different buffer systems could be caused by a higher degree of ionization of the polymer free carboxyl end groups in pH 7.4, which would make the matrix more hydrophilic and enhance water uptake.

PLGA copolymers degrade *in vitro* by hydrolytic cleavage of ester bonds, and the degradation rate is usually higher in an acidic environment than in physiological pH due to acid catalysis [22, 23]. The hydrolysis of ester bonds can be catalyzed by free carboxyl end groups within the same molecule (intramolecular catalysis) or by other acidic moieties, such as lactic and glycolic acids, products of PLGA degradation that accumulate within hydrated ms (intermolecular catalysis) [18].

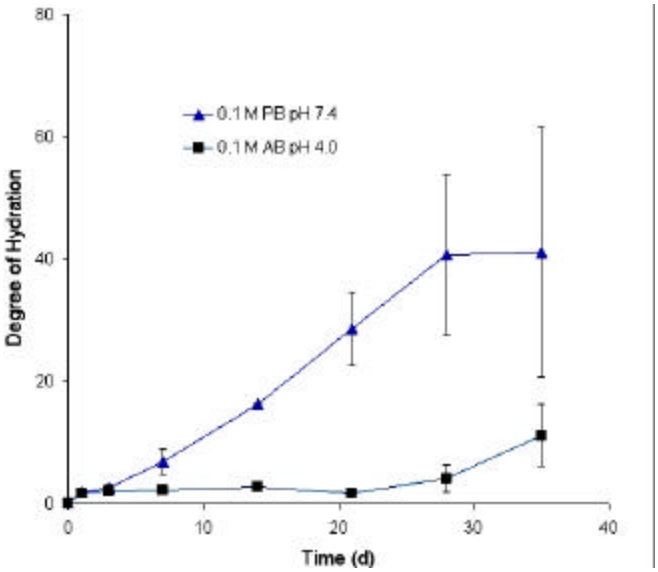

Figure 8. *In vitro* hydration of Orntide PLGA microspheres in phosphate and acetate buffers at 37 C (n = 2).

Peptide adsorption to PLGA microspheres

Previously, nonspecific adsorption of bovine serum albumin (BSA) to the surface of degrading PLGA ms during *in vitro* release study in phosphate buffer saline (PBS) was shown to severely limit the amount of protein available for release, resulting in slow and incomplete release profiles [15, 16]. Therefore, the possibility of peptide nonspecific adsorption to degrading ms during an *in vitro* release study should be minimized in order to obtain a complete release profile.

As shown in Figure 9, there was no significant adsorption of Orntide acetate to blank PLGA ms in 0.1 M AB, pH 4.0, at 37° C, and the amount of peptide remaining in the supernatant throughout 12 hours was 100 ± 2% of the initial amount. In contrast, the amount of peptide adsorbed to the blank ms in 0.1 M PB, pH 7.4, increased with time and approximately 128 μ g of the peptide was adsorbed to 10 mg of the ms after 12 hours (29% of the initial amount). There was no further adsorption at 24 and 48 hours.

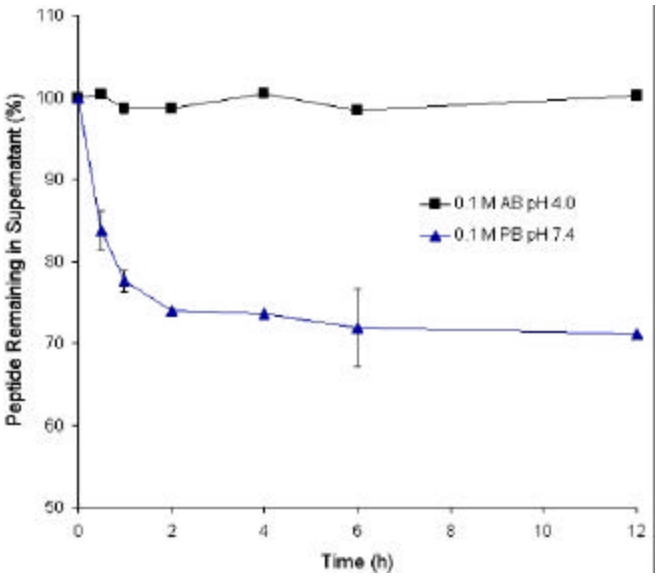

Figure 9. Peptide adsorption to blank PLGA microspheres in phosphate and acetate buffers at 37 C (n = 2).

These results indicate that the nonspecific adsorption of Orntide to PLGA ms was greater at a pH closer to the isoelectric point (pI) of Orntide, which is in the basic region. At a pH below its pI, the peptide is positively charged and exists in unfolded form. This results in repulsion between peptide molecules thereby requiring a larger interspace to overcome the repulsive forces. At pH close to the pI, peptide molecules with a zero net charge approach each other more closely and form a more compact conformation resulting in more effective adsorption [24].

In a similar study, nonspecific adsorption of peptide drugs (salmon calcitonin and triptorelin) to PLGA ms was found to be affected by ionic strength, polarity, temperature, and acidity of the release medium [24, 25]. The maximum adsorption occurred near the pI of the peptides and almost no adsorption was observed at pH < 6.

In vivo studies

Serum Orntide levels in rats after administration of 30-day Orntide ms (3 mg Orntide/kg) are shown in Figure 10. Initially, nearly 22 ng/mL of Orntide acetate was detected in rats' serum after 6 hours and the maximal drug concentration in serum was observed on day 6 (C_{max}=87 ng/mL). After that the level of Orntide in serum started to decrease gradually and only 3 ng/mL of Orntide were detected on day 28.

Testosterone suppression in rats treated with Orntide ms occurred within 24 hours (0.3 ng/mL) and the levels of testosterone remained below 0.5 ng/mL (castration level) for 39 days, with the exception of day 4 when a slight elevation to 0.8 ng/mL was observed (Figure 10).

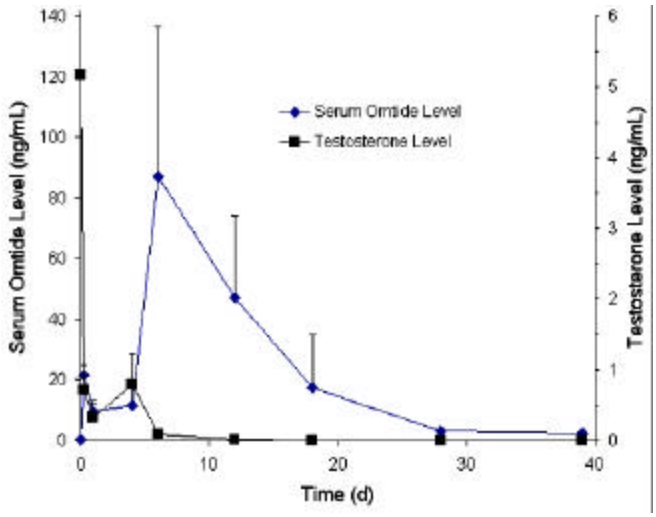

Figure 10. Serum Orntide and testosterone levels in rats after a single administration of Orntide microspheres (3 mg Orntide/kg, n = 6).

These results suggest that the initial *in vivo* release from the ms and the initial serum Orntide levels (10-12 ng/mL) were not high enough to produce and maintain a complete castration in rats between days 1 and 4. A complete testosterone suppression occurred on day 6 when serum Orntide levels rose to almost 90 ng/mL. Interestingly, testosterone suppression was maintained between days 25 and 39, although the serum drug levels decreased at this point to below 10 ng/mL. A similar behavior was observed previously with other LHRH analogues when, after higher initial blood levels of peptide and suppressed testosterone level, only a low level of the drug was necessary to sustain the pharmacological effect [26].

Figure 11 shows a comparison of the *in vitro* release profiles obtained with the extraction and dialysis

methods, and the *in vivo* data plotted as cumulative area under serum Ornitide curve normalized as percent of the total area between days 0 and 39 (total area under the curve $(AUC)_{0\text{-}39\ d}$ = 874 ng d/mL). Assuming that 1) the peptide has a high clearance and a short biological half-life, and 2) Ornitide does not stimulate an antibody reaction, then the serum levels of the drug should correspond to *in vivo* release from the ms. The estimated initial *in vivo* release was approximately 1.7% after 24 hours and was higher than the initial *in vitro* release in AB (~1% per 24 hours), but not as high as that observed in PB (~6%/24 h). The *in vivo* release reached 10% after 5 days (t_{10}=5 days), 50% after 10 days (t_{50}=10 days), and finally 90% of the initial drug load was released *in vivo* after 21 days (t_{90}=21 days). In contrast, t_{10} for the *in vitro* release in PB (extraction method) was approximately 10 days but only an additional 8% was released over the following 25 days. A better correlation between the *in vivo* results and the *in vitro* release profile was seen with the dialysis method, where 10% of the initial drug load was released within 9 days (t_{10}=9 days), 50% within 20 days (t_{50}=20 days), and approximately 90% after 33 days (t_{90}=33 days) (Figure 11).

The solubility and stability of Ornitide acetate, and the nonspecific adsorption to PLGA ms were found to be pH-dependent and consequently influenced markedly the *in vitro* release kinetics. Also, PLGA degradation and ms hydration were influenced by pH of the test medium. Substantially higher solubility and better stability of the peptide in 0.1 M AB pH 4.0 increased the *in vitro* release from Ornitide ms and enhanced the total drug recovery (approximately 100% after 39 days of incubation at 37° C). The release medium used with the dialysis method decreased the nonspecific adsorption of the peptide to PLGA ms and accelerated hydrolytic degradation of the ms. The better correlation between the *in vivo* data and *in vitro* release profiles obtained with the dialysis method in AB may be ascribed to several factors exclusive to the *in vivo* environment. For example, the *in vivo* solubility of Ornitide acetate may be higher than in PB pH 7.4 due to the presence of plasma proteins. BSA and HSA were reported to enhance the solubility of another peptide, vapreotide pamoate (somatostatin analogue), by the factor of 10 [14].

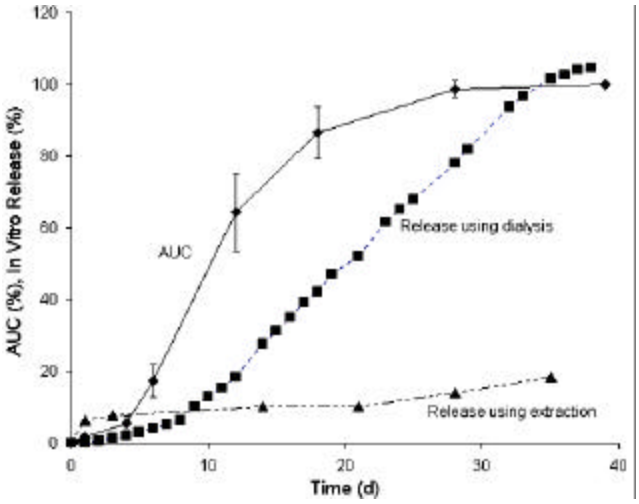

Figure 11. Area under serum Ornitide curve and correlation to *in vitro* release profiles for the extraction and dialysis methods.

Also, formation of a highly acidic microenvironment within the degrading ms and the presence of esterolytic enzymes in the interstitial fluids may result in faster degradation of the polymeric matrix *in vivo* than that seen *in vitro* in PB pH 7.4 [18, 27, 28].

The experimental setup used with the dialysis method provided a complete cumulative *in vitro* release profile from the same sample of ms (10 mg-20 mg). Enclosure of the ms sample in a dialysis tube facilitated replacement of the contents of the outer tube if necessary during sample removal. This arrangement accommodated any peptide degradation and maintained sink conditions without any loss due to incomplete recovery of the remaining ms, as can occur with the extraction method. The *in vitro* release profile obtained with the dialysis method permitted a better prediction of the *in vivo* release onset and duration compared with that obtained with the extraction method, although the overall *in vitro* release rate was still somewhat slower than the estimated *in vivo* release. Further optimization of the dialysis method and its evaluation with different peptide-containing PLGA ms is necessary to develop and validate a standard method for *in vitro* release testing.

REFERENCES

1. Okada, H, Toguchi, H. Biodegradable microspheres in drug delivery. Critical Reviews in Therapeutic Drug Carrier Systems. 1995;12:1-99.
2. Nitsch, MJ, Banakar, UV. Implantable drug delivery. J Biomaterial Applications. 1994;8:247-284.
3. Spenlehauer, G, Veillard, M, Benoit, JP. Formation and characterization of cisplatin loaded poly(d,l-lactide) microspheres for chemoembolization. J Pharm Sci. 1986;75:750-755.
4. Zhou, MX, Chang, TMS. Control release of prostaglandin E_2 from polylactic acid microcapsules, microparticles and modified microparticles. J Microencapsulation. 1988;5:27-36.
5. Beck, LR, Cowsar, DR, Lewis, DH, et al. A new long-acting injectable mirocapsule system for the administration of progesterone. Fertil Steril. 1979;31:545-551.
6. Tsai, DC, Howard, SA, Hogan, TF, Malanga, CJ, Kandzari, SJ, Ma, JKH. Preparation and in vitro evaluation of polylactic acid-mitomycin C microcapsules. J Microencapsulation. 1986;3:181-193.
7. Schrier, JA, DeLuca, PP. Recombinant human bone morphogenetic protein-2 binding and incorporation in PLGA microsphere delivery systems. Pharm Dev Technol. 1999;4:611-621.
8. Rojas, J, Pinto-Alphandary, H, Leo, E, Pecquet, S, Couvreur, P, Fattal, E. Optimization of the encapsulation and release of beta-lactoglobulin entrapped in poly(D,L-lactide-co-glycolide) microspheres. Int J Pharm. 1999;183:67-71.
9. Saltzman, WM, Mak, MW, Mahoney, MJ, Duenas, ET, Cleland, JL. Intracranial delivery of recombinant nerve growth factor: release kinetics and protein distribution for three delivery systems. Pharm Res. 1999;16:232-240.
10. Capan, Y, Woo, BH, Gebrekidan, S, Ahmed, S, DeLuca, PP. Preparation and characterization of poly (D,L-lactide-co-glycolide) microspheres for controlled release of poly(L-lysine) complexed plasmid DNA. Pharm Res. 1999;6:509-513.
11. Ando, S, Putnam, D, Pack, DW, Langer, R. PLGA microspheres containing plasmid DNA: Preservation of supercoiled DNA via cryopreparation and carbohydrate stabilization. J Pharm Sci. 1999;88:126-130.
12. Wang, D, Robinson, DR, Kwon, GS, Samuel, J. Encapsulation of plasmid DNA in biodegradable poly(D, L-lactic-co-glycolic acid) microspheres as a novel approach for immunogene delivery. J Controlled Release. 1999;57:9-18.
13. Blanco-Prieto, MJ, Besseghir, K, Orsolini, P, et al. Importance of the test medium for the release kinetics of a somatostatin analogue from poly(D,L-lactide-co-glycolide) microspheres. Int J Pharm. 1999;184:243-250.
14. Hora, MS, Rana, RK, Nunberg, JH, Tice, TR, Gilley, RM, Hudson, ME. Release of human serum albumin from poly(lactide-co-glycolide) microsperes. Pharm Res. 1990;7:1190-1194.
15. Crotts, G, Park, TG. Stability and release of bovine serum albumin encapsulated within poly(D,L-lactide-co-glycolide) microparticles. J Controlled Rel. 1997;44:123-134.
16. Crotts, G, Sah, H, Park, TG. Adsorption determines in-vitro release rate from biodegradable microspheres: Quantitative analysis of surface area during degradation. J Contr Rel. 1997;47:101-111.
17. Hernadez, RM, Igartua, M, Gascon, AR, Calvo, MB, Pedraz, JL. Influence of shaking and surfactants on the release of bsa from plga microspheres. Eur J Drug Metab Pharmacokinet. 1998;23:92-96.
18. Mader, K, Bittner, B, Li, Y, Wohlauf, W, Kissel, T. Monitoring microviscosity and microacidity of the albumin microenvironment inside degrading microparticles from poly(lactide-co-glycolide) (PLG) or ABA-triblock polymers containing hydrophobic poly(lactide-co-glycolide) A blocks and hydrophilic poly(ethyleneoxide) B blocks. Pharm Res. 1998;15:787-793.
19. Jeyanthi, R, Thanoo, BC, Metha, RC, DeLuca, PP. Effect of solvent removal technique on the matrix characteristics of polylactide/glycolide microspheres for peptide delivery. J Controlled Release. 1996;38:235-244.
20. Mehta, RC, Thanoo, BC, DeLuca, PP. Peptide containing microspheres from low molecular weight poly(d,l-lactide-co-glycolide). J Controlled Release. 1996;41:249-257.
21. Tan, MM, Corley, CA, Stevenson, CL. Effect of gelation on the chemical stability and conformation of leuprolide. Pharm Res. 1998;15:1442-1448.

22. Park, TG, Lu, W, Crotts, G. Importance of in vitro experimental conditions on protein release kinetics, stability and polymer degradation in protein encapsulated poly(D,L-lactic-co-glycolic acid) microspheres. J Controlled Release. 1995;33:211-222.
23. Sah, H, Chien, YW. Effects of H^+ liberated from hydrophilic cleavage of polyester microcapsules on their permeability and degradability. J Pharm Sci. 1995;84:1353-1359.
24. Tsai, T, Mehta, RC, DeLuca, PP. Adsorption of peptides to poly(D,L-lactide-co-glycolide): 1. Effect of solution properties on the adsorption. Int J Pharm. 1996;127:43-52. 25. Tsai, T, Mehta, RC, DeLuca, PP. Adsorption of peptides to poly(D,L-lactide-co-glycolide): 1. Effect of physical factors on the adsorption. Int J Pharm. 1996;127:31-42.
26. Tunn, UW, Bargelloni, U, Cosciani, S, Fiaccavento, G, Guazzieri, S, Pagano, F. Comparison of LH-RH analogue 1-month depot and 3-month depot by their hormone levels and pharmacokinetic profile in patients with advanced prostate cancer. Urol Int. 1998;60:9-17.
27. Holland, SJ, Tighe, BJ, Gould, PL. Polymers for biodegradable medical devices. 1. The potential of polyesters as controlled macromolecular release systems. J Controlled Release. 1986;4:155-180.
28. Makino, K, Arakawa, M, Kondo, T. Preparation and in vitro degradation properties of polylactide microcapsules. Chem Pharm Bull (Tokyo). 1985;33:1195-1201.