Journal of Peptide Science
J. Pept. Sci. 2007; **13**: 649–654
Published online 29 August 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.894

JOURNAL OF PEPTIDE SCIENCE

Zinc–leuprolide complex: preparation, physicochemical characterization and release behaviour from *in situ* forming implant

REYHANEH ASTANEH,[a]* NASTARAN NAFISSI-VARCHEH[b] and MOHAMMAD ERFAN[a]

[a] Department of Pharmaceutics, School of Pharmacy, Shaheed Beheshti University of Medical Sciences, P.O. Box 14155-6153, Tehran, Iran
[b] Pharmaceutical Sciences Research Center, Shaheed Beheshti University of Medical Sciences, P.O. Box 14155-3817, Tehran, Iran

Received 28 May 2007; Accepted 2 June 2007

Abstract: Leuprolide acetate (LA) has been accepted as treatment for prostatic cancer and is currently also being evaluated in phase II clinical trials for the treatment of Alzheimer's disease. In this study, the zinc complex of leuprolide was prepared and its structure determined by Fourier-transform infrared (FTIR), differential scanning calorimetry (DSC), UV, X-ray diffraction (XRD), atomic absorption spectroscopy, elemental analysis, and compared with these parameters for leuorolide acetate. Also, the *in vitro* release profile of leuprolide and its complex form *in situ* forming implant (ISFI) in comparison to a commercial formulation (Eligard) was investigated. These studies indicate that the zinc complex can be effectively synthesized and influenced on tri-phasic pattern after burst release of LA from the ISFI and shifts this trend to a continuous release profile. Non-linear regression test confirmed this transformation as a zero-order release profile as well. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: zinc–leuprolide complex; *in situ* forming implant; modification of release; non-linear regression

INTRODUCTION

Leuprolide acetate (LA) belongs to the general class of drugs known as hormone agonists. It is a synthetic nanopeptide similar to the luteinizing hormone-releasing hormone (LHRH) that regulates the production of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) produced by the pituitary gland. With continued use, LA causes pituitary desensitization and down-regulation, leading to suppressed circulating levels of gonadotrophins and sex hormones [1]. It is used to treat advanced prostate cancer, uterine fibroids and endometriosis [2–5]. Currently, LA is also being evaluated in phase II clinical trials for the treatment of Alzheimer's disease [6,7].

LA has no oral bioavailability, so various non-oral routes have been considered for administration of LA, such as parenteral, pulmonary, nasal, sublingual and transderamal [8,9].

LA was marketed as a 1-mg-daily subcutaneous (Lupron) injection in 1985. The inconvenience of chronic repetitive injections was later eliminated in 1989 by development of 1–6 months' sustained-release depot product based on poly(DL-lactide-co-glycolide) microspheres (Lupron Depot) [10–13]. However, the manufacture of these systems is complex and expensive; also stabilization of the peptide can be problematic, both during manufacture and for extended delivery durations. In addition, removal of intramuscularly injected microspheres in case of an adverse drug reaction is not easy [14,15]. Another formulation of LA is an osmotically driven implantable system (Viadur) that delivers it for one year with zero-order release kinetics. It could maximize therapeutic effects by the long-term continuous dosage of LA [14].

It is known that zinc salts can potentate or retard the action of certain proteins. Two known examples for successful prolongation of biological activity by such methodology are the precipitation of insulin [16] and corticotrophin [17] with zinc salts. Recently, precipitation of hirudin (Hir) by zinc salts at neutral pH was reported to result in Hir–Zn suspensions with prolonged activity in rats [18,19]. Also, the addition of divalent cations to a lyophilized human growth hormone formulation is known to decrease its solubility and dissolution [20]. We suggested that LA could be quantitatively precipitated to form a low solubility product, zinc–leuprolide (Zn–LA), which may be suitable as a slow-release form of LA for sustained parenteral delivery. On the other hand, biodegradable, injectable, *in situ* forming implant (ISFI) presents a better alternative to microspheres and implants [21]. The concept of ISFI based on polymer precipitation was first developed by Dunn and co-workers in 1990 [22,23]. The controlled release of bioactive macromolecules via semisolid *in situ* forming systems has a number of advantages, such as ease of administration, less complicated fabrication and less stressful manufacturing conditions for sensitive drug molecules [24]. Eligard, containing LA, is the

* Correspondence to: R. Astaneh, Department of Pharmaceutics, School of Pharmacy, Shaheed Beheshti University of Medical Sciences, P.O. Box 14155-6153, Tehran, Iran; e-mail: rastaneh@yahoo.com

most advanced product using this technology [25–28]. However, these controlled-release devices often exhibit high bursts of drug release initially and minimal drug release thereafter. To our knowledge, there has been no report on the preparation of the Zn–LA complex. The Zn–LA complex can be used for improving the sustained release of this valuable drug in controlled-release systems such as microspheres or *in situ* forming devices. In this study, Zn–LA was prepared, characterized with different analysis methods and evaluated by *in vitro* release study in comparison to the commercial ISFI formulation of LA (Eligard) as well.

MATERIALS AND METHODS

Poly(DL-lactide co-glycolide) (PLGA) 50 : 50 copolymers, Resomer RG 504H (an end-uncapped PLGA with an average molecular weight of 48 000 kDa), was obtained from Bohringer Ingelheim, Germany. LA was purchased from Bachem Inc., Switzerland. *N*-Methyl-2-Pyrrolidone (NMP), zinc choloride and acetonitrile (HPLC grade) were purchased from Merck, Germany. Other chemicals were obtained commercially and all were analytical grade reagents.

Preparation of Zinc–Leuprolide Complex

An appropriate amount of zinc chloride solution was added to the LA solution (8 mg/ml). After mixing, the pH was adjusted by adding a small amount of 3 N sodium hydroxide. The sample was then quickly shaken manually for about 15–30 s and then incubated for 1 h at room temperature to form a Zn–LA complex. The resulting Zn–LA suspension was centrifuged at 14000×g for 10 min. The precipitate of Zn–LA complex was washed with cold water and then freeze-dried for further experiments [18].

Determination of Leuprolide Acetate Content of the Complex

The percentage of precipitated peptide was determined using UV-vis spectrophotometry indirectly. The supernatant was assayed by UV for determining LA. The percentage of leuprolide in precipitate, LA_{perc}, was calculated by:

$$LA_{perc} = \frac{LA_{total} - LA_{supernatant}}{LA_{total}} \times 100\%$$

where LA_{total} is the total leuprolide content of the solution based on the amount of LA stock solution used, and $LA_{supernatant}$ is the leuprolide content in the supernatant [19].

Characterization of Leuprolide and Its Zinc Complex

Fourier-transform infrared (FTIR) spectra up to 500 cm^{-1} were recorded on a Bruker EOUINOXX55 FRA 10615 instrument using potassium bromide pellets. Differential scanning calorimetry (DSC) was conducted on a Shimadzu DSC-60 instrument.

The operating conditions in the closed-pan system were as follows: (i) sample weight, 2 mg; (ii) heating rate, 10 °C min^{-1}; and (iii) N_2 gas flow rate, 30 ml min^{-1}. Elemental analysis was performed on a Perkin Elmer 2400 CHN analyser. The zinc content was determined using a Perkin Elmer 1100B atomic absorption spectrophotometer. Ultraviolet spectra (UV) were recorded on a Shimadzu 1201 UV-vis spectrophotometer. The amorphous or crystallinity of complex and peptide was observed using X-ray diffraction (XRD) patterns.

Solution Preparation of *in situ* Forming Implants

Each solution was composed of 33% (w/w) polymer and 3% (w/w) LA or Zn–LA dissolved in NMP. These formulations are in the liquid form and solidify when they come into contact with aqueous media.

Membrane Preparation of *in situ* Forming Implants

Solutions were cast at room temperature on the very smooth surface of a home-made holding cell. The polymer solution on the holding cell was quickly immersed in the coagulation bath before any phase inversion in air took place.

Membrane Performance of *in situ* Forming Implants

The performance of the prepared membranes was measured on the basis of drug release. The experiments for release studies were carried out in a polypropylene vial for lowest peptide adsorption. In each release study, 0.2 g of the solution was placed in the receptor phase which was separated from it by a mesh [29]. This mesh allows the solvent to exchange (by diffusion), which is necessary for matrix formation. Following published methods, 10 ml phosphate buffer (0.03 M, pH 7.4) containing 0.01% (w/v) sodium azide and 0.02% (w/v) Tween 80 was used as the receptor medium [30,31]. The receptor phase was stirred at 100 rpm throughout the experiment.

At predetermined time intervals, 1 ml of the receptor phase was withdrawn using a 0.2 μm Watman filter assisted by a 21 g needle/2 cc plastic syringe assembly. The withdrawn receptor phase was replaced with 1 ml of fresh medium. Release studies were performed at 37 °C for 28 days. The samples were kept frozen until analysis. The amount of drug in samples was determined by high-performance liquid choromatography (HPLC) operated under the following conditions: reversed phase C-18 column (Waters); isocratic elution of a mobile phase composed of 68 : 32 volume ratio of deionized water : acetonitrile containing 0.1% (w/v) trifluroacetic acid; UV detection at 220 nm [32].

Statistical Analysis

The compiled data were presented as mean ± SD. For comparing the burst release, data were analysed for statistical significance by unpaired students' *t*-test supported by SPSS 10 for Windows (SPSS, Inc, USA). For this purpose the level of significance was set at $P < 0.05$. Also, a non-linear regression method was used to analyse whether statistical differences were seen between the release rates of all systems.

DOI: 10.1002/psc

RESULTS

Characterization of Leuprolide and its Zinc Complex

The structure of LA is shown in Figure 1. The percentage of precipitation was 93.95 ± 1.73 (mean $\pm$ SD, $n = 5$) and standard error (RSD) was 1.84%, which shows the acceptable yield, accuracy and precision of this method. The FTIR spectra of LA have been reported [33]. Similar spectra were observed for its zinc complex except for the shift of the N–H stretching (3600–2500 cm^{-1}) (Figure 2).

In DSC, the endothermic peak of the pure drug at 167 °C disappeared completely in the case of its zinc complex with the appearance of a new peak at 112 °C (Figure 3). The drug showed almost no XRD peaks, but there were several peaks in the diffraction pattern of its zinc complex (Figure 4). The complex showed two absorption maxima at $\lambda = 227.5$ and 280 nm in its ultraviolet spectra which lie in a similar region as in the drug ($\lambda = 225.5$ and 281.5 nm)(not shown) [33].

The complex was also investigated to get information about the interaction of zinc and leuprolide. According to the results of elemental analyses of the complex, the ligand–metal ratio was 1:30, which suggests that 30 Zn molecules interact with one molecule LA. Anal: calculated for [($C_{61}H_{130}N_{16}O_{65}$; (LA) $Zn_{30}.51H_2O$), 4149.93 g/mol] C: 17.65; H, 4.61; N, 5.40 found C: 17.40, H: 3.37 and N: 5.13.

In vitro Release Studies

The rate of drug release from a delivery system is critical and has to be investigated to achieve an optional system with the desired release characteristics. Furthermore, *in vitro* release studies are often performed to predict how a delivery system might work in ideal situations, which might give some indication of its *in vivo* performance. In our current study, the release of leuprolide was analysed in three ISFI formulations with LA and its zinc complex in phosphate buffer in

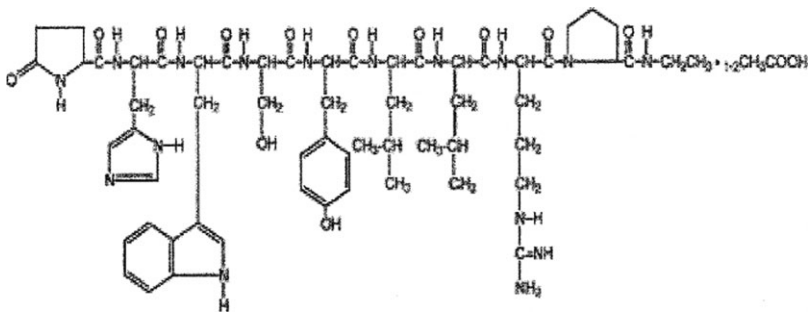

Figure 1 The chemical structure of leuprolide.

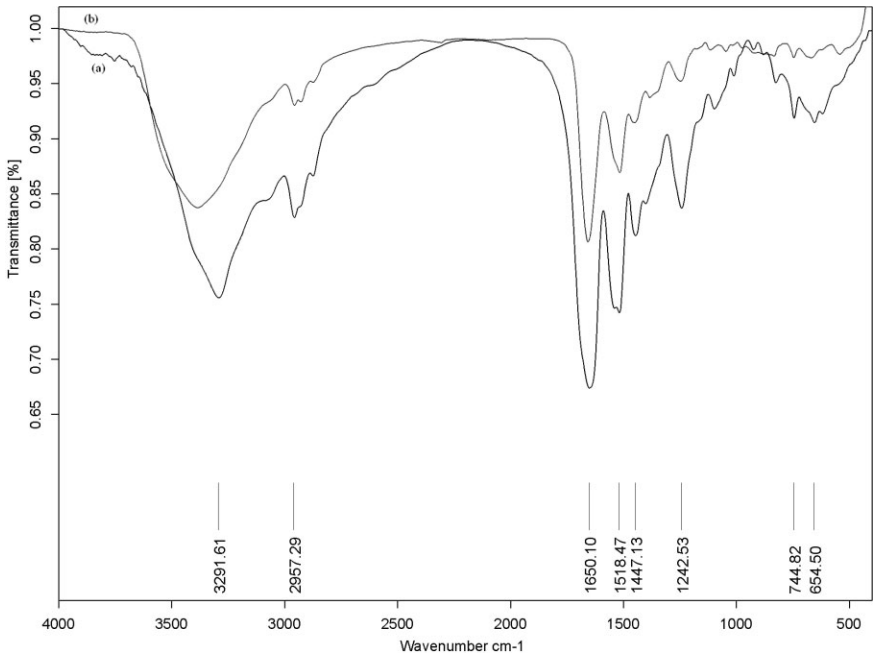

Figure 2 FTIR spectra of leuprolide acetate (a) and the zinc–leuprolide complex (b).

DOI: 10.1002/psc

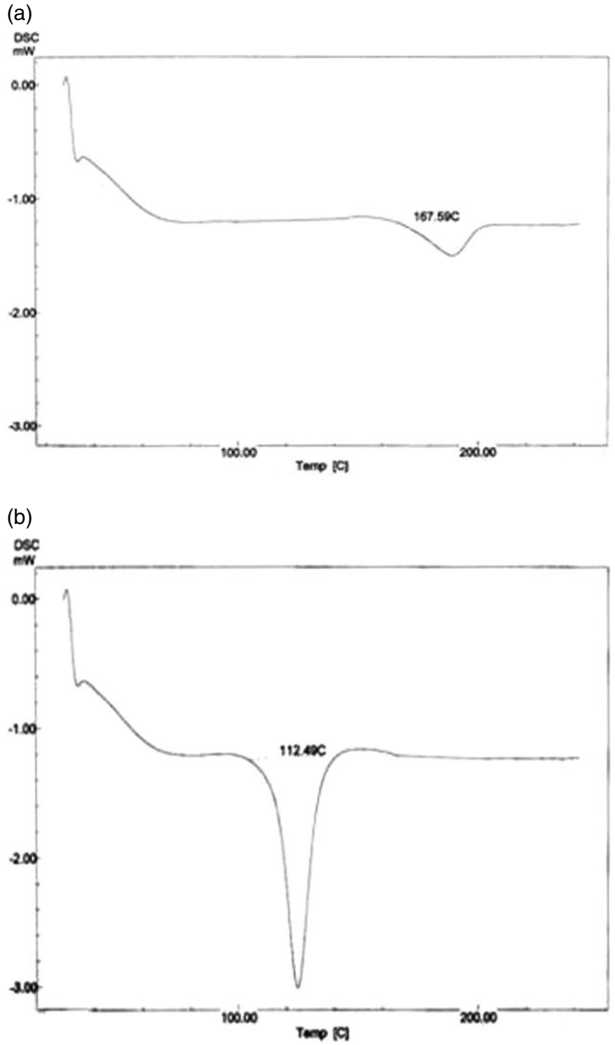

Figure 3 DSC thermograph of leuprolide acetate (a) and the zinc–leuprolide complex (b).

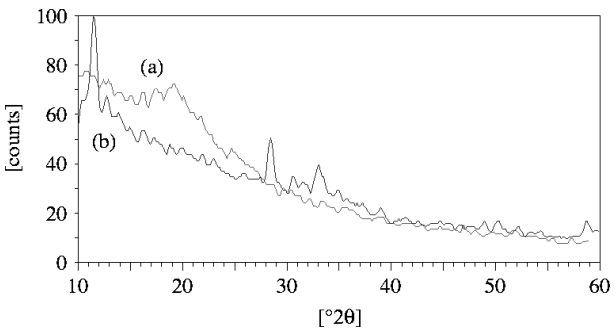

Figure 4 XRD pattern of leuprolide acetate (a) and the zinc–leuprolide complex (b).

comparison with Eligard (a commercial formulation). The mean release profiles are shown in Figure 5.

The ISFI made from the complex had a burst drug release (25.19 ± 1.75%), followed by a slower, continuous and uniform release. But ISFI made from LA showed a lower initial drug release (13.19 ± 0.078%),

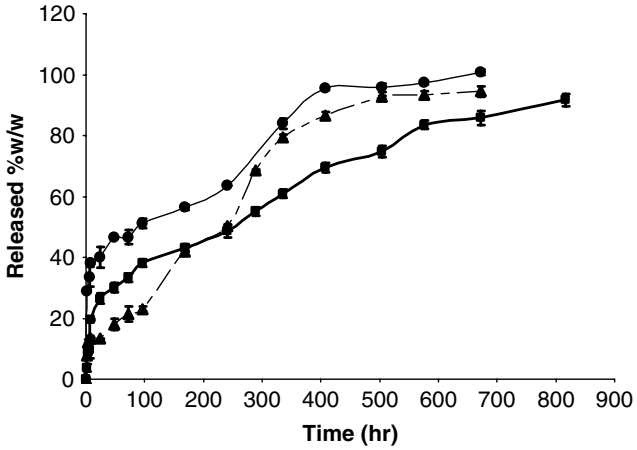

Figure 5 Profiles of release from *in situ* forming implants, leuprolide acetate (▲) zinc–leuprolide complex (■) and Eligard (●). Data are mean ± SD, $n = 3$.

Table 1 Non-linear regression of leuprolide released between 168 and 408 h from all systems

Group	Slope (release rate, % hr^{-1})			
	Estimate	Asymptotic Std. Error	Asymptotic 95% Confidence Interval	
			Lower	Upper
Eligard™	0.168	0.013	0.140	0.197
Zinc–leuprolide complex	0.114	0.017	0.079	0.149
Leuprolide	0.202	0.017	0.167	0.237

Table 2 Non-linear regression of ratio of slopes based on complex formulation between 168 and 408 h

Group	Ratio of slopes			
	Estimate	Asymptotic Std. error	Asymptotic 95% Confidence Interval	
			Lower	Upper
Slope of Eligard™/slope of complex	1.474	0.250	0.956	1.991
Slope of leuprolide/slope of complex	1.767	0.302	1.144	2.390

followed by a slow release until day 4 and a rapid release thereafter [29]. At the same release condition, drug release from Eligard was very rapid, with approximately 40 ± 3.47% of drug being released within 24 h. A one-way ANOVA for burst release

DOI: 10.1002/psc

(maximum delivered percentage of leuprolide over 24 h) from all systems yielded a significant difference ($F(2, 4) = 62.728$, $P < 0.05$). Turkey, LSD and Scheffe *post-hoc* analysis revealed that this difference is significant between each pair of groups as well. Since the burst release is needed for the down-regulation of the receptor, the formulation containing the complex revealed this characteristic as well.

Non-linear regression analysis of the release rate (slope of the phase between 168 and 408 h) for all systems is shown in Table 1. As seen, there is no significant difference between the release rate of Eligard and the complex formulation at this phase (Table 2).

Also, the drug release mechanism between 24 and 504 h was fitted with a linear regression ($y = 0.1013x + 25.884$, $r^2 = 0.9905$) with ISFI containing zinc–leuprolide complex. This zero-order equation after burst release is the best model for a controlled delivery system.

DISCUSSION

Different attempts have been made to change the release profile of LA, especially from microshperes and ISFI. Solvent strength [34] and polymer type (polymer molecular weight, concentration and polymer blending) [26–28,35,36], addition of additives [37,38], hydrophobic ion pair complexation [31], heterosterocomplexes of D-PLA and leuprolide [39] and spray-dried OED microparticles [40] are some examples of these attempts.

On the other hand, the stability, solubility and biological activity of proteins and peptides can be affected in widely different ways by salts [41]. At low concentrations, salts can stabilize proteins and other polyelectrolytes through non-specific electrostatic interactions, depending only on the ionic strength of the medium [42]. At high concentrations, however, proteins can be affected by salts, resulting in either the stabilization or denaturation of the proteins, as well as in their salting in or salting out (either precipitation or crystallization) [43]. To our knowledge, there is no report of the zinc complexation of LA. So the potential of zinc complexation of LA was investigated in this study. For this reason a complex of LA with zinc was prepared. Then, characterization of the complex in comparison with a pure drug was found with different instrumental methods. The FTIR spectra of the complex showed a shift at 3500–2000 cm^{-1}. This shift may be due to donation of electrons to the metal, which produces lower excitation states and therefore shifts to longer wavelengths after complexation. A significant difference between DSC thermograms shows the formation of a new compound and might be due to melting with decomposition of the complex. XRD of LA and its complex suggested that complexation enhanced the crystalline form of the drug or inhibited its amorphous form. The UV absorption spectrum for LA above 240 nm could have been due to summation of the absorption spectra from tyrosin and the tryptophan segment of the nanopeptide. But there is no difference in the maximum wavelength of absorption of Zn–LA and LA. It shows that there are no structural differences of the amino acids between them.

In the next step, to account for the possibility of this complex for use as a drug, and to investigate the amount as well as release rate, a controlled release device such as an ISFI was investigated for 1 month and compared to the same parameter of a commercial formulation (Eligard).

In contrast to the commercial formulation that lost its drug content rapidly, the ISFI, especially that with Zn–LA, retained the release rate of the drug effectively.

The tri-phasic release pattern was influenced by the formulation that was prepared from Zn–LA. Thus, the complexation of LA successfully shifted the tri-phasic pattern to a continuous-release profile.

CONCLUSIONS

A zinc–leuprolide complex was successfully prepared and different characterization methods such as FTIR, XRD, DSC and elemental analysis were carried out in comparison with LA. The zinc–leuprolide complex can effectively influence the tri-phasic pattern of LA released from an ISFI and shift this trend to a continuous-release profile. The results of this study revealed that the zinc–leuprolide complex can be used for improving the sustained release activity of this valuable drug, especially in controlled release systems, such as, in ISFIs.

Acknowledgements

The authors would like to thank Dr M. Ghasemzadeh for her guidance and assistance in elemental analysis, Dr H. Mobedi for encouragement and Dr H.R. Moghimi for his advice. We also wish to sincerely thank the Ministry of Health and Medical Education of Iran for financial support.

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