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Hydrophobic ion pair formation between leuprolide and sodium oleate for sustained release from biodegradable polymeric microspheres

Seung Ho Choi, Tae Gwan Park*

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1 Kusong-dong, Yusong-gu, Taejon 305-701, South Korea

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Abstract

Leuprolide acetate, an analogue of luteinizing hormone-releasing hormone (LH-RH), was hydrophobically ion paired with a long chain fatty acid, sodium oleate, in an aqueous solution. Solution behaviors of the complex formed between leuprolide and sodium oleate were investigated in terms of aqueous solubility, turbidity, particle size, and zeta potential as a function of molar ratio between the two species. It was found that with increasing the stoichiometric molar amounts of sodium oleate to leuprolide approached up to 2.5–3, the solution became gradually turbid with increasing particle sizes, indicating leuprolide precipitation as a result of hydrophobic ion pairing. On the other hand, beyond that critical molar ratio range, the solution turned into clear with much reduced particle size, indicative of micelle formation. The hydrophobically modified leuprolide–oleate complex was lyophilized and directly encapsulated within biodegradable poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres via a single oil-in-water (O/W) emulsion method. Microsphere morphology, leuprolide release behavior, and polymer mass erosion profiles were examined in comparison to the PLGA microspheres prepared with free leuprolide.

Keywords: Leuprolide acetate; PLGA; Microspheres; Hydrophobic ion pairing

1. Introduction

Leuprolide acetate is a synthetic analogue of luteinizing hormone-releasing hormone (LH-RH), which acts as a potent agonist towards anterior pituitary receptor (Plosker and Brogden, 1994; Kutscher et al., 1997). For its sustained release purpose, the formulations of biodegradable microspheres were developed to treat prostate cancer and endometriosis for 1- or 3-month treatment by a single subcutaneous or intra-muscular injection (Redding et al., 1984; Okada et al., 1988, 1991; Okada et al. 1994a,b). Commercially available formulation was based on a double emulsion solvent evaporation method. Since leuprolide acetate

* Corresponding author. Tel.: +82-42-8692621; fax: +82-42-8692610.

E-mail address: tgpark@sorak.kaist.ac.kr (T.G. Park).

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is a water-soluble peptide, leuprolide was first dissolved in a small volume of aqueous solution, emulsified in an organic solvent phase containing polymer, and then re-emulsified in a large volume of aqueous phase (Ogawa et al., 1988). Its limited solubility in various organic solvents could be disadvantageous with respect to microsphere formulation development. It is desirable to prepare PLGA microspheres by using a single oil-in-water emulsion method, in which the drug to be encapsulated and polymer should be dissolved together in the same organic solvent.

The hydrophobic ion pairing of various protein molecules with surface-active agents was reported (Kendrick et al., 1997; Meyer and Manning, 1998). It has been used for non-aqueous enzyme reactions, selective bioseparations, and enhanced protein stability in organic solvent phase. Major advantages of hydrophobic ion pairing are to increase the lipophilicity of hydrophilic macromolecules (Matsuura et al., 1993; Meyer et al., 1995). By ionic pairing between peptides or proteins, and amphipathic molecules, it was suggested that hydrophobically modified peptides or proteins might increase an absorption efficiency through mucosal membrane present in a nasal or oral route (Imanidis et al., 1995). Leuprolide is composed of nine amino acids and has two ionizable basic side chains, imidazole group of His ($pK_a \sim 6.0$) and guanidine group of Arg ($pK_a \sim 13.0$). Thus, hydrophobic ion pairing of leuprolide with various acidic amphipathic molecules such as a series of alkyl sulfonates with different carbon numbers was attempted to enhance its lipophilic partition coefficient (Adjei and Hsu, 1993).

In this study, leuprolide and sodium oleate, chosen as an acidic amphipathic surface-active agent, were combined to increase the lipophilicity of the leuprolide via hydrophobic ion pairing. The ion pairing behaviors in aqueous solution were investigated by aqueous solubility change, particle size of water insoluble fraction, its zeta potential as a function of molar ratio between leuprolide and sodium oleate. After the optimization of ion pairing process, the resultant leuprolide–oleate complex was freeze-dried. The complex dissolved in a mixture of organic solvents was encapsulated within PLGA microspheres by using a single oil-in-water emulsion solvent evaporation method. Release profile of leuprolide from microspheres, stability, microsphere morphology, and erosion profile were compared to those of PLGA microspheres prepared by the encapsulation of free leuprolide acetate.

2. Materials and methods

2.1. Materials

Leuprolide acetate was obtained from Dong Kook Pharmaceutical Co. (Seoul, Korea). Two poly(D,L-lactic-co-glycolic acid) having lactic/glycolic molar ratio of 50/50, Resomer RG502 and RG502H, were purchased from Boehringer Ingelheim. RG502 was an end-capped PLGA with weight average molecular weight of 12 000 and RG502H was an end-uncapped PLGA with that of 8600. Polyvinyl alcohol (PVA), 88% hydrolyzed, average molecular weight of 31 000–50 000 and sodium oleate were from Sigma. All other chemical reagents were of analytical grade.

2.2. Hydrophobic ion pairing

Various concentrations of sodium oleate in deionized water ranging from 0.21 to 1.65 mg/ml were slowly added in a dropwise manner into 5 ml of 1.67 mg/ml of leuprolide acetate dissolved in deionized water, which corresponds to the sodium oleate/leuprolide molar ratio range from 0.5 to 4.0. Under continuous agitation at room temperature, a cloudy solution was spontaneously developed as a result of the complex formation.

2.3. Characterization of hydrophobically ion-paired complex

The formation of water insoluble ion paired complex between leuprolide and sodium oleate at various oleate/leuprolide molar ratios was traced by monitoring the transmittance of solution at 500 nm by using a spectrophotometer. After the hydrophobic ion pairing, water-soluble fraction of leuprolide (aqueous solubility) was determined by measuring its concentration remaining in the su-

pernatant after centrifugation at 3000 rpm. The concentration of leuprolide was determined by measuring UV absorbance at 280 nm based on a series of leuprolide acetate concentrations as calibration standards. Size and zeta potential of ion paired complex particles in aqueous solution were determined by a dynamic light scattering instrument (Zetaplus, Brookhaven, New York) equipped with a He–Ne laser at a wavelength of 632.2 nm.

2.4. Microsphere preparation

Suspended leuprolide–oleate complex particles in the aqueous solution, which was formed at 1:1 molar ratio, were directly freeze-dried. PLGA microspheres were prepared by an oil-in-water (O/W) single emulsion technique. For the encapsulation of leuprolide–oleate complex, 124.8 mg of the freeze-dried complex dissolved in 0.75 ml of a water miscible organic co-solvent, *N*-methyl-2-pyrrolidinone, was combined with 700 mg of the PLGA blend mixture (RG502H:RG502 = 3:1 weight ratio) dissolved in 2.25 ml of methylene chloride. For the encapsulation of free leuprolide acetate, 100 mg of leuprolide acetate was used. The above clear solution was briefly emulsified in 250 ml of 0.5% (w/v) PVA solution for 5 min by homogenization using a PowerGen 700 (Fisher Scientific) and subsequently stirred magnetically for 3 h at room temperature to evaporate the methylene chloride. The hardened microspheres were collected, washed three times with deionized water, and then freeze-dried.

2.5. Characterization of microspheres

The amount of leuprolide acetate encapsulated within PLGA microspheres was determined by dissolving 10 mg of freeze-dried microspheres in 1 ml of dimethylsulfoxide (DMSO). Leuprolide concentration in the solution was then measured by detecting UV absorbance at 280 nm. A standard calibration curve was constructed by dissolving different leuprolide acetate amounts dissolved in DMSO. Surface and cross-sectional morphologies of PLGA microspheres were observed by scanning electron microscopy (SEM, Philips 535M). Dry microspheres were coated with gold particles by using a sputter-coater (Hammer V, Technics, USA).

2.6. In vitro release studies

Seven mg of freeze-dried microspheres, in duplicate, was suspended in 1.5 ml of 33 mM phosphate buffered saline (PBS) solution, pH 7.4, containing 0.01% (w/v) sodium azide and 0.02% (w/v) Tween 80. They were incubated in a polypropylene tube at 37°C in a shaking incubator. The microspheres incubated for predetermined time intervals were recovered after removing supernatant by centrifugation and then freeze-dried. The remaining leuprolide amount within the microspheres was determined by dissolving them in DMSO as described above. The mass erosion of microspheres during the release period was determined based on the dry weight difference of microspheres before and after the incubation as a function of time. The gravimetrical measurements were performed in duplicate with the accuracy of ± 0.1 mg. Stability of the released leuprolide in the supernatant was analyzed by high performance liquid chromatography (HPLC) operated by 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) trifluoroacetic acid; UV detection at 220 nm (Shameem et al., 1999).

3. Results and discussion

The complex formation between leuprolide acetate and sodium oleate was carried out in deionized water (pH 5–6) at room temperature to minimize the effect of buffer salt ions on the ionic interaction between the two species. Because the nature of ion pair formation between the two species was weak acid–base interactions, the complex formation was very sensitive to environmental conditions such as temperature, pH, and ionic strength. The slow addition of sodium oleate to the leuprolide acetate solution gradually de-

creased the medium pH with concomitant formation of white precipitates in the solution. Fig. 1 shows the transmittance change of the solution as a function of molar ratio between sodium oleate and leuprolide acetate. It can be seen that the transmittance, as a measure of solution turbidity observed by scattering intensity of the solution at 500 nm, sharply decreases at the relative molar ratio of oleate to leuprolide at 0.5 and maintains the lowest values until the molar ratio reaches to 2.5. Then, transmittance value increases up to 3

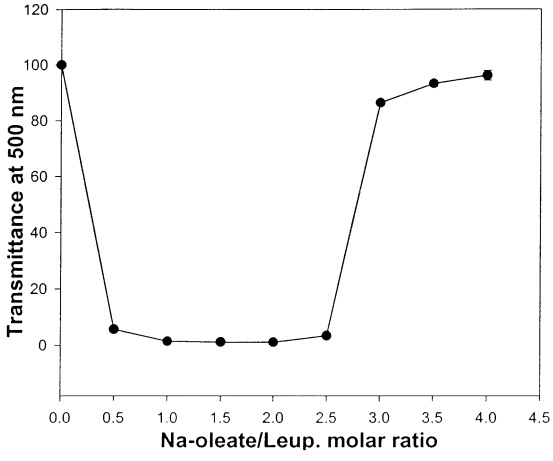

Fig. 1. Transmittance change of the solution in which leuprolide acetate and Na–oleate were complexed.

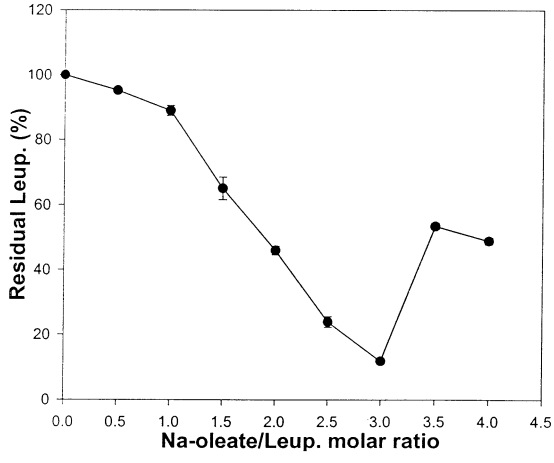

Fig. 2. The amount of free leuprolide acetate which was not hydrophobically ion paired with Na–oleate with increasing the Na–oleate/leuprolide molar ratios.

and the clear solution was observed thereafter. This clearly indicates that oleate was bound to leuprolide by ionic interaction between oppositely charged groups. Leuprolide has two basic amino acid groups in the peptide chain, to which a negatively charged carboxylic acid group in oleate was bound in a stoichiometric manner of two oleate molecules per leuprolide (Adjei et al., 1993). Approaching the gradual occupation of the binding sites in leuprolide with oleate, aqueous solubility of the complex was gradually reduced. As a result, water insoluble complexes tended to aggregate with each other and formed light scattering particulates. Upon further addition of excessive oleate molecules beyond the binding saturation point, the complex particulates being hydrophobically ion paired tended to dissociate into individual micelles. The micelle formation appears to complete above the oleate/leuprolide molar ratio value of 3.

Aqueous solubility of leuprolide as a function of oleate/leuprolide molar ratio is shown in Fig. 2. The leuprolide solubility decreases until reaching the molar ratio value of 3 and then regained after that point. This is in agreement with the solution turbidity result as shown in Fig. 1. Obviously, the micellization of leuprolide–oleate complex occurred with sufficient addition of oleate, which resulted in the enhanced aqueous solubility again. Although the stoichiometric binding number of oleate molecules to leuprolide molecule is expected to be two, the regaining aqueous solubility was observed on increasing the oleate/leuprolide molar ratio value above three. This might be due to the fact that for an efficient micellar solubilization, additional amount of sodium oleate are needed above its critical micelle concentration (3.5 mM at 50°C) in the aqueous solution (Taylor and Princen, 1979).

The formation of complex, existing as water insoluble suspended particulates in aqueous medium, was traced by measuring their size and zeta potential at different oleate/leuprolide molar ratios. This is shown in Fig. 3. It can be seen that the variation of particle sizes depends on the oleate/leuprolide molar ratio in accordance with the results of turbidity and solubility as shown in Figs. 1 and 2. The average size of particles formed

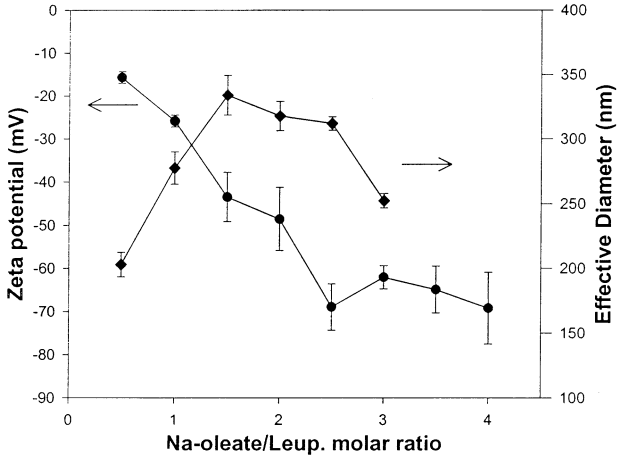

Fig. 3. Zeta potential (mV) and dynamic light scattering (nm) of leuprolide–oleate ionic complex as a function of Na–oleate/leuprolide acetate molar ratio. ●, zeta potential; ◆, dynamic light scattering.

at stoichiometric binding between oleate and leuprolide is ~300 nm. Observing a decreasing trend in particle size can be clear evidence of micellar solubilization at higher oleate/leuprolide molar ratios. Zeta potential as a measure of particle surface charge decreases concomitantly with increasing the gradual increment of oleate amount in the aqueous medium. It should be mentioned that it was difficult to obtain reliable size results of the expected micelles above the leuprolide/oleate molar ratio value of 3, although the solution became transparent. Effective diameter values of micelles as measured by dynamic light scattering fluctuated to a great extent. This is probably because the spontaneously generated micelles did not have a spherical shape with a defined size below 100 nm, but formed large particles in the shape of cylindrical rods or lamellar disks, and additionally they were very unstable in aqueous solution due to the incorporated leuprolide (Hiemenz, 1986). A schematic representation of leuprolide/oleate complex formation is shown in Fig. 4.

The leuprolide–oleate complex prepared upon mixing at 1:1 molar ratio in aqueous solution was directly freeze-dried and then used for the microencapsulation within PLGA microspheres. At this leuprolide–oleate mixing ratio, leuprolide was not completely ion paired by oleate molecules and may exist mainly as a soluble complex in the solution as shown in Fig. 2. Using an excess amount of sodium oleate for the formation of ion-paired complex was not desirable for the formulation of PLGA microspheres because of its surfactant action. Thus, 1:1 mixing ratio was selected for the microencapsulation. A PLGA blend system consisting of RG502H and RG502 at the weight ratio of 3:1 was used to prepare a 1-month leuprolide release system by controlling the polymer erosion rate, which was established in our preliminary experiments. The microspheres were prepared by a single oil-in-water emulsion method, in which oil phase was composed of a binary co-solvent mixture of nonpolar methylene chloride and a water miscible polar solvent, *N*-methyl-2-pyrrolidinone. Table 1 shows formulation conditions and characteristics of two PLGA microspheres prepared by using leuprolide–oleate complex and free leuprolide. The PLGA micro-

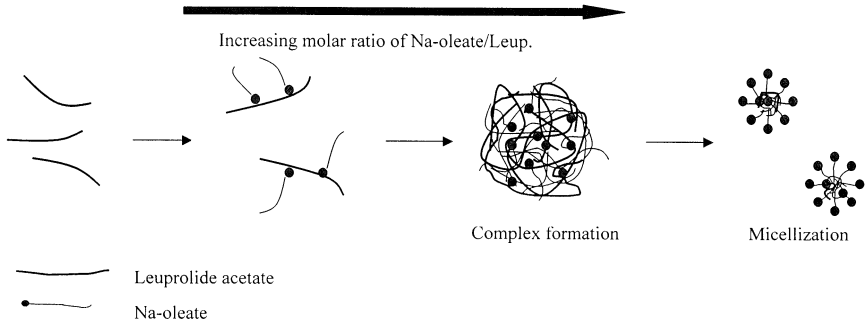

Fig. 4. Schematic illustration of leuprolide acetate — Na–oleate hydrophobic ion pairing.

Table 1
Preparation and characterization of PLGA microspheres

	Polymer Weight RG502H/RG502 (mg)	Leup. (mg)	Na–oleate (mg)	CH_2Cl_2 (ml)	Cosolvent[a] (ml)	Average particle size (μm)[b]	Drug loading (%, w/w)	Encapsulation efficiency (%)[c]
Free leup.	525/175	100	–	3.00	0.60	5.6	9.4	75.3
Leup.–oleate complex	525/175	100	24.84	2.25	0.75	10.5	11.7	96.3

[a] *N*-methyl-2-pyrrolidinone.
[b] Determined by scanning electron microscopy (SEM).
[c] Actual loading/theoretical loading # 100.

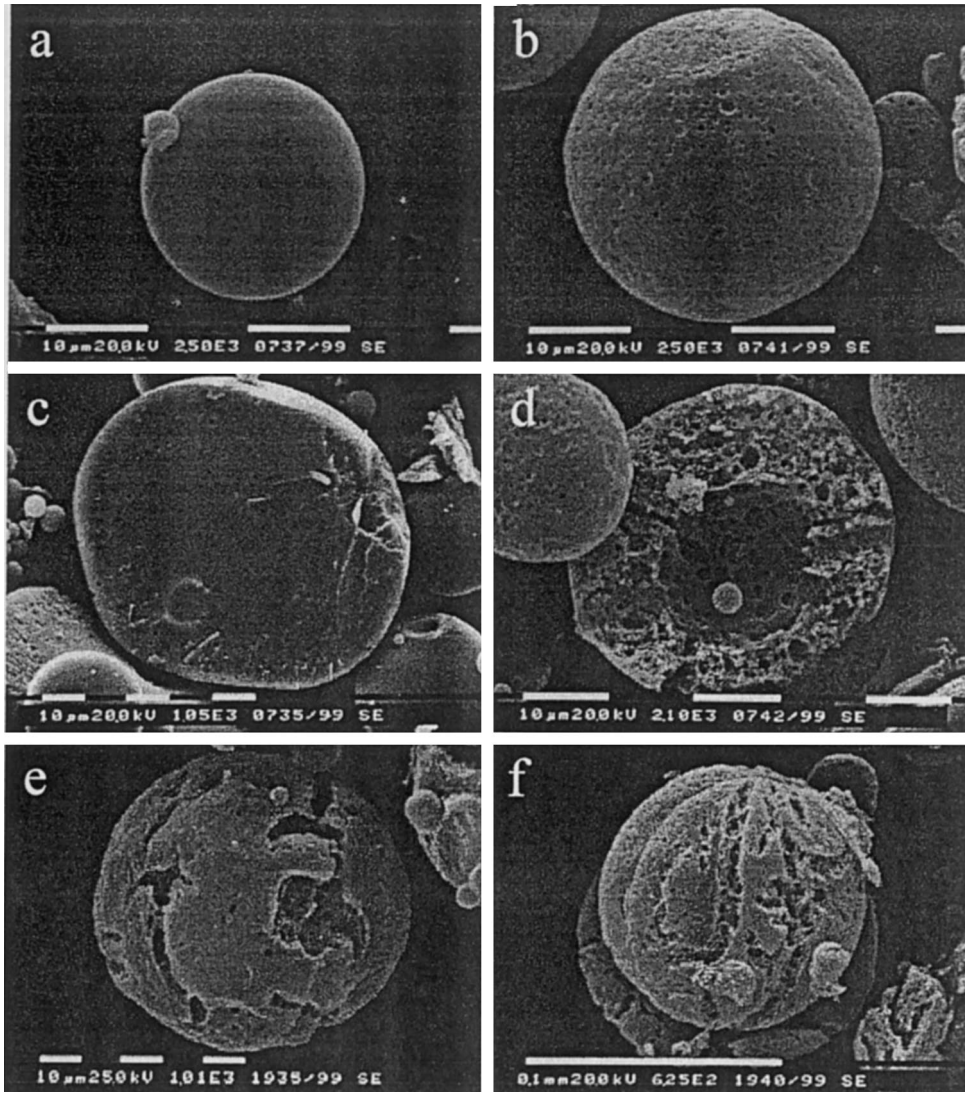

Fig. 5. SEM pictures of free leuprolide acetate (left panel) and leuprolide–oleate ionic complex (right panel) encapsulated microspheres. Surface (a, b), cross-sectional view (c, d), degradation for 8 days in the aqueous media (e, f).

spheres encapsulated with the complex leuprolide exhibited higher loading amount and greater encapsulation efficiency than those with free leuprolide. This may be due to the limited water solubility of the leuprolide/oleate complex in water, which prevented the diffusion escape of water-soluble leuprolide from the embryonic microspheres during the solvent evaporation process. Both of the PLGA microspheres have similar size distribution as determined by SEM.

Fig. 5 shows SEM pictures of the two PLGA microspheres encapsulated with the complex and free leuprolide. Surface morphologies of the two microspheres degraded for 8 days in the aqueous medium are also shown. Surface and cross-sectional morphologies of the microspheres encapsulated with free leuprolide are very smooth and dense, whereas those of the microspheres encapsulated with the complex are porous and rugged internal structure with smooth surface, although

both of the microspheres were prepared by a single emulsion method. The internal morphological difference between the two microspheres can be attributed to the different volume ratios of the two solvents, methylene chloride and water miscible *N*-methyl-2-pyrrolidinone in the organic phase used for the generation of single emulsion. The use of water miscible solvents in the organic phase tends to generate more porous microspheres as a result of preferential escaping tendency of the polar solvent into the aqueous medium to water immiscible solvent, methylene chloride. The effect of solvent composition on the morphology of microspheres was described in detail in our previous study (Crotts and Park, 1995).

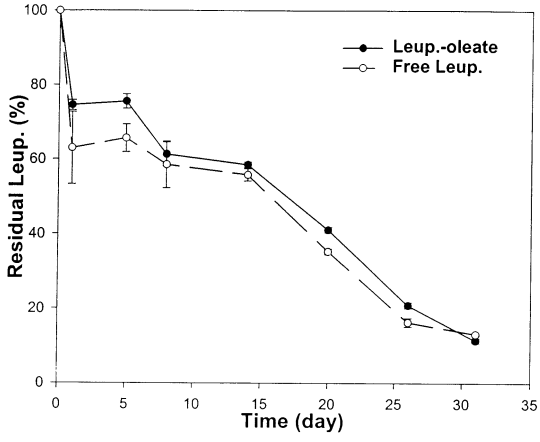

Fig. 6. Leuprolide acetate release profiles from free leuprolide acetate encapsulated microsphere and leuprolide–oleate ionic complex microspheres.

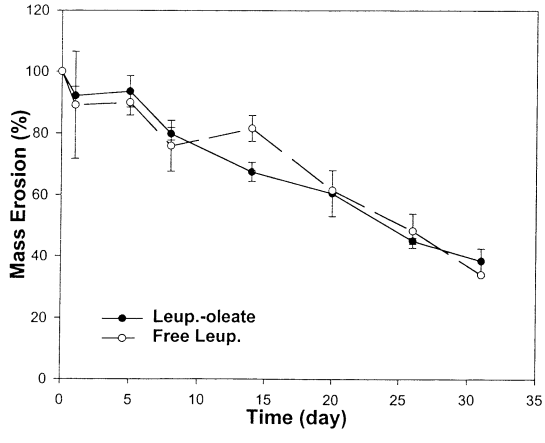

Fig. 7. Mass erosion profiles from free leuprolide acetate encapsulated microspheres and leuprolide–oleate ionic complex microspheres.

Release profiles of leuprolide from the two microspheres are shown in Fig. 6. The release profiles were determined by measuring remaining leuprolide amount within the microspheres after dissolving them in DMSO. It can be seen that the formulation prepared with the leuprolide–oleate complex significantly suppresses the initial burst release of leuprolide ($\sim$ 10% after one day incubation) from the microspheres in contrast to that prepared with free leuprolide. This result can be easily understandable because hydrophobically modified leuprolide in the complex had much smaller aqueous solubility than free leuprolide. However, both of the formulations exhibit similar leuprolide release profiles after the burst and show fairly constant release patterns up to 30 days. It can be surmised that for the microspheres prepared with the complex, oleate molecules initially ionically bound to leuprolide were slowly leached out in the aqueous medium upon incubation. This might lead to the no discernible difference in the release profiles after the early stage of incubation. Weak interactions between leuprolide and oleate species within microspheres might be prone to dissociate by slight changes of microenvironment pH and ionic strength induced by polymer degradation. The constant leuprolide release profiles can be explained by passive diffusion mechanism in the early stage, which was coupled with enhanced matrix erosion in the later stage (O'Donnell and McGinity, 1997). Additionally, ion exchange mechanism between basic side chain groups of amino acids in leuprolide and terminal carboxylic acid groups of hydrolyzed PLGA chains might play an important role in controlling the release as suggested earlier (Okada et al., 1994a; Okada et al. 1994b). Mass erosion profiles of the two microspheres are shown in Fig. 7. There are no apparent differences in the mass erosion rate, suggesting that the incorporation of oleate within PLGA microspheres did not play a role in affecting the PLGA degradation. Both of the microspheres exhibit an initial latent lag period of 5 days before starting to the mass erosion

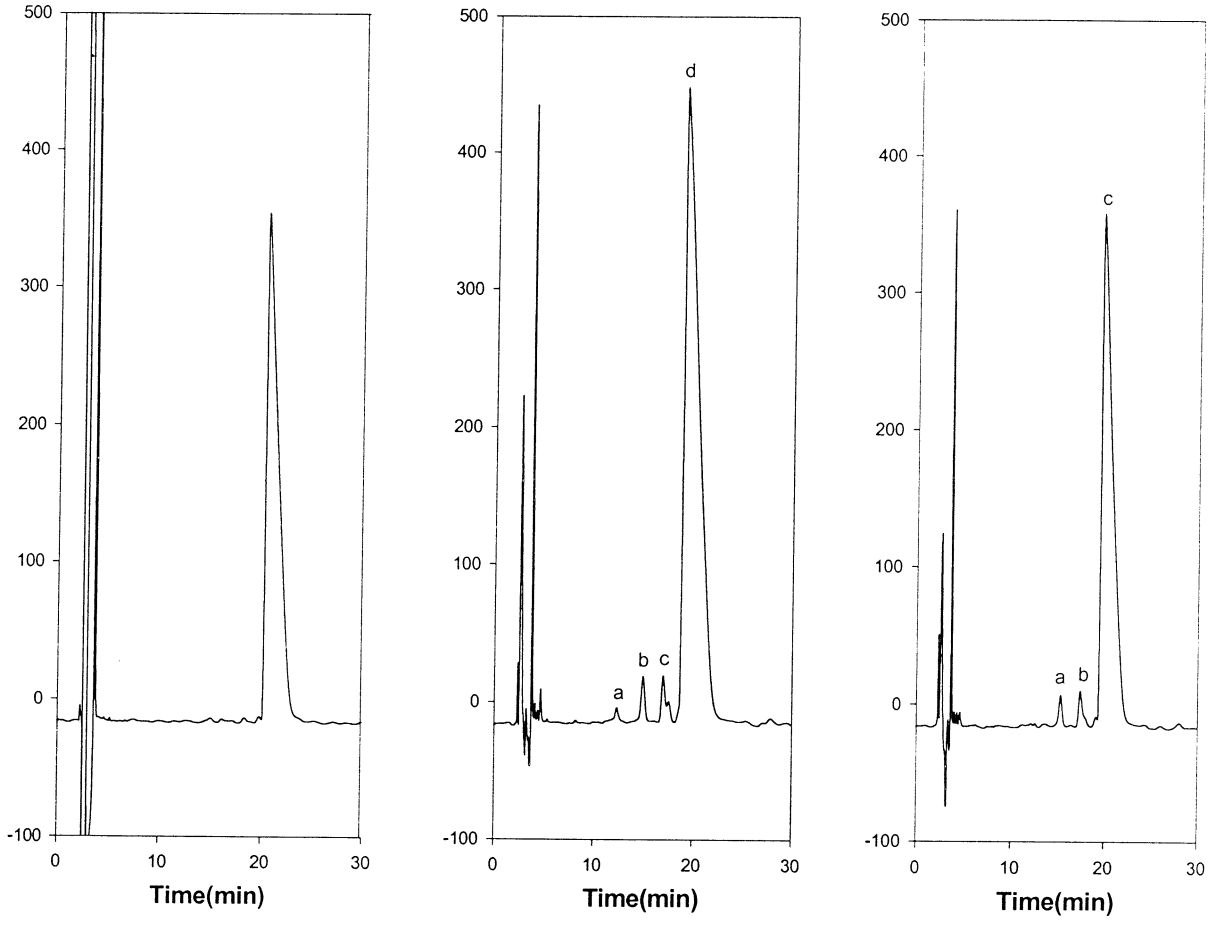

Fig. 8. Free leuprolide acetate RP-HPLC result (100 μg/ml) (left), released leuprolide from free leuprolide acetate encapsulated microspheres between day 20 and day 27 (a, 0.12%; b, 1.49%; c, 2.25%; d, 96.14%) (middle), released leuprolide from leuprolide/oleate complex encapsulated microspheres between day 20 and day 27 (a, 1.12%; b, 1.16%; c, 97.27%) (right).

in accordance to a typical degradation behavior of PLGA matrices as previously reported (Park, 1994). After 30 days of incubation, ~60 wt.% of the polymer mass was eroded, while about 90% of initially loaded leuprolide within the microspheres was released in the same period. The leuprolide release profiles presented in this study are almost similar to those of commercially available formulation. Fig. 8 shows the HPLC chromatograms of released leuprolide in the incubation medium, which was collected at day 27. Both of the formulations show similar chromatograms: 2–3 small minor peaks and a major intact peak of leuprolide. The small peaks are thought to be caused by chemical degradation reactions of leuprolide during the release period. However, it seems that the amount of the degradation products relative to that of intact leuprolide is small enough and to accept.

In conclusion, this study shows that leuprolide could be complexed with a fatty acid salt, sodium oleate in a manner of stoichiometric binding between the two species. The hydrophobically modified oleate–leuprolide complex gained more lipophilicity and could be formulated into biodegradable PLGA microspheres based on a single oil-in-water emulsion method. The microspheres encapsulated with the complex demon-

strated reduced burst release, but showed a similar leuprolide release behavior and stability compared to those encapsulated with free leuprolide. Hydrophobic modification of biologically active molecules based on ion pairing mechanism can be potentially applied for the sustained delivery of other peptides and proteins, particularly reducing an initial burst effect.

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