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Research Article

PREPARATION AND CHARACTERIZATION OF SUSTAINED RELEASE PLGA- LOADED MICROPARTICLES CONTAINING LEUPROLIDE ACETATE

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ABSTRACT

The objective of the present work was to formulate and evaluate sustained release leuprolide acetate microspheres for treating prostate and breast cancer effectively. In order to improve patient compliance with fewer side effects like pain and hot flashes from the marketed product of implants. Sustained release Leuprolide acetate microspheres for two months depot which had been designed based on disease condition were prepared in the form of microspheres for subcutaneous administration for sustained release. Different formulations were prepared by following Solvent Evaporation technique (double emulsion) using synthetic biodegradable polymer like Poly (Lactide-co-Glycolide) acid. The formulations were evaluated for percentage yield, entrapment efficiency, surface morphology (SEM), particle size analysis, In-vitro drug release and stability studies. The in-vitro release of drug from the formulations were studied in pH 7.4 saline phosphate buffer solution, and it was found that the prepared microspheres were able to sustained the release of the drug upto two months of about 95.6%. The release of the drug from the microspheres was found to follow zero order kinetics. The optimized formulation of the microspheres containing polymer and drug was found to be compatible from FTIR studies.

Keywords: Leuprolide acetate, Sustained release, PLGA, Microspheres, Implants.

INTRODUCTION

Leuprolide acetate is a synthetic nonapeptide analogue of naturally occurring gonadotropin releasing hormone (GnRH or LH-RH). The analogue possesses greater potency than the natural hormone. Administration of leuprolide acetate results in an initial increase in circulating levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH), leading to a transient increase in levels of the gonadal steroids. However, continuous administration of leuprolide acetate results in decreased levels of LH and FSH. In males, testosterone is reduced to castrate levels. The effect of cancer is reversible upon discontinuation of drug therapy.

The objective of the present work was to formulate and evaluate sustained release leuprolide acetate microspheres for treating prostate and breast cancer effectively. In order to improve patient compliance with fewer side effects like pain and hot flashes.

MATERIALS AND METHODS

Materials

Leuprolide acetate was obtained from Hemmo pharmaceuticals, china. Poly (Lactide-co-glycolic) acid 75:25 lactide to glycolide ratio (Resomer

RG755) was supplied by Boehringer-Ingelheim, Germany. Poly vinyl alcohol 1% (PVA) was obtained from S.D fine chemicals, Mumbai. Dichloromethane (DCM), Ethyl Acetate was supplied from Qualigens.

Preparation of Microspheres

Leuprolide acetate-loaded microspheres were prepared by a double emulsion-solvent evaporation technique. Briefly, 500 mg of polymer PLGA 75:25 was dissolved in 2 ml of organic phase dichloromethane (organic or oil phase). 100mg of drug (Leuprolide acetate) was dissolved in 0.2ml of water for injection was prepared separately (inner aqueous phase or w^1). To the organic phase, 0.2ml of aqueous drug solution was added drop by drop with the help of micropipette and emulsified by using high speed homogenizer (IKA) operating around 9000 rpm for about 5min. at a temperature of 2-8^{0}c to prepare water^1 /oil (w^1/o) primary emulsion. This primary emulsion was added to 50ml of external aqueous phase containing surfactant (1% poly vinyl alcohol (w^2) was used to prepare w^1/o/w^2 emulsion) at homogenizer speed around 6000 rpm for 3 minutes. The secondary emulsion was stirred at 1000rpm using digital overhead stirrer for 30min. at 2-8 ^{0}c and next 30min. at room temperature for the evaporation of DCM. The microspheres get hardened and wet microspheres are washed with chilled WFI for the removal of free drug and retained microspheres are collected.

As the polymer is commonly used in the preparation of microspheres, only three concentrations were attempted based on the literature survey. The composition of various formulations of leuprolide acetate prepared using different concentrations of PLGA, DCM and sodium chloride are shown in table 1.

The effect of different primary and secondary homogenization speeds on the formulation was also investigated in Formulations F7 to F12 as shown in the table 2.

Table 1: The composition of various formulations of leuprolide acetate microspheres

COMPOSITION		FORMULATIONS				
		F1	F2	F3	F4	F5
Drug (mg)		100	100	100	100	100
Water for Injection (ml)		0.2	0.2	0.2	0.2	0.2
PLGA 75:25 (mg) (Resomer RG755)		**800**	**600**	**500**	500	500
DCM (ml)		1	1.5	2	-	-
PVA 1% (ml)		50	50	50	50	50
Sodium chloride		0.1%	0.1%	0.2%	0.1%	0.2%
Temperature (^{0}c)		2-8	2-8	2-8	2-8	2-8
Homogenization speed (rpm)	Primary(5min)	9000	9000	9000	9000	9000
	Secondary(3min)	6000	6000	6000	6000	6000

Table 2: The composition of various formulations of Leuprolide acetate microspheres

COMPOSITION		FORMULATIONS						
		F6	F7	F8	F9	F10	F11	F12
Drug (mg)		100	100	100	100	100	100	100
WFI (ml)		0.2	0.2	0.2	0.2	0.2	0.2	0.2
PLGA 75-25 (mg) (ResomerRG755)		500	500	500	500	500	500	500
DCM (ml)		2.5	2	2	2	2	2	2
PVA 1% (ml)		50	50	50	50	50	50	50
Sodium chloride		0.3%	0.2%	0.2%	0.2%	0.2%	0.2%	0.2%
Temperature (^{0}C)		2-8	2-8	2-8	2-8	2-8	2-8	2-8
Homogenization speed (rpm)	Primary(5min)	9000	9000	9000	9000	9000	11,000	13,000
	Secondary(3min)	6000	3000	4000	5000	6000	6000	6000

Preformulation parameters

FTIR studies

FTIR studies were carried out for leuprolide acetate plain drug, PLGA 75:25, drug with polymer and scanned from 4000 cm-1 to 400 cm-1 in Bruker IR spectrophotometer and checked for any shifts in functional peaks.

Characterization of Microspheres

Determination of percentage yield

Microspheres dried at room temperature were weighed and the yield of microspheres was calculated using the formula.

Percentage yield = Practical yield (gm) / Theoretical yield × 100

Drug entrapment efficiency

The amount of drug entrapped was estimated by dissolving the 100mg of microspheres in DCM and water in 3:1 ratio ,under vigorous shaking for 1hr, the resultant solution was centrifuged, both layers were separated, leuprolide acetate was soluble in water but not in DCM. The drug content in aqueous solution was analyzed by using HPLC at 220nm with further dilutions against appropriate blank. The amount of the drug entrapped in the microspheres was calculated using the formula:

$$\text{Drug entrapment efficiency (\%)} = \frac{\text{Amount of drug actually present}}{\text{Theoretical drug load expected}} \times 100$$

Particle size analysis

Determination of average particle size of leuprolide acetate microspheres with carrier was very important characteristic. It was measured by using MALVERN INSTRUMENTS, STARTECH LABS PVT. LTD.

Scanning electron microscopy

Microspheres were observed and photographed with scanning electron microscopy (SEM) (JEOL JSM-6701F, Japan). Scanning electron microscopy was carried out to study the morphological characteristics of leuprolide acetate PLGA microspheres. The samples for the SEM analysis were prepared by sprinkling the microspheres on one side of adhesive stub. Then the microspheres were coated with gold (100A°) before microscopy. Finally the morphology of the microspheres was observed with the scanning electron microscopy.

In-vitro drug release

The in-vitro release of drug from the microsphere formulation was carried out by using Float-A-Lyzer which is a ready to use dialysis device. 2 ml of microspheres suspension containing known amount of drug was placed in the Float-A-Lyzer and this was placed in 50ml of 7.4p^{H} saline phosphate buffer solution, maintained at 37^{o}C and stirred with the help of a magnetic stirrer. Aliquots (2ml) of release medium were withdrawn at different time intervals and the sample was replaced with fresh saline phosphate buffer (pH 7.4) to maintain constant volume. The samples were analyzed for drug content by HPLC at 220nm. After every 10days the complete medium was withdrawn and replaced by fresh medium to avoid saturation of the medium.

In-vitro drug release kinetics

The obtained data were fitted into mathematical equation zero order, first order, higuchi model and korsemeyer equation/ peppa's model in order to describe the kinetics and mechanism of drug release from the microsphere formulations.

Stability studies

To assess the physical and chemical stability of the microspheres, accelerated stability studies were conducted for 3 months under the storage conditions mentioned in ICH guidelines. The sample containing optimized formulations were place in vials and stored at 25±2^{o}C / 60% RH. After 90 days the formulations were checked for physical appearance and drug content.

RESULT AND DISCUSSION

In the preparation of microspheres, as the amount of polymer increased (F1, F2 and F3), the particle size of microspheres was found to increase and encapsulation efficiency was found to decrease (Table 3). The organic solvent dichloromethane was used when the polymer was dissolved in DCM (BP- 40˚C), high encapsulation efficiency was obtained indicating faster rate of solvent removal. Low temperature (2-8^{o}C) was maintained to improve the formation of microspheres with high encapsulation efficiency.

When the secondary homogenization speed (rpm) was decreased as seen in Table 3 in case of F7, F8,

F9 and F10 formulations, the particle size was also found to increase. When homogenization speed was increased to 8000 rpm, particle size was found to be 80.7µm which was optimized. In F11 and F12 formulations, as the primary homogenization speed was increased from 9000 rpm to 13,000 rpm, further reduction in the particle size of microspheres was observed.

FTIR studies

The FTIR spectra of the plain drug, polymer and drug with polymer are shown in Fig 1, 2 and 3. The position of peak in FTIR spectra of pure drug was compared with those in FTIR spectra of drug with polymer. It was observed that there was no disappearance or significant shift in the peak position of drug in any spectra of drug with polymer which proved that the drug and polymers used for the study are compatible.

Evaluation parameters

Percentage Yield, Entrapment Efficiency & mean particle size

The percentage yield, encapsulation efficiency and mean particle size were determined for all the formulations from F1 to F12 (Table 3). The percentage yield for optimized formulation (F10) was found to be 74.3%, encapsulation efficiency was found to be 86.3% and rounded mean particle size was found to be 80.7µm.

Mean particle size distribution

The obtained mean particle size distribution values for all the formulations were rounded. As the polymer concentration decreased the particle size also was found to decrease. The organic solvent DCM was selected based on the %Entrapment efficiency. However F10 was optimized based on the particle size of 80.7µm as shown in Fig.5 and highest entrapment efficiency of 86.3%.

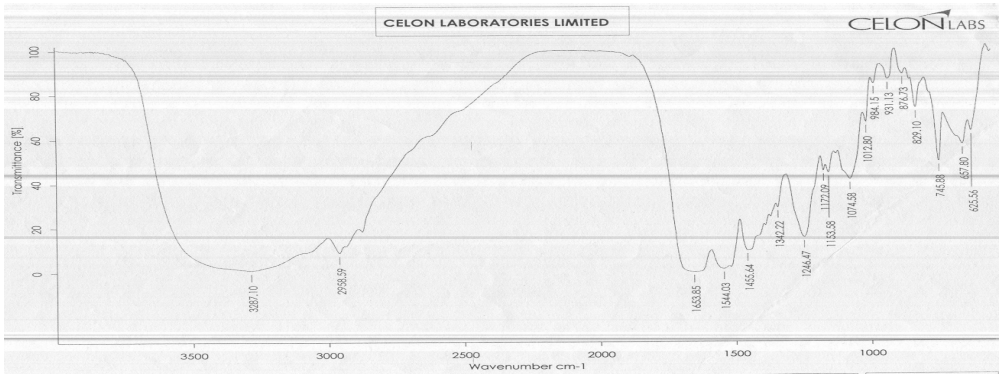

Fig. 1: FTIR Spectrum of Leuprolide acetate plain drug

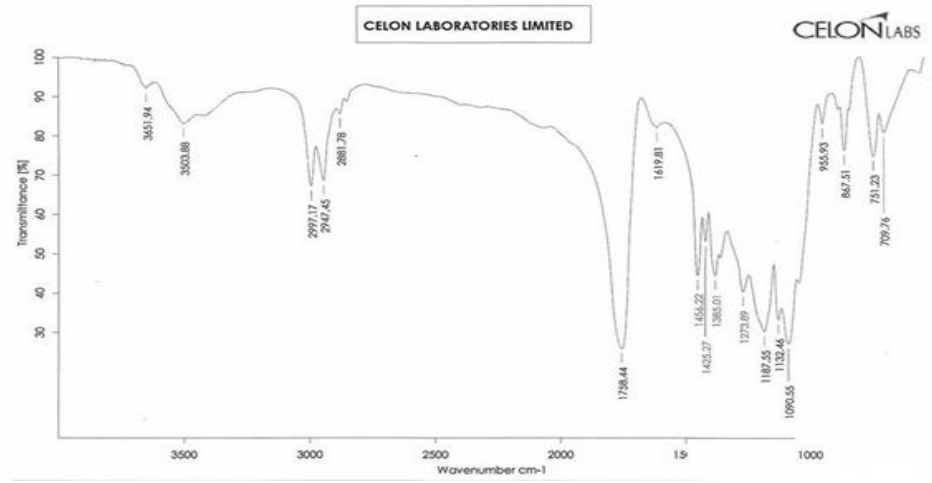

Fig. 2: FTIR Spectrum of PLGA 75:25

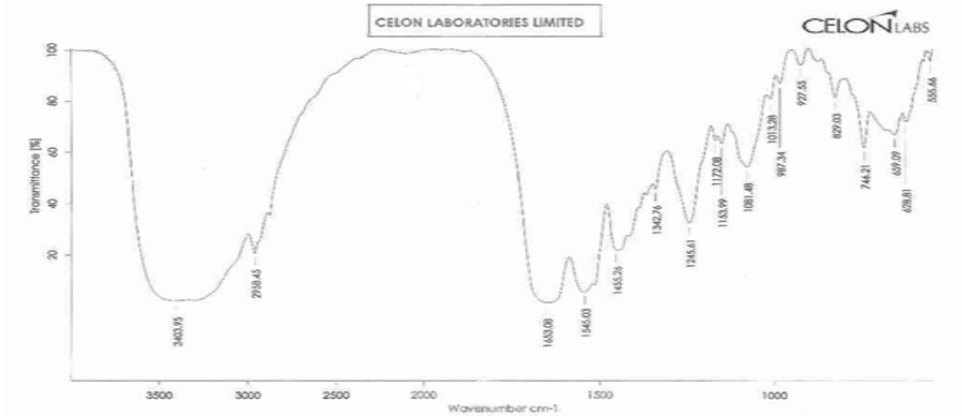

Fig. 3: Spectra of physical mixture of leuprolide acetate and PLGA 75:25

Table 3: Percentage yield, Entrapment efficiency and Rounded mean particle size of all the formulations

BATCHES	PERCENTAGE YIELD (%)	ENTRAPMENT EFFICIENCY (%)	ROUNDED MEAN PARTICLE SIZE
F1	71.6	79.1	85.2
F2	69.8	78.3	83.5
F3	62.3	80.1	79.3
F4	49.3	68.5	54.8
F5	52.6	78.2	65.4
F6	69.8	79.6	78.5
F7	70.6	80.4	104.0
F8	70.2	81.5	90.6
F9	71.8	83.1	84.8
F10	**74.3**	**86.3**	**80.7**
F11	68.1	73.6	61.2
F12	59.3	64.2	53.8

Scanning electron microscopy

SEM was performed on optimized leuprolide acetate microspheres at 120X magnification. The SEM picture showed that the shape of the microspheres was spherical and the coated surface was clearly visible as seen in Fig 4.

In vitro release studies

The release profiles showed a characteristic initial burst release followed by a lag period and further initiation of controlled release. After the initial lag, a nearly linear and continuous release was observed over 35- 48 days, around 95.6% at day 60. Comparison of *in vitro* drug release profiles for some formulations F8, F9 and F10 are shown in Fig 6 and the data is shown in Table 4.

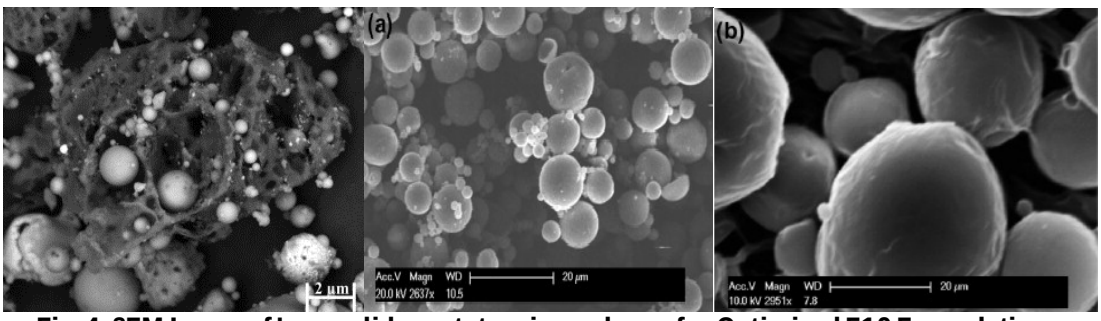

Fig. 4: SEM Image of Leuprolide acetate microspheres for Optimised F10 Formulation

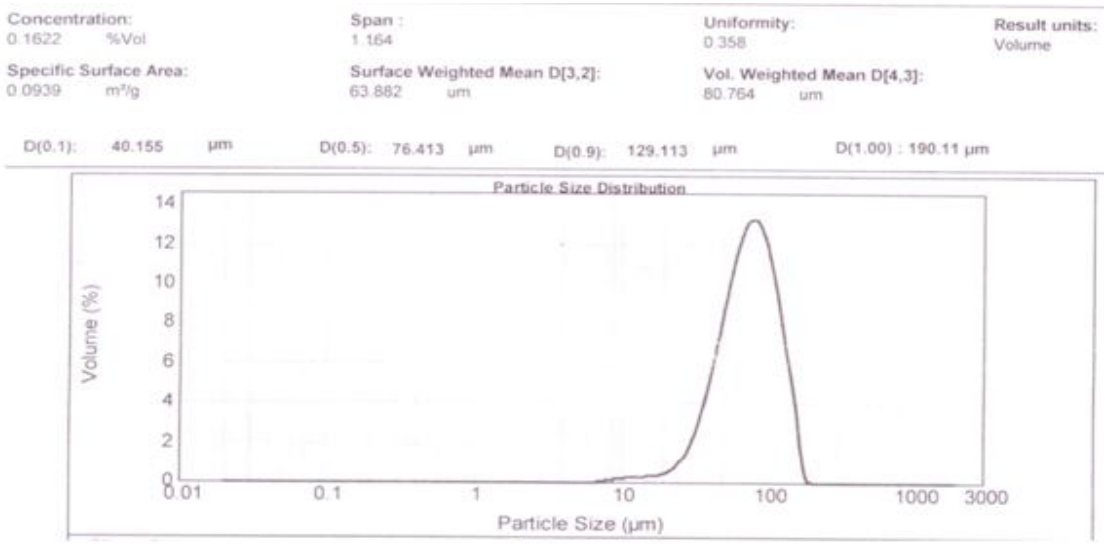

Fig. 5: Mean Particle Size of Leuprolide acetate microspheres for Optimised Formulation (F10).

Table 4: Comparison of in-vitro drug release profile of Leuprolide acetate from the formulation F8, F9 and F10

Time (Days)	Cumulative% drug release		
	F8	F9	F10
0	0	0	0
1	9.4	10.8	8.5
7	23.2	21.2	19.7
15	41.3	35.7	31.9
25	55.5	54.3	45.5
35	73.7	69.5	69.6
45	85.5	81.4	88.4
55	89.2	89.2	92.3
60	90.6	92.4	95.6

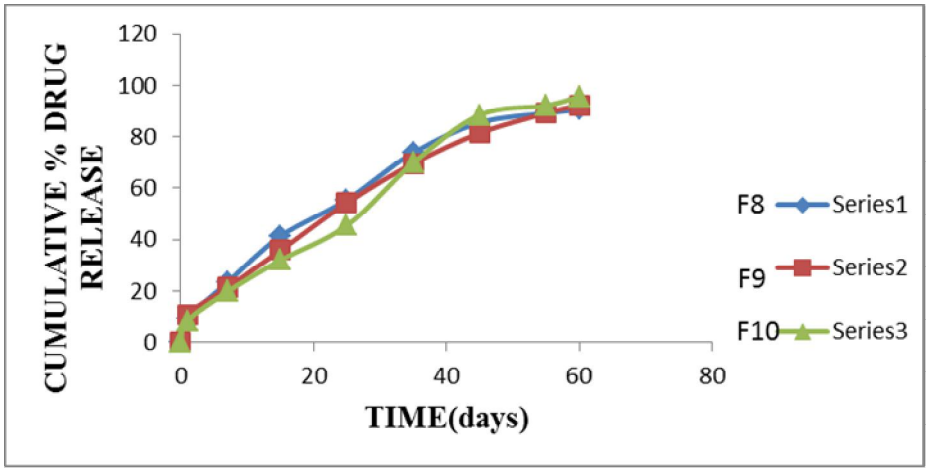

Fig. 6: Comparison of in-vitro drug release profile of Leuprolide acetate from the formulation F8, F9 and F10

Table 5: Data of drug release kinetic profiles for formulations F8, F9 and F10

FORMULATION CODE	ZERO ORDER (R^2)	FIRST ORDER (R^2)	HIGUCHI (R^2)	KORSMEYER PEPPAS (n)
F8	0.952	0.982	0.986	0.991
F9	0.978	0.986	0.983	0.972
F10	**0.977**	0.958	0.959	0.970

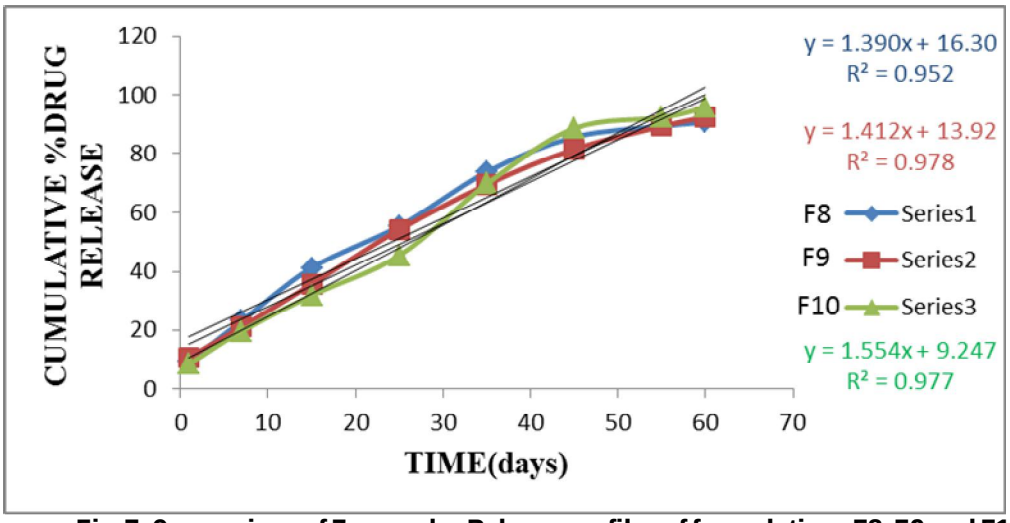

Fig. 7: Comparison of Zero order Release profiles of formulations F8, F9 and F10

Table 6: Accelerated stability data for Leuprolide acetate microspheres at 25±20C/ 60±5% RH

Test	0 days	15 days	30 days	45 days	60 days	75 days	90 days
Description	White to almost white	White to almost white	White to almost white	White to almost white	White to almost white	White to almost white	White to almost white
Assay of F8 formulation	82.3%	81.5%	79.2%	78.3%	76.9%	75.1%	74.3%
Assay of F9 formulation	83.4%	82.2%	81.4%	80.1%	79.2%	78.4%	77.3%
Assay of F10 formulation	85.8%	85.2%	84.5%	83.9%	82.3%	81.9%	81.1%

In vitro release kinetics

The release kinetics of F8, F9, F10 formulations was studied and fitted into various models such as Zero Order, First Order, Higuchi and Korsmeyer Peppas. From the data as seen in table 5 and the zero order release is shown in Fig 7, it can be said that F10 formulation followed zero order kinetics based on the R^2 value.

Stability studies

The stability studies of Leuprolide acetate microspheres were evaluated after storage at 25±2^{0}c/60±5% RH. The assays and appearance of samples were determined as a function of the storage time. There was no colour change in the physical appearance and the assay of microspheres of formulation F10 was found to be 81.1% after 90 days. Hence leuprolide acetate microsphere was found to be stable.

CONCLUSION

In conclusion, the uniform-sized biodegradable PLGA microspheres containing leuprolide acetate were successfully prepared by double emulsion solvent evaporation method. Various factors related to the preparation process, influenced the drug encapsulation efficiency, stirring speed and the cumulative drug release was subsequently investigated. The results indicated that the drug encapsulation efficiency and the cumulative drug

release rates were affected by the presence of NaCl (in the outer water phase, inner water phase volume), and concentration of co-encapsulated surfactant. Ultimately, spherical PLGA microparticles with encapsulation efficiency higher than 74% and prolonged leuprolide acetate release upto two months of about 95.6% were obtained.

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