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Research Article

PREPARATION AND CHARACTERIZATION OF BUSERELIN ACETATE LOADED MICROSPHERERS

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ABSTRACT

The objective of the present work was to formulate and evaluate Buserelin acetate microspheres for treating the palliative treatment of patients with hormone-dependent advanced carcinoma of the prostate gland in the form of biodegradable polymers. The marketed preparation of Buserelin acetate is available in the form of solid implant which has less patient compliance because of severe pain during injection; cost of product is more and need special precautions during injection. Buserelin acetate was prepared in the form of microspheres to overcome all these drawbacks for subcutaneous or intramuscular administration over a period of one month controlled release delivery. The influence of various formulation and process parameters using synthetic biodegradable polymer such as polymer concentration, molecular weight of polymer, end group of polymer, effect of solvents, volume of inner and outer aqueous phase, homogenization time, homogenization speed, solidification temperature etc on encapsulation efficiency, particle size, surface morphology and release were investigated. The formulation and process variables parameters were affected significantly on particle size, entrapment efficiency and release rate of buserelin microspheres. The Scanning Electron Microscopy (SEM) image showed that the shape and morphology of microsphere was spherical having porous structure with 80 ± 5 µm particle sizes. The *in-vitro* drug release for optimized formulation was found to be controlled release of drug over a period of one month. From the experimental results, it is evident that the controlled release of Buserelin acetate loaded microspheres can be a suitable alternative of solid implant in the treatment of patients with hormone-dependent advanced carcinoma of the prostate gland.

Keywords: Buserelin acetate, Microspheres, Controlled release, PLGA polymer, SEM

INTRODCUTION

Many proteins currently being developed are aimed at chronic conditions where therapy may be required over months or years. Alternative administration by frequent injections to keep the protein drug at effective concentrations is tedious, expensive, and has poor patient compliance. Therefore, development of sustained release injectable dosage forms becomes necessary to improve the efficacy of peptide drugs and eliminate the need for frequent administration [1].

Buserelin acetate is a synthetic peptide analogue of the natural gonadotropin releasing hormone (GnRH/LH-RH). The substitution of glycine in position 6 by D-serine, and that of glycinamide in position 10 by ethylamide, leads to a nonapeptide with a greatly enhanced LH-RH effect. The effects of buserelin on follicle stimulating hormone (FSH) and luteinizing hormone (LH) release are 20 to 170 times greater than those of LH-RH. Buserelin also has a longer duration of action than natural LH-RH.

The marketed preparation of Buserelin acetate in the form of solid implant is available on the name of SUPREFACT® DEPOT manufactured by sanofi-aventis Canada Inc. It is available with two different strengths i.e. 6.3 mg for 2 months and 9.45 mg for 3 months duration therapy.

The aim of the present research was to formulate and evaluate Buserelin acetate microspheres for subcutaneous or intramuscular administration for period on one month controlled release delivery system. Biodegradable microspheres were shown to improve the bioavailability of peptides by protecting them from physical degradation and proteolysis in body fluids. Poly (D,L-lactide) (PLA) and poly (D,L-lactide-co-glycolide) (PLGA) are the most widely used and well-characterized materials for the preparation of biodegradable microspheres.

MATERIALS AND METHODS

Materials

Buserelin acetate was purchased from Hemmo pharmaceutical PVD (Mumbai). Poly (D, L-Lactic-co-glycolic acid) 50:50 (PLGA 50:50) Resomer®RG 504 and Resomer®RG 504H were supplied by Evonik/Boehringer-Ingelheim (Germany). Poly (vinyl alcohol) (PVA) (MW 22000, 88% hydrolyzed) and ethyl acetate were supplied by Merck (India). Dichloromethane, Tween 80, Sodium chloride, Potassium chloride, di-sodium hydrogen phosphate, Potassium dehydrogenate phosphate and sodium azide were obtained from J. T. Baker (India).

Preparation of Buserelin acetate microsphere

Buserelin acetate-loaded microspheres were prepared by a double emulsion-solvent evaporation technique. Briefly, 500 mg PLGA 5050 was dissolved in 5 mL dichloromethane (oil phase). An aqueous solution containing 50 mg of Buserelin acetate in 1 ml of phosphate buffer pH 7.4 was prepared separately (inner aqueous phase or W1). The first aqueous (W1) phase was emulsified into the oil phase (containing PLGA), using a high-speed homogenizer (T18 basic, IKA, Germany) at 2-8 °C using different speeds and time durations to form water in oil primary emulsion. This primary emulsion was added in to 100 ml of external aqueous phase containing 1 % PVA solution to form secondary emulsion at 6000 rpm speed for 3 mins at 2-8°C temperature. The wet microspheres were then stirred at 1000 rpm for 2 hrs at 2-8°C to permit evaporation of DCM and solidification of microspheres. The wet microspheres obtained were collected by centrifugation followed by filtration and Lyophillization [2-5].

Different formulation variables like volume of DCM (F1, F2 & F3), volume of PVA solution (F2, F4 & F5), Volume of inner aqueous phase (F2, F6 & F7) and effect of solvents (F2 & F8) were carried out as below mentioned table 1:

Evaluation of Buserelin acetate microsphere

Determination of percentage yield

Microspheres were weighed and the yield of microspheres was calculated using the formula:

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

Determination of drug entrapment efficiency (EE)

The amount of drug entrapped was estimated by dispersing 50 mg of microspheres in DCM and water in 3:1 ratio, under vigorous shaking for 1hr, the resultant solution was centrifuged. Both layers were separated. As the buserelin acetate was soluble in water but not in DCM, the drug content in aqueous solution was analyzed by using HPLC at 220 nm with further dilutions against appropriate blank.

Table 1: Formulation variable of buserelin acetate microspheres

Compositions	Formulation variables							
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**
Buserelin acetate (mg)	50	50	50	50	50	50	50	50
Phosphate buffer pH 7.4	1	1	1	1	1	**0.5**	**1.5**	1
PLGA 5050 (mg)	500	500	500	500	500	500	500	500
DCM (ml)	**2.5**	**5**	**7.5**	5	5	5	5	5
Ethyl acetate (ml)	-	-	-	-	-	-	-	**5**
1 % PVA (ml)	100	100	100	**50**	**150**	100	100	100
1° Homogenization speed (rpm)	10000	10000	10000	10000	10000	10000	10000	10000
1° Homogenization time (mins)	1	1	1	1	1	1	1	1
1° Homogenization temperature (°C)	5	5	5	5	5	5	5	5
2° Homogenization speed (rpm)	6000	6000	6000	6000	6000	6000	6000	6000
2° Homogenization time (mins)	3	3	3	3	3	3	3	3
2° Homogenization temperature (°C)	5	5	5	5	5	5	5	5
Stirring speed (rpm)	1000	1000	1000	1000	1000	1000	1000	1000
Stirring time (hrs)	2	2	2	2	2	2	2	2
Stirring temperature (°C)	5	5	5	5	5	5	5	5

Different process variables like sped of primary homogenization (F9, F10 & F11), time of primary homogenization (F12 & F13) and effect of temperature for solidification of microspheres (F14 & F15) were carried out as below mentioned table 2:

Table 2: Process variable of buserelin acetate microspheres

Compositions	Process variables						
	F9	**F10**	**F11**	**F12**	**F13**	**F14**	**F15**
Buserelin acetate (mg)	50	50	50	50	50	50	50
Phosphate buffer pH 7.4	1	1	1	1	1	1	1
PLGA 5050 (mg)	500	500	500	500	500	500	500
DCM (ml)	5	5	5	5	5	5	5
1 % PVA (ml)	100	100	100	100	100	100	100
1° Homogenization speed (rpm)	**6000**	**10000**	**14000**	10000	10000	10000	10000
1° Homogenization time (mins)	1	1	1	**3**	**5**	1	1
1° Homogenization temperature (°C)	5	5	5	5	5	5	5
2° Homogenization speed (rpm)	6000	6000	6000	6000	6000	6000	6000
2° Homogenization time (mins)	3	3	3	3	3	3	3
2° Homogenization temperature (°C)	5	5	5	5	5	5	5
Stirring speed (rpm)	1000	1000	1000	1000	1000	1000	1000
Stirring time (hrs)	2	2	2	2	2	2	2
Stirring temperature (°C)	5	5	5	5	5	**25**	**40**

The amount of the drug entrapped in the microspheres was calculated using the formula:

% EE = Actual weight of drug in sample/ Theoretical weight of drug in sample x 100.

Particle size analysis

The mean diameter of microspheres was determined by laser diffractometer (Mastersizer X, Malvern Instrument, UK). Microparticles were suspended in 0.3% aqueous solution of Tween 80 and sonicated for 15 s prior to particle size determination.

Scanning electron microscopy (SEM)

The morphology of microparticles was examined by scanning electron microscopy (MW2300, Cam Scan-England). Samples were mounted on metal stubs and sputter-coated with gold for 4 min prior to examination under.

***In-vitro* drug release**

The in-vitro drug release from the microspheres was carried out by using a regenerated cellulose membrane dialysis apparatus Float-A-lyzer.2ml of microspheres suspension containing known amount of drug was placed in the Float-A-lyzer and this was placed in 250 ml of PBS (pH 7.4), maintained at 37°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 PBS (pH 7.4) to maintain constant volume. The samples were analyzed for drug content by HPLC at 220nm. Upon completion of one week, the complete medium was withdrawn and replaced by fresh medium to avoid saturation of the medium.

Stability studies

To assess the physical and chemical stability of the microspheres, stability studies were conducted for 3 months under various storage conditions mentioned in ICH guidelines. The optimized formulation was placed in vials and stored at 25±20c/ 60±5% RH. After 90 days the formulations were checked for physical appearance and drug content.

RESULTS AND DISCUSSIONS

Summarized results of formulation and process variable are given in below table 3 & 4 respectively:

Effect of DCM volume\Polymer concentration

As the volume of DCM increased or decreased the polymer concentration, the viscosity of polymer was decreased. Encapsulation efficiency of buserelin loaded microspheres was increased with increasing polymer concentration and the particle size was decreased. No significant difference was observed between F1 & F2 formulation in terms of particle size, encapsulation efficiency and initial burst release but there was significant difference between F2 & F3 formulation in term of particle size and encapsulation efficiency. The contribution of a high polymer concentration to the encapsulation efficiency can be interpreted in two ways. First, when highly concentrated, the polymer precipitates faster on the surface of the dispersed phase and prevents drug diffusion across the phase boundary. Second, the high concentration increases viscosity of the solution and delays the drug diffusion within the polymer droplets. In-vitro release of the microspheres with high polymer concentration or lower DCM volume was lower than the higher volume of DCM [6].

Table 3: Results of formulation variables of buserelin acetate microspheres

Formulation code	Particle size (µm)	Encapsulation efficiency (%)	Initial burst release (%)
F1	88	87%	17%
F2	80	85%	18%
F3	60	70%	25%
F4	55	60%	27%
F5	125	80%	18%
F6	75	86%	18%
F7	100	65%	30%
F8	95	73%	22%

Table 4: Results of process variables of buserelin acetate microspheres

Formulation code	Particle size (µm)	Encapsulation efficiency (%)	Initial burst release (%)
F9	100	72%	19%
F10	80	85%	18%
F11	50	68%	26%
F12	60	80%	24%
F13	58	82%	27%
F14	95	80%	21%
F15	130	65%	18%

Effect of PVA volume

An increase in the volume of PVA solution resulted in an increase the encapsulation efficiency and particle size of buserelin loaded microspheres. Formulation F5 has shown more particle size than formulation F2. The increase in the particle size was attributed to a reduction in agitation that occurred because of a decrease in mixing efficiency associated with higher volumes. A reduction in mixing efficiency probably produced as increase in the size of the emulsion droplets during the preparative process, which would result in the formulation of large microparticles [7]. As a result of increased particle size, there is as associated increase in particle volume, which enables more buserelin to be incorporated into the microparticles.

Effect of solvent

Microspheres prepared with DCM (F2) were given higher entrapment efficiency and higher particle size than microsphere prepared with ethyl acetate (F8). Because, boiling point of DCM (40°C) is lower than ethyl acetate (77°C) so that solidification of microspheres was faster with lower boiling point. Because of longer time of solidification, entrapment efficiency was decreased [6].

Effect of primary homogenization speed

The particle size of microspheres was decreased with increase in speed of primary homogenization. Formulation F9 having 100 µm particle size as compare to formulation F11 having 60 µm. As the homogenization speed increases, the shear stress increases and the established balance between tangential stresses at the droplet interface impacted by the homogenizer and interfacial tension is going to be altered. The larger tangential stress leads to a reduction in droplet size, while the homogenization speed affects the relative viscosity of the emulsion. Typically, the viscosity reduction at a higher rotational speed is responsible for a decrease in particle size [8,9,10,11].

Effect of primary homogenization time

The particle size of microspheres was decreased with increase in time of primary homogenization [[8,9,1][0,11]]. Formulation F10 having 80 µm particle size as compare to formulation F13 having 58 µm. Mechanism follows the same as mentioned above parameter.

Effect of temperature on solidification of microspheres

The microspheres prepared at 2-8°C and 40°C (F2 & F8) has shown higher entrapment efficiency and lower initial burst release than microspheres prepared at 25°C (F7). Microspheres prepared at 40°C solidified rapidly, forming a dense thin skin, indicating high drug encapsulation efficiency. Although faster skin formulation may reduce drug loss, the increase in solubility of drug at higher temperature and faster mass transfer may also increase the amount of buserelin leaving the dispersed phase during formation. The microspheres fabricated at lower temperature solidify slower, the lower solubility and mass transfer of buserelin at that temperature probably compensate for the relative ease of diffusing through the softer and less dense skin. The activity also remains fairly intact at this lower temperature. The resultant encapsulation efficiency for microspheres prepared at higher and lower temperature is almost similar. Initial burst release of microspheres prepared at higher temperature is slightly higher than microspheres prepared at 5°C because higher porosity of microspheres [12].

From above results of all parameters, it was observed that the formulation F10 was given desired particle size, entrapment efficiency and initial burst release.

Characterization of buserelin loaded microspheres

Formulation F10 was chosen for characterization of buserelin loaded PLGA microspheres.

Mean particle size distribution

The mean diameter of microspheres was determined by laser diffractometer (Mastersizer X, Malvern Instrument, UK). Microparticles were suspended in 0.3% aqueous solution of Tween 80 and sonicated for 15 s prior to particle size determination. The mean particle size of formulation F10 was shown below in Fig 1.

Scanning electron microscopy (SEM)

The morphology of microparticles was examined by scanning electron microscopy (MW2300, Cam Scan-England). Samples were mounted on metal stubs and sputter-coated with gold for 4 min prior to examination under. The SEM picture showed in Fig 2 that the shape of the microspheres was spherical and smooth surface with less porosity. SEM picture was compared with formulation F7 and it was observed that porosity of microspheres was more as inner aqueous phase volume increased.

^{1}H NMR study

^{1}H NMR analysis has been done to determine the co-monomer ratio of lactide and glycolide polymer [14]. Co-monomer ratios (Fig 3) were determinate by integrating the methane group of the lactide unit at 4.75 ppm (1.9287) and for the methylene group of the glycolide unit at 5.25 ppm (1.0). These integral values, LA and GA respectively, were converted into co-monomer ration, R LA and R GA using the following equations:

R LA = ILA + IGA / ILA = 1.9287 + 1 / 1 = 1.9287

RGA = IGA + ILA / IGA = 1 + 1.9287 / 1.9287 = 2

So, from ^{1}H NMR spectrum and co-monomer ratio, we can conclude that PLGA polymer has 50% lactide and 50% glycolide.

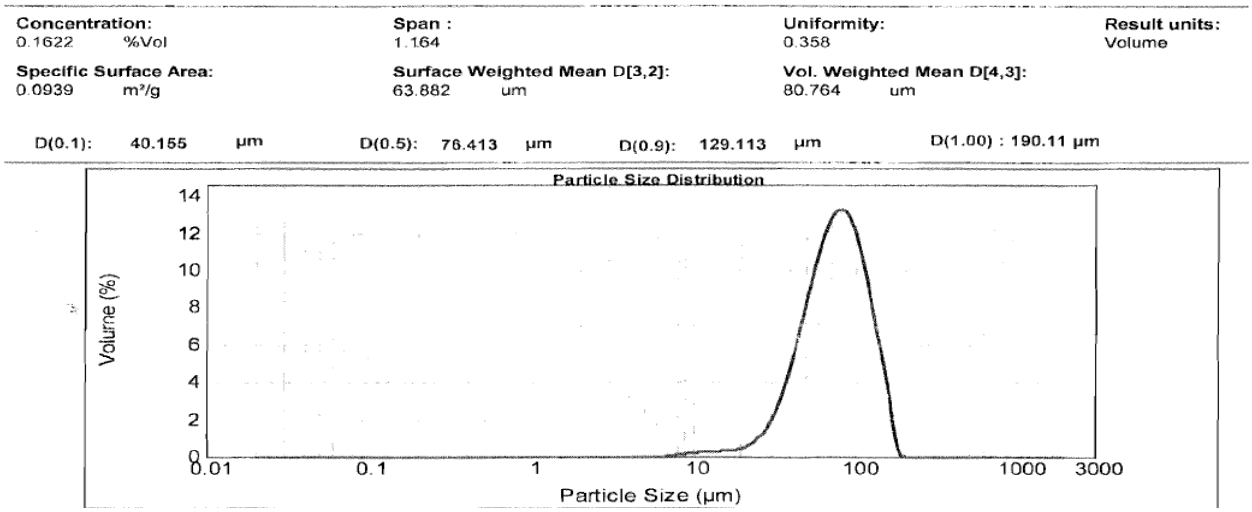

Fig. 1: Mean Particle size of formulation F10

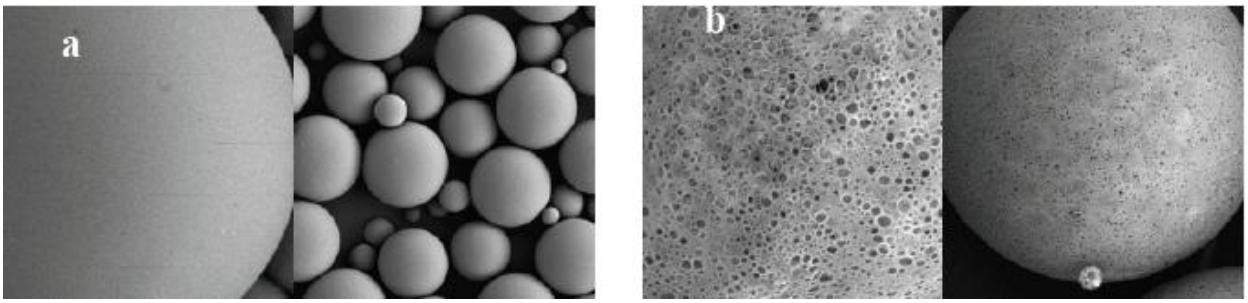

Fig. 2: a) SEM picture of formulation F10. b) SEM picture of formulation F7.

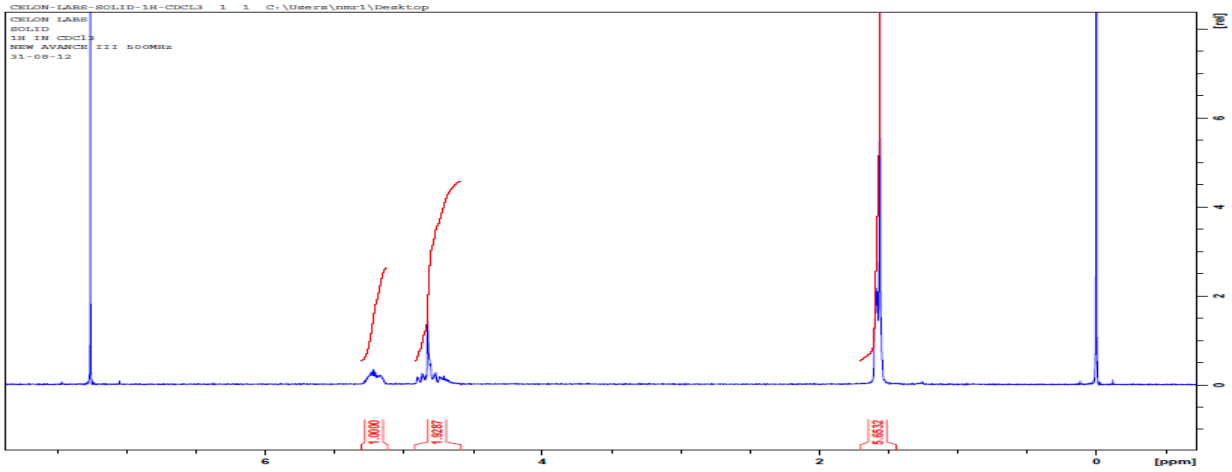

Fig. 3: ^{1}H NMR spectrum of buserelin acetate microspheres

***In-vitro* drug release**

The in-vitro drug release from the microspheres was carried out by using a regenerated cellulose membrane dialysis apparatus Float-A-lyzer [12,14,15]. 2ml of microspheres suspension containing known amount of drug (50 mg microspheres) was placed in the Float-A-lyzer and this was placed in 250ml of PBS (pH 7.4), maintained at 37°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 PBS (pH 7.4) to maintain constant volume. The samples were analyzed for drug content by HPLC at 220nm. Upon completion of one week, the complete medium was withdrawn and replaced by fresh medium to avoid saturation of the medium. Initial burst release means release of drug within 24 hrs and F10 has shown 18% IBR.

The cumulative percent release of F1, F6 & F10 formulations at various time intervals was calculated. The cumulative percent drug release in F1, F6 & F10 formulations was plotted against time in Figure 4.

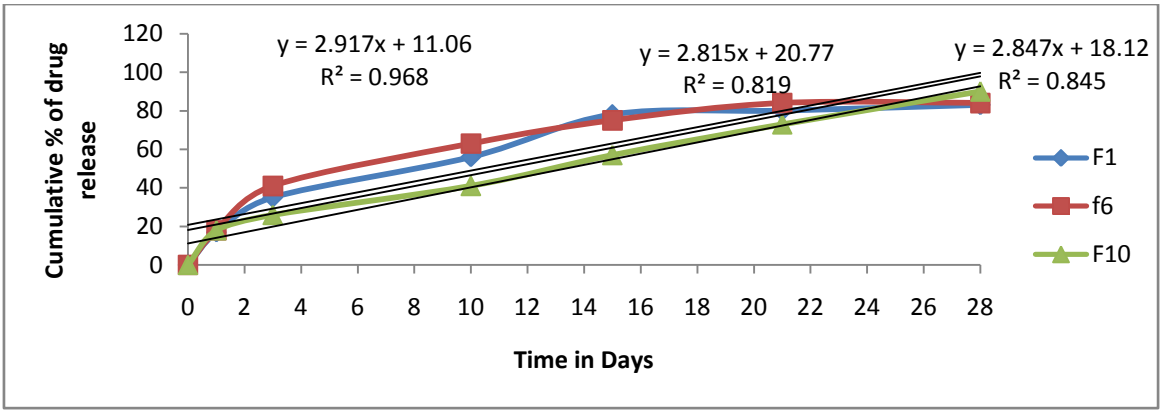

Fig. 4: *In- vitro* release studies for optimized formulations - F1, F6 & F10

Release Kinetics

The release kinetics of F1, F6 & F10 formulations was studied. All formulations follow Higuchi release kinetics and follow Anomalous (non-Fickian) diffusion when it applied to the Korsmeyer-Peppa's Model for mechanism of drug release. F10 formulation has better kinetic results when compared to F1 and F6 formulations. The results are shown in Figure 5, 6, 7 & 8 and in Table 5.

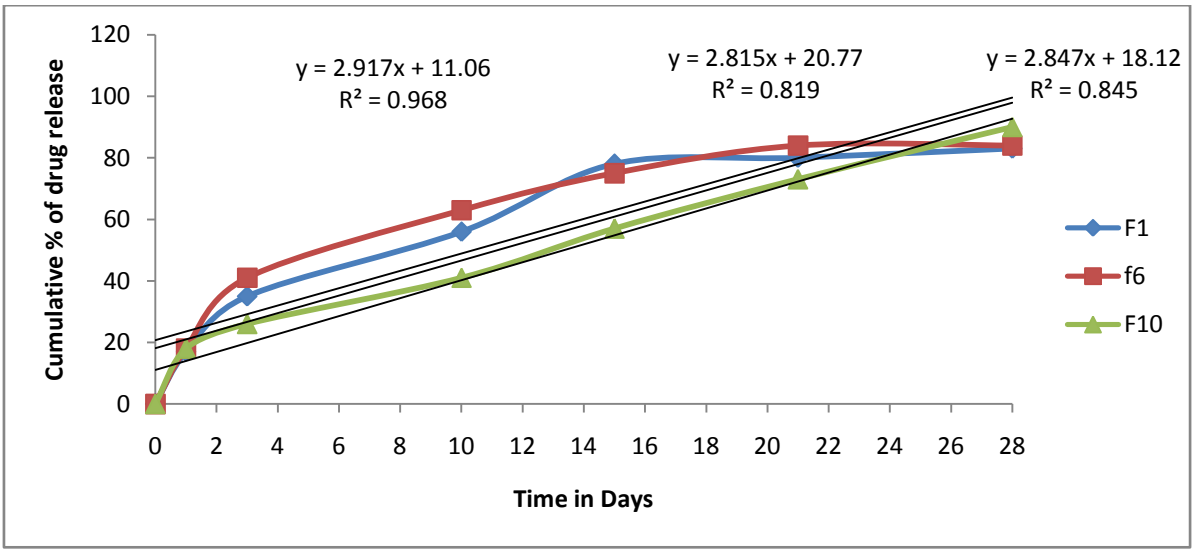

Fig. 5: Zero order release studies for optimized formulations F1, F6 & F10

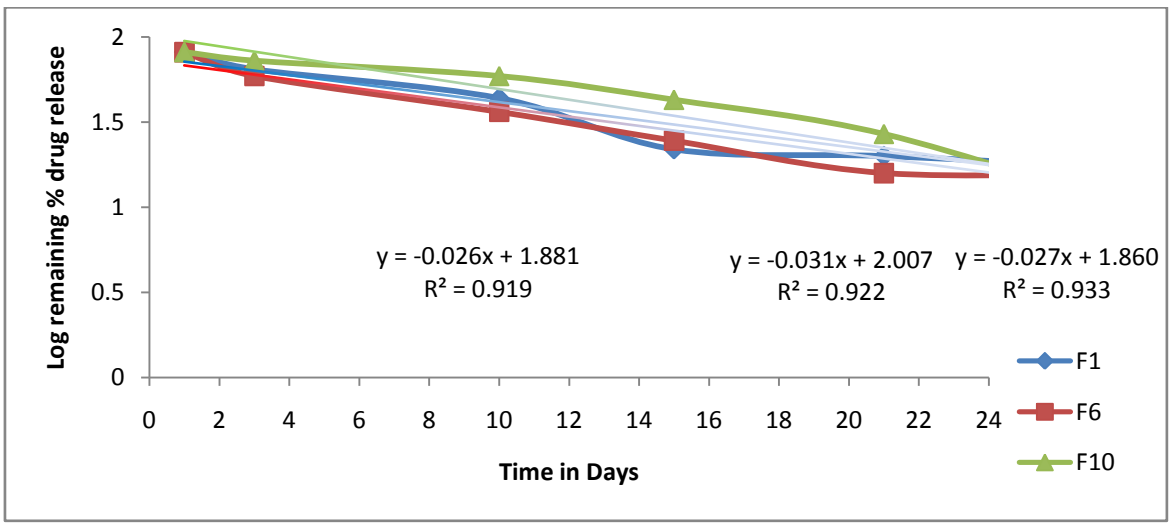

Fig. 6: First order release studies for optimized formulations F1, F6 & F10

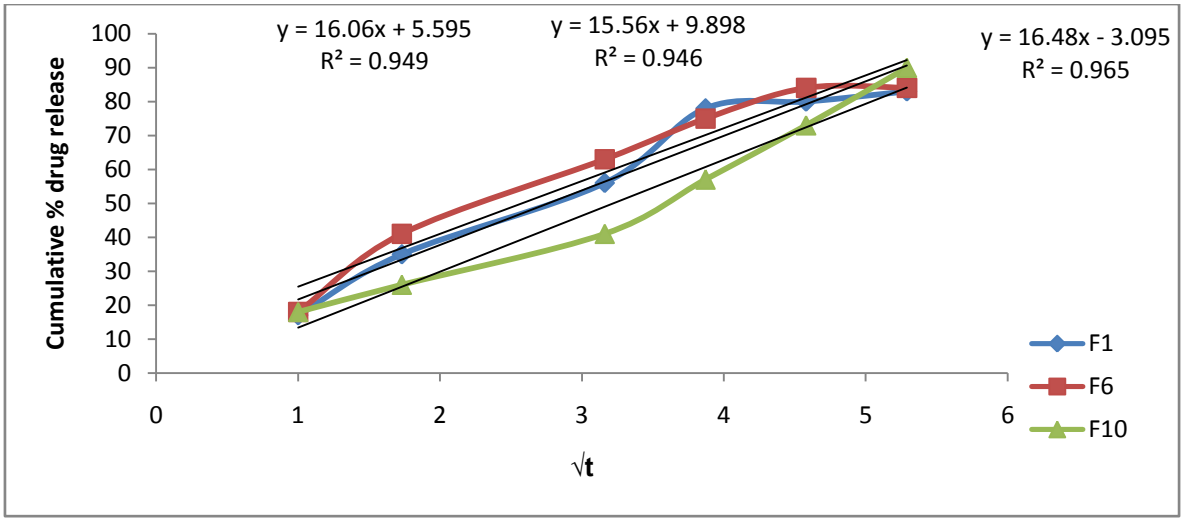

Fig. 7: Higuchi's order plot for optimized formulations F1, F6 & F10

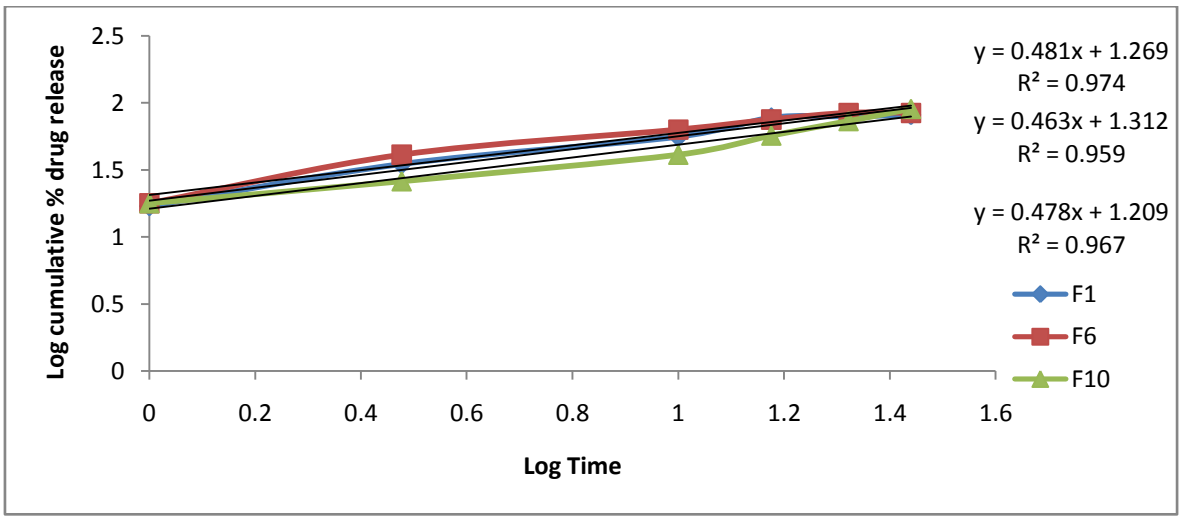

Fig. 8: Korsmeyer –Peppa's model for optimized formulations F1, F6 & F10

Table 5: Release rate profile of Formulations F1, F6 & F10

Type of Formulation	Zero order (R^2)	First-order (R^2)	Higuchi (R^2)	Korsmeyer –Peppas (n)
F1	0.845	0.919	0.949	0.481
F6	0.819	0.933	0.946	0.463
F10	0.968	0.922	0.965	0.478

Stability studies

Accelerated stability studies of Buserelin acetate microspheres at temperature 25±20C/60±5% RH as per ICH guidelines were studied for 90 days. The assays and appearance of samples were determined as a function of the storage time. There was no color change in the physical appearance, particle size was not change significantly and assay was found to be 95 % after 90 days. From the data, it is observed that there was negligible change in the drug content indicating chemical stability. The results of stability data has shown in below table 5:

Table 5: Accelerated stability (25±2°C / 60±5% RH) data of Buserelin acetate microspheres

Test	Time points				
	Initial	**15 days**	**30 days**	**60 days**	**90 days**
Description	White to off white colored lyophilized free flowing powder	White to off white colored lyophilized free flowing powder	White to off white colored lyophilized free flowing powder	White to off white colored lyophilized free flowing powder	White to off white colored lyophilized free flowing powder
Assay (%)	98.25	97.5	96.3	95.2	95.3
Particle size (µm)	81	80	78	76	77

CONCLUSIONS

In the present study, attempts were made to prepare buserelin acetate microspheres for controlled release by double emulsion solvent evaporation technique using PLGA 50-50 polymer. The selection of organic solvent, concentration of polymer, speed of primary homogenization and solidification temperature were found to have played a predominant role in the preparation. The formed microspheres were found to be uniform and spherical in shape. The optimized formulations exhibited 90% in vitro controlled release for one month. From the experimental results it is evident that the controlled release microspheres of buserelin acetate can be successfully formulated for subcutaneous or intramuscular administration in the treatment of patients with hormone-dependent advanced carcinoma of the prostate gland.

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