

Development and Characterization of Leuprolide Acetate Encapsulated PLGA Microspheres for Parenteral Controlled Release Depot Injection

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ABSTRACT

Objectives: Polylactic acid (PLA) and copolymer polylactic-co-glycolic acid (PLGA) are the most versatile drug carriers for long acting release injectable (LAI) formulations for small molecules, peptides and macromolecules such as proteins and nucleic acids. The present research work consists of a PLGA based one-month release microsphere formulation of GnRH agonist leuprolide acetate, using double emulsion ($W_1/O/W_2$) technique. **Materials and Methods:** Microparticles were prepared by double emulsion solvent evaporation technique and critical quality attributes of finished products such as drug loading, entrapment efficiency, particle size distribution (PSD), surface porosity, drug distribution within microparticles were analysed. **Results and Conclusion:** The microspheres have shown the mean particle size of 15.2 μm and suitable particle size distribution pattern ideal for syringe ability. The microparticles were spherical in shape with uniform pore distribution and uniform distribution of API in the polymer matrix. The drug release has been sustained over a month for the microsphere formulation and could be correlated with the testosterone suppression.

Key words: Leuprolide, PLGA Microspheres, Double-emulsion technique, Prostate cancer, Controlled release depot Injection.

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INTRODUCTION

Prostate cancer is one of the primary causes of cancer related mortality among men. The difficulty in early detection and improper screening techniques, make the primary stage unnoticed.^{1,2} The treatment strategies include a combination of surgery, radiation therapy, hormone therapy or chemotherapy.³ However, the exponential rise in prostate cancer cases globally witnessed the evidence of the failure of current therapy which left the formulation scientist to think for alternative therapeutics. In this context PLGA microspheres are gaining increasing attention due to ease in fabrication and ability of releasing the active ingredient from several weeks

to months by modulating formulation aspect. The application of controlled drug delivery carriers is offering many exciting possibilities in the management and successful treatment of prostate cancer due to their much superiority over conventional formulations. Microparticulate drug delivery system is the most reliable and widely accepted delivery platform for parenteral controlled release depot injections. Hence, it has been commercialized in more than a dozen of marketed formulations for different therapeutic segments.⁴

Gonadotropin-releasing hormone (GnRH) agonists have a scientifically and clinically established role in the therapeutic regimen

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of hormone-sensitive cancers such as prostate cancer and breast cancer. When injected, these analogues stimulate the luteinizing hormone (LH) and follicle stimulating hormone (FSH) production, which in turn send message to hypothalamus and anterior pituitary to suppress the sex hormones in both males and females. Thus these agonists when administered at a higher dose for a longer period of time act by negative feedback mechanism to suppress the levels of testosterone and dihydro testosterone, responsible for the growth of prostate cancer cells in males and similarly oestrogen and progesterone, responsible for the growth of endometriosis in females. This down regulation can be maintained for a long period with medication and reversible in nature when the treatment is stopped. The discovery and development of GnRH agonist was a great help for patients suffering from cancers caused by increased levels of hormones, such as prostate cancers.⁵⁻¹⁰ One of such highly effective and widely used GnRH agonist is leuprolide acetate which holds significant market share in the controlled release peptide injectable.¹¹⁻¹³ But it suffers from the disadvantages of short elimination half-life of 3-4 h and frequent administration via subcutaneous or intramuscular route.¹⁴ Lupron Depot® (LD) containing hydrophilic leuprolide acetate in PLGA microspheres was the first, once a month depot injectable launched in the US market to overcome this issue.¹⁵ Although the patent got expired for a long period of time, no generic formulation has been approved in the USA due to complexity in the manufacturing techniques and process optimization. Thus, the present research work aimed to manufacture and characterize once a month release formulation containing leuprolide acetate loaded PLGA microspheres as the platform for parenteral controlled release depot injection. One-month release injectable microspheres of leuprolide injection have been published earlier (1989 and 1991) but *in vitro* drug release study has not been reported in the papers.^{16,17} *In-vivo* drug release has only been estimated at 24 h and onward (1989).¹⁶ The availability of the drug *in vivo* within first 24 h is very important in sustained release product and that is missing in the paper. The objective of this research article is to focus on how raw materials and manufacturing process variables affect the critical quality attributes of the finished formulation such as residual solvent, particle size distribution and *in vitro* release etc. Residual solvent has not been reported earlier publications of Ocada.^{16,17} Subsequently, the results described here could help on the development of a generic version of LD as well as PLGA based new controlled release formulations containing peptides.

MATERIALS AND METHODS

Materials

Leuprolideacetate and PLGA (copolymer ratio 75:25; molecular weight 12 kDa; inherent viscosity 0.17 dL/g) were obtained as gift sample fromExcimed, Hyderabad. Other excipients such as gelatine, polyvinyl alcohol 8-88 (mW- 67,000), D-mannitol, methylene chloride, polysorbate 80, glacial acetic acid, carboxymethylcellulose sodium were procured from Merck Life Science Private Limited, Mumbai. All these raw materials were of pharmaceutical grade.

Preparation of microspheres

Multiple Emulsion ($W_1/O/W_2$) also known as double emulsion solvent evaporation technique was used to prepare the leuprolide acetate loaded microspheres. This manufacturing technology is highly sophisticated, complex and dozens of critical process parameters need to be optimized to get desired product profile.¹⁸ The information regarding the quantities of PLGA, Leuprolide acetate and other raw materials for a single unit is mentioned in the pack insert and quantity for a 100 unit batch is calculated accordingly.¹⁵

Preparation of Oil Phase (O)

PLGA polymer was accurately weighed (3310 mg) in a 20 mL beaker and 5 mL of dichloromethane was added inside the beaker through a Pipette. The beaker was closed immediately to prevent evaporation. It was soaked for 2 hr with occasional sonication to make it homogeneous and clear. This phase is known as oil phase (O).

Polymer concentration is of paramount significance to manipulate emulsion viscosity, particle size and encapsulation efficiency, hardening time, porosity pattern and initial burst release.¹⁹⁻²³

Preparation of External aqueous Phase (W_2)

Distilled water (1.5 L) was taken and 3 g of polyvinyl alcohol (PVA) was added into it. It was mixed well at moderate speed for 20-30 min. It was placed at 15°C for 2 hr (until the temperature reaches in between 18–20°C). This phase was known as outer water phase (W_2). PVA was used in the outer water phase as a colloidal protector to make a stable double emulsion by preventing the aggregation of the semi dry microspheres. The concentration of PVA influences emulsion stabilization, particle size and sphericity index of the finished microspheres.²⁴⁻²⁸

Preparation of internal aqueous phase (W_1)

In a test tube containing 9 mL of water 1.2 g gelatine was heated at 50°C with occasional vortexing for homogenous mixing. It was filtered through a syringe driven filter (0.22 μ PVDF filter). Gelatin solution containing 525.5 mg was taken in a wide mouth test tube and 387.5 mg of leuprolide acetate (equivalent to 375 mg leuprolide base) was added into the test tube and vortexed until it becomes homogenous. This phase was known as internal aqueous phase (W_1). Gelatine plays a critical role by increasing the viscosity of the internal aqueous phase which led to the high entrapment efficiency, thus the grade and bloom strength is carefully selected. The volume of W_1 is very important for the stabilization of primary emulsion.^{29,30}

Preparation of Primary emulsion (W_1/O)

Oil phase (O) was added into the aqueous phase (W_1) and homogenization was done for 1 min through bench top homogenizer at rpm 10,000. This was known as primary emulsion (W_1/O). It was covered immediately with lid to prevent evaporation and kept at 15°C for stabilization for half an hour. The cooling of primary emulsion increased the viscosity of the formulation. This higher viscosity led to the higher encapsulation efficiency.^{6,7} Volume of primary emulsion influences the surface porosity and loss of peptide during secondary emulsification.^{29,30}

Preparation of Double emulsion ($W_1/O/W_2$)

The stabilized water-in-oil emulsion (W_1/O) was checked for temperature to be maintained in the range of 14 \pm 2°C. This was injected into the outer water phase (W_2) very slowly for 2-3 min to form the multiple emulsion or double emulsion ($W_1/O/W_2$). Injection time and post injection stirring time were critically observed during each trial and optimized. Solvent removal was carried out by continuous nitrogen purging and increasing temperature gradually from 20-35°C. Filtration was carried out by 5 μ poly ether sulphone (PES), 127 mm diameter Millipore filter through a Buchner funnel and bench top filtration assembly. On the top of the Buchner funnel 75 μ m sieve (prefilter) was used to remove the coarser microparticles. It was cleaned with enough water (approximately 2-3 L) for complete PVA removal. The microspheres were collected and dried. The yield of the microsphere was found to be 80 %. Mannitol was weighed 528 mg instead of 660 mg considering 80 % yield of microsphere and dissolved it in 100 mL of water. After dispersing the microsphere in mannitol, dispersion was transferred into the SS 316 made round lyophilization tray. After lyophilization, the product was filled into vials at low humidity area under Laminar Air

Flow (LAF) and sealed properly.³¹ Methylene Chloride (DCM), Polyvinyl Alcohol (PVA) and distilled water were used in the manufacturing process and hence not a part of the final formulation.

Pre-formulation studies

Fourier transforms infrared spectroscopy (FTIR)

The pure drug, individual raw materials and a mixture of both of them, were mixed (physical mixture) with IR gradedried potassium bromide in the proportion of 1:100 and to make a transparent pellet compressed at 20,000-25,000 psi in a hydraulic press. Then the sample was scanned in the range of 4000-450 cm^{-1} using FT-IR spectrophotometer (Perkin Elmer-Spectrum 100 is the software used for data collection).³²

DSC analysis

Interactions can be detected in DSC based on presence and absence of endothermic peaks. DSC experiments were performed by a DSC mode 1821 (Mettler-Toledo, Switzerland). All accurately weighed samples of polymer and API at different proportions were placed properly inside sealed pans made up of aluminium. Heating was performed under nitrogen flow at 20 mL/min and scanning at the rate of 10°C per min. DSC thermograms of microspheres, polymers and complexes were recorded.³³

Physicochemical characterization of the drug loaded microspheres

SEM analysis

For surface topography characterization, scanning electron microscopy was used for the finished microparticles (Hitachi, TM3030Plus). The reconstituted microparticles were properly distributed on the surface of carbon tape over the SEM stub. Once the vacuum-drying was accomplished, the sample was coated with a very fine layer of gold through an ion sputting device. Then the samples were vacuum dried and scanning was performed on the microspheres surface and photomicrographs were produced by electron microscope.

High resolution Scanning Electron Microscopy

High resolution scanning electron microscopy (JEOL JSM-7800FLV) was performed and colour images with proper contrast and resolution was obtained. This was immensely helpful for understanding the API distribution in the polymer matrix, PLGA distribution and the intense drug domains inside the microspheres.

Determination of particle size

A small amount of freeze-dried microsphere was redispersed in distilled water and their size was measured

in Master sizer 3000 (Make: Malvern) by using the laser diffraction technique. The samples were dispersed in a medium volume liquid cell. When the laser beam was passed inside the cell, the dispersed particles scattered the light with a pattern. The helium neon laser beam calculates the intensity and angles of scattered light from which particle size distribution was measured in micrometre range.

Determination of Percentage yield and Entrapment efficiency

Microsphere prepared were weighed after lyophilization and percent yield was measured using the following formula.

$$\text{Percentage yield} = \left(\frac{\text{Actual weight}}{\text{Theoretical weight}} \right) \times 100$$

In order to determine the entrapment efficiency, micro-particles equivalent to 10 mg of leuprolide acetate were mixed with 10 mL of 6.8 pH phosphate buffer. This mixture was placed on a mechanical shaker for 24 hr. The resulting solution was removed and filtration was carried out through a polyether sulphone (PES) membrane filter (0.45 µm pore size). One mL of the solution was appropriately diluted using 6.8 pH phosphate buffer and analyzed by HPLC (Waters with Empower 2.0 software).

The following formula is used to calculate the Entrapment efficiency

$$\text{Encapsulation efficiency} = \left(\frac{\text{Estimated drug content}}{\text{Theoretical drug content}} \right) \times 100$$

Drug loading study

About 20 mg of microparticles was dissolved with 2 mL of dimethylsulphoxide (DMSO) and sonicated for 2 min. Then 2 ml of purified water was added and sonicated for another 2 min. Volume was made up to 20 mL with purified water, filtered and loaded on HPLC. For drug loading running time 20 min and column used was inert sustain 4.6 × 100 3.5µ C₁₈.

In vitro drug release study

Phosphate buffer (pH 7) 9 mL and 1mL diluent (earlier prepared) were added in one of the bottle of rotating bottle apparatus. Entire content of one vial containing leuprolide acetate microspheres was added inside the bottle and closed. Different samples in different rotating bottle for different time intervals were placed in triplicate at 0.071 (12 h), 1, 2, 3 and 4 week (properly labelled). After completion of each time point the content of the bottle was centrifuged for 5 min at 10,000 rpm. The

supernatant liquid from the bottle was discarded and 2 mL of dimethyl sulphoxide (DMSO) was added in the same bottle and sonicated for 2 min with the addition of 2 mL water. Then sonicated it for 2 min, volume made up to 20 mL with distilled water. The content was filtered (with 0.45µ PVDF) and analysed on HPLC.

Residual Solvent

Finished formulations were analysed for residual solvent of methylene chloride present by using Agilent 7890 GC with head space sampling option. DMSO was used as diluent for the preparation of sample and standards. Different levels of methylene chloride standard were prepared in DMSO from 1 to 1000 ppm not less than 6 levels. Leuprolide microspheres were dissolved in DMSO at concentration level 5 mg/mL. Nitrogen was used as carrier gas, Nitrogen/air/hydrogen were used as 25/340/30 mL/min and detector temperature was 260°C, sample and standard injection volume 2 mL with split ratio of 25. Temperature gradient for column used as 0-15 min 40°C and each minute increase by 10°C up to 260°C.

RESULTS AND DISCUSSION

Pre-formulation studies

FTIR study

Fourier transform infrared spectroscopy (FTIR) analysis was performed as a preformulation study to evaluate if there was any interaction between the active ingredient and the excipients. The results of the FTIR data showed that the characteristics absorption peak of the drug obtained at 3267.59 of N-H stretching, 1744.79 of C=O stretching and 1458.55 cm⁻¹ of C-N stretching were found to be unaltered in physically mixed sample of active and raw materials. It was also observed that there was no traceable shifting of existing peaks and no additional peaks were also found. From the above result it can be concluded that there was no interaction in between active ingredient and inactive raw materials (Figure 1).

DSC analysis

The DSC experiments is run 0-220°C temperature range and at 10 or 20°C/min. The heat flow against temperatures was plotted and the DSC curves were obtained (Figure 2). The melting range can be found out from the onset, peak and endpoint. The Tg of PLGA was found to be 53.06 and that of the microsphere formulation was 56.88°C. From the results of the DSC data of the final formulation it can be concluded that,

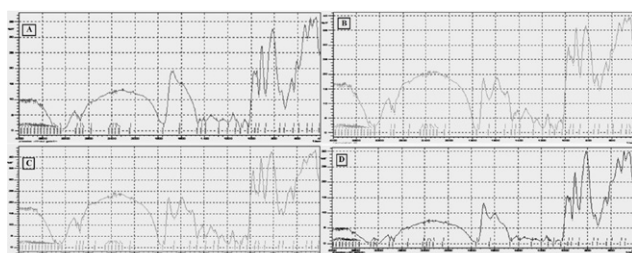


Figure 1: FTIR spectra of A) Leuprolide acetate; B) PLGA (75:25) and Leuprolide acetate mixture; C) Leuprolide acetate and Mannitol; D) Leuprolide acetate and Gelatin.

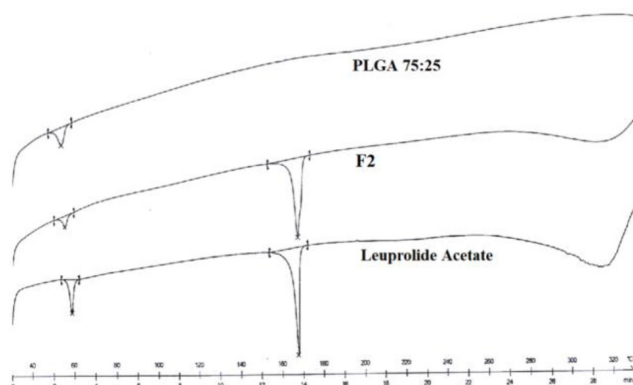


Figure 2: Differential Scanning Calorimetry (DSC).

no chemical interaction exists between the polymers and the drug, thus they are compatible.

Characterization of Microspheres

F2 showed better drug loading (8.44 %) as compared to F1 (7.8). The entrapment efficiency was 99 and 92 % respectively.

SEM analysis

SEM photograph of (Figure 3) during initial microspheres, shows that in F2 the surface porosity is uniform compared to that of F1 this may be due to the higher amount of external phase taken in F2.

Advanced SEM analysis

Drug distribution pattern as per colour coding

Different colour codes such as red and black were used. Red colour code represented polymer and black was designed to represent the API distribution. From the images, according to the given colour codes more or less uniform drug distribution pattern was observed in the experimental sample. However in final (F2) sample the drug distribution was uniform throughout and in other (F1) sample the distribution pattern was slightly more intense towards periphery. This can be explained possibly due to the higher amount of external phase taken during the in water drying resulting in the slower migration of DCM.

Intense Drug Domains

This is a cross section of microsphere with different colour codings instead of microscopy of intact microsphere. In that the colour black represented API, sky blue for polymer, purple colour for surface drug responsible for burst release and yellow for void space respectively. From the Figure 4 it was observed that the distribution pattern of API in polymer was more or less similar in both the batches; however pockets of low drug concentration observed in the F1 batch compared to the F2 final batch.

PLGA Distribution

Similarly the PLGA distribution was observed and found to be quiet similar in both F1 and F2 batches.

Particle size analysis

Figure 5 shows the particle size distribution of freeze-dried formulation F1 and F2. The particle size distribution pattern of freeze dried formulation F1 exhibited as 2.48, 13.37 and 41.96 μm of d10, d50 and d90 respectively

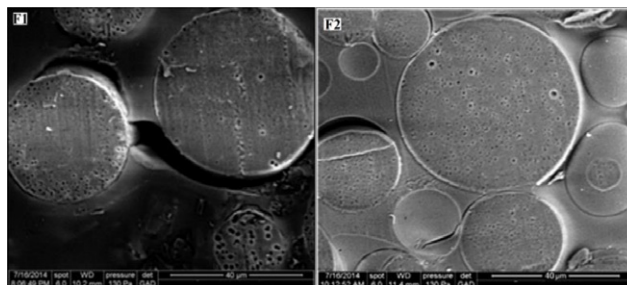


Figure 3: Scanning electron microscopy (SEM) of leuprolide acetate microspheres of F1 and F2.

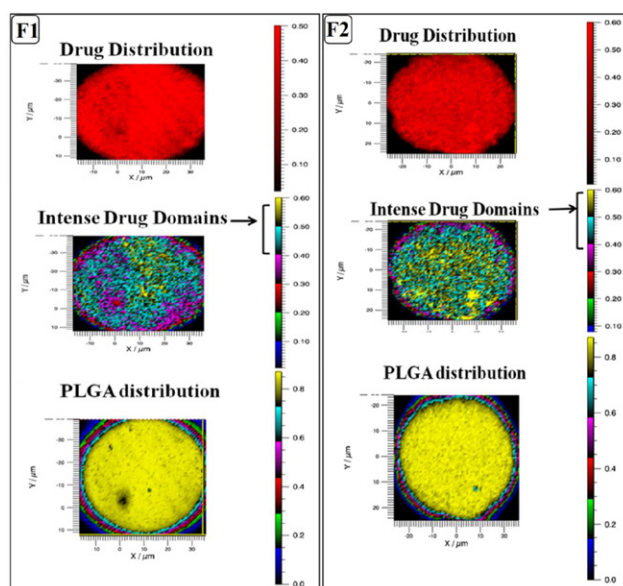


Figure 4: Advanced SEM analysis of leuprolide acetate microspheres F1 and F2.

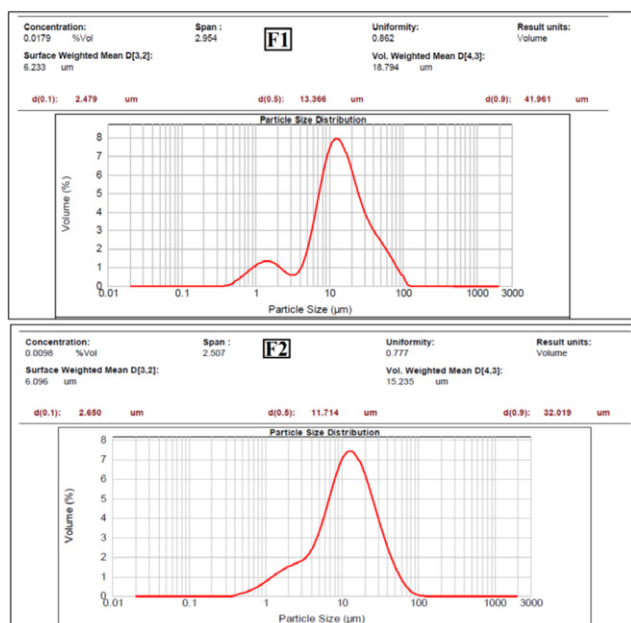


Figure 5: Particle size distribution pattern of freeze-dried formulation F1 and F2.

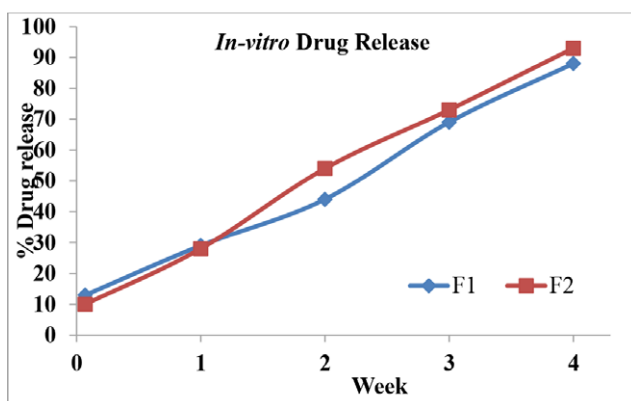


Figure 6: *In vitro* release profile of leuprolide from encapsulated PLGA microspheres of formulation F1 and F2.

with the mean particle size by volume of 18.79 micron. The mean particle size of by volume of F2 was 15.23 micron with the distribution of were 2.65, 11.71 and 32.02 μm of d10, d50 and d90 respectively. Distilled water were used in the manufacturing process and hence not a part of the final formulation.

***In vitro* drug release study**

Real time *in vitro* release study showed 93 and 88 % drug release from the formulation batches F2 and F1 respectively after 4 week time (Figure 6). Drug release of F1 was found to be slower as compared to formulation F2.

Residual solvent analysis

Residual solvents analysed for methylene chloride using Agilent GC system has shown very trace amount of

Table 1: Formulation and characteristics of leuprolide encapsulated PLGA microspheres prepared by double emulsion solvent evaporation technique.

Formulation ^a	F-1	F-2
Leuprolide acetate(mg)	375	375
PLGA (mg)	3310	3310
Gelatin ^b	65	65
Mannitol (mg)	660	660
DCM (mL)	5	5
PVA 8-88 (g)	3	4.5
Purified water (mL)	1000	1500
d value(μm)	d 10	2.48
	d 50	13.37
	d 90	41.96
Drug loading (%)	7.80	8.44
Entrapment Efficiency (%)	92	99
Residual solvent ^c (DCM in ppm)	< 1	< 1

^aFormulation for 100 unit;

^bBloom of Gelatin = 100;

^cICH limit as per guideline = 600 ppm;

DCM = methylene chloride;

PVA = Polyvinyl Alcohol.

almost no detectable (Table 1). Residual solvent was well below the ICH limit as per guideline of 600 ppm.

DISCUSSION

Determination of drug excipient interactions is of paramount significance for any preformulation studies as this provides an indication of compatibility and stability.³⁴ The commonly available methods which are employed to determine the interaction between API and raw materials are FTIR, XRD and DSC analysis.^{35,36} In the present study, we evaluated the drug-excipient interaction by FTIR spectroscopy. FTIR spectra assess the drug-excipient interaction at the level of functional groups by determining their vibrational patterns. Here the spectra of leuprolide acetate, mixture of API and PLGA, mixture of API and mannitol, mixture of API and gelatin have been depicted in the results section. After analysing the result, no interaction observed between API and raw materials. Thus the drug and excipients are concluded to be compatible to each other. Here DSC study was conducted to select suitable polymer for microencapsulation. It was observed that T_g of PLGA (75:25) is 53°C, which is very close to the T_g of the reference listed product earlier reported to be 48.6°C.³⁵ Therefore PLGA 75:25 was selected so that higher temperature (40°C) can be applied during water evaporation. From the SEM it was also obvious that the microparticles were properly formed without any sticking

to the solvent evaporation tank. DSC data of the finished formulation was found to be 55°C which was higher than that of the PLGA, due to the interaction between the drug and the excipient. In some literature T_g was reported to increase with the increase of drug loading.^{37,38}

Size, shape and surface characteristics of the microsphere were analysed by SEM. Scanning electron micrograph (Figure 3) of the selected microspheres, F2 and F1 showed overall smooth and spherical surface morphology. In case of F2, the freeze dried microsphere were found to be associated with more homogeneous pores compared to F1. It may be considered to be attributed to the higher amount of outer water phase (W₂) taken in F2 which lead to easily partitioning DCM in the PVA solution resulting in uniform pore size.

The particle size plays an important role in dispersibility, syringe ability and drug release. Data obtained from particle size analysis (Table 1) indicate that the entire freeze dried formulations were in micro size range, where F2 had the narrowest range of distribution. Most importantly the d₉₀ value in all the trails was less than 50 μ which will impart good syringe ability. The results suggested that the microparticles were well formed and within the desired distribution range in order to impart one month drug release.

When subjected to high resolution SEM study (Figure 4), it was observed that the distribution pattern of API in polymer was homogenous. However the surface API distribution was abundant in F2. Pockets of low drug concentrations observed in PLGA distribution (F1) presumably because of void spaces.

Loading of leuprolide acetate and the entrapment efficiency within microparticles were estimated to be 8.44 and 99 % for F2 and were 7.8 and 92 % for that of F1 respectively. The encapsulation of API inside polymer core is directly related to the physical and chemical properties of API, solubility of the drug in the solvent, concentration of API, ratio of API to polymer and the temperature during primary and secondary emulsions. Similarly burst release (12 h release) was significantly lowered in F2 compared to F1 due to the higher continuous phase to dispersed phase (CP/DP) ratio. In all the experimental trials the rpm of Polytron PT 4000 during primary emulsification was kept at 10,000, rpm of Megatron MT 3000 was kept at 8500 and, Injection time of Primary emulsion into the external aqueous phase through the injector port was kept 4 min and post injection stirring time prior to solvent evaporation was kept as 6 min. Similarly solvent evaporation time was also kept constant for all the cycles. Variation was performed in the composition and results were summarized.

The solubility of methylene chloride in water is very less of around 2 % (v/v). As the quantity of continuous phase was less than the optimal range, methylene chloride diffusion or partitioning rate into the aqueous phase have become slow. So there was great opportunity for the peptide to migrate to the surface of the microsphere. On the contrary if the water phase was large enough to achieve the optimal level, methylene chloride immediately partitioned limiting the migration of active substance to the microsphere surface significantly.

The active ingredient near the surface or its vicinity diffuses out from the microparticles quickly causing initial burst release. Burst release significantly reduced when the continuous phase volume was higher, because the continuous phase containing optimal water concentration led to the quicker precipitation of polymer. Thus the surface of the microspheres F2 became relatively smoother and pores were smaller and uniform compared to F1 as observed in photomicrographs.

CONCLUSION

Leuprolide acetate loaded PLGA microparticles were formulated using adouble emulsification solvent extraction technique. The finished microparticles have shown the mean particle size by volume was 15.2 μm and a suitable PSD range and the pattern of ideal syringe ability with high entrapment efficiency of above 90 %. The residual solvent was well within the limit specified by ICH and the particles were spherical in shape with uniform pore distribution and the distribution of API in the polymer matrix was found optimum. The results suggest that the drug released over a month for the microsphere formulation (F2) could be correlated with the testosterone suppression. Therefore, the formulation (F2) will also be tested for *in vivo* efficacy in an appropriate animal model.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

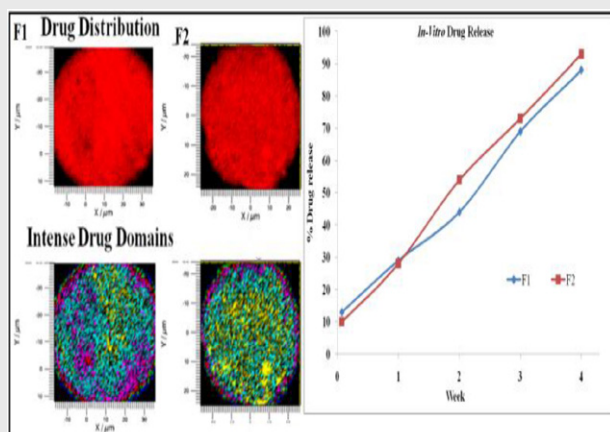
ABBREVIATIONS

PLA: Poly Lactic Acid; **PLGA:** Poly lactic-co-glycolic acid; **LAI:** Long acting release Injectable; **SEM:** Scanning electron microscopy; **PDI:** Poly dispersity Index.

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PICTORIAL ABSTRACT



SUMMARY

Leuprolide acetate loaded PLGA spherical micro-particles were produced formulated using a double emulsification solvent extraction technique. With the finished microparticles have shown high entrapment efficiency of above 90 % and the mean particle size distribution of suitable range and the pattern of ideal syringe ability. Uniform pore distribution and the distribution of API in the polymer matrix was found in the produced microspheres. The drug release uniformly sustained over a month and could be correlated with the testosterone suppression.

About Authors



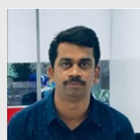
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