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Research Article

DESIGN, DEVELOPMENT AND OPTIMIZATION OF LETROZOLE NANOPARTICLES FOR BREAST CANCER TREATMENT

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ABSTRACT

The objective of the present research work was to design, develop and optimize Letrozole loaded polymeric nanoparticles in order to potentially maximize the therapeutic efficiency, to decrease toxicity as well as to minimize the drug side effects. Pectin and Tween 80 were used as polymer and surfactants respectively. Letrozole polymeric nanoparticles (LTZ-PNPs) were prepared by precipitation method. The drug - polymer compatibility was studied using Fourier Transform Infrared (FTIR) and Differential Scanning Microscopy (DSC). The formulations' optimization was done by design expert. The drug content (DC) and entrapment efficiency (EE) of nanoparticles (NPs) were evaluated by UV-Vis microscopy. The particle size of NPs were investigated using dynamic light scattering technique. The Franz diffusion cell was used for In-Vitro drug release studies. Accelerated stability studies were performed as per ICH guidelines. FTIR and DSC studies revealed compatibility between Letrozole and pectin. The DC was found to be uniform within all formulations (95%) and 96.87 % for the optimized formulation (F9). The E.E was found to be in the range of 68.87 to 95.11%. The NPs particle size was between 194 nm and 333 nm; F9 showed the particle size of 218.5 nm. Drug release from all formulated NPs followed non - fickian transport, thus the release mechanism was diffusion. The optimized formulation did not show a large variation in DC and EE when stored at 40 °C/75% RH as per ICH guidelines.

Keywords: Letrozole, Polymeric Nanoparticles, Pectin, Tween 80.

INTRODUCTION

In 2012 Breast Cancer cases were estimated to be 1.7 million with 521,900 deaths and therefore is the most frequently diagnosed cancer and the worldwide leading cause of cancer death among women[1]. The risks factors and the availability of screening are reflected both by the global breast cancer incidence patterns. North America, Australia/New Zealand, and Northern and Western Europe have the highest cancer incidence rates while the lowest are in Africa and Asia[2].

The major barrier to get cures for malignant breast cancers is the evolution of resistances to therapeutic treatments by tumors[3]. Letrozole (LTZ) is the among the promising aromatase inhibitors in the field of breast cancer management. The drug has attracted researcher attention after showing relatively high effectiveness and safety profile compared to tamoxifen[4]. The USFDA has approved the drug as an adjuvant for hormonally positive local or metastatic breast cancers treatment in postmenopausal women[5]. Like various drugs, in addition to drug resistance, LTZ causes several adverse effects such as arthralgia, hypercholesterolemia, hot flashes, bone fractures and cardiac failure[6,7]. Research of integrating drugs into NPs-based formulations have clearly demonstrated substantial improvements in biodistribution, tissue selectivity, and superior pharmacokinetic profiles of drug[8,9]. Particularly studies show that Pectin is biocompatible and exhibit low toxicity; citrus pectin and modified citrus pectin are useful in the prevention and treatment of metastatic cancer, cancers of prostate, colon and breast[10,11].

The main aim of the present research work was to formulate and optimize Letrozole loaded polymeric nanoparticles in order to potentially maximize the therapeutic efficiency, to decrease toxicity and minimize the side effects of the drug.

MATERIALS AND METHODS

Letrozole was gifted sample from INM technology, Bangalore, India. Pectin was gifted from Yarochem Pvt Ltd, Mumbai, India. PEG-400 was procured from Karnataka fine chem, Bangalore, India. Tween 80 was purchased from Aka Fine Chem, Bangalore, India. Acetone was purchased from SD Fine Chemicals, Mumbai, India. Ethanol and sodium acetate were purchased from Rolex Chemical Industries, Mumbai.

Preformulation studies

- Infrared (IR) absorption spectroscopy

Physical mixture of the drug and the polymer (about 10 mg) were scanned in FTIR spectrophotometer in the range of 4000-400 cm^{-1} and any incompatibility associated with the drug and excipients were studied.

- Differential Scanning Calorimetry[12]

The DSC thermogram was recorded using differential scanning calorimeter (Shimadzu DSC-60 Calorimeter, Tokyo, Japan). Thermogram of Letrozole, pectin and mixture of Letrozole and pectin (10mg) was obtained at a scanning rate of 25°C/min

conducted over a temperature range of 25- 250° C in the environment of liquid nitrogen.

Formulation of Letrozole polymeric nanoparticles

Letrozole nanoparticles were prepared by polymer dispersion method**.** Briefly, 0.6% of pectin was dispersed in purified water containing 1% of tween 80 and sodium acetate using magnetic stirrer at ambient temperature for 5 hours at 150 rpm. Then 50 mg of Letrozole (50mg) was dissolved in a non-aqueous phase acetone: polyethylene glycol 400 in the ratio of 1:1. To this phase, a non aqueous phase was added slowly at the rate of 2.5 mL/ min during homogenization using an Ultra-Turrax homogenizer at 20000 rpm/20 min. 1% of Sodium chloride was added at the end of homogenization.

Characterization and evaluation of Letrozole polymeric nanoparticles

- Particle size analysis[13]

The size of nanoparticles was determined by dynamic light scattering technology of the zetasizer (Nano ZS 3600, Malvern Instruments, Malvern, UK), at 90 degree. The dispersant used was distilled water. Disposable sizing cuvette was used for determination.

- Determination of drug content[14]

About 50 mg of Letrozole equivalent suspension nanoparticles was dissolved in 50 mL of ethanol and sonicated for 45 minutes. Then 10 mL of the above solution was diluted to 100 mL with ethanol. 1 mL of this solution was taken and diluted to 10 mL with acetonitrile and measured at 240 nm under UV spectrophotometer. The percentage drug content in the nanoparticle was calculated from the equation shown below.

$$\%\ drug\ content = \frac{analysed\ weight\ of\ drug\ in\ nanoparticles}{theoritical\ weight\ of\ the\ drug\ loaded\ in\ the\ system} * 100$$

Determination of Entrapment efficiency[14]

About 50 mg of letrozole equivalent suspension nanoparticles was dissolved in 50mL of ethanol and filtered using Whitman filter paper (#1). The above solution was centrifuged at 12600 rpm for 20 minutes. Supernatant liquid was collected, filtered and from the filtrate 1 mL was taken and makes up to 10 mL with ethanol and measured at 240 nm UV spectrophotometer. The percentage drug entrapment for the formulation was calculated by the equation:

$$\%\ Entrapment\ Efficiency = \frac{amount\ of\ drug\ entrapped\ in\ nanoparticles}{total\ amount\ of\ drug\ in\ nanoparticle} * 100$$

- *In-vitro* diffusion studies[15]

The diffusion study of prepared nanoparticles was performed using cellulose dialysis membrane with a molecular weight cut-off of 12,000 Da and HCl buffer (pH 6.8) at 37 °C ± 0.5° C. The Franz diffusion cell of 140 mL capacity was used. The composition equivalent to 5 mg of topotecan hydrochloride was applied on the membrane and diffusion study was performed over a period of 08 h. The samples (3 mL) were withdrawn at 1, 2, 3, 4, 5, 6, 7 and 8, hour and fresh medium were added to maintain sink condition. The collected samples were filtered through 0.22 µl filter and analysed at 240 nm by using UV/VIS Spectrophotometer. The obtained data were subjected to release kinetics studies by applying four kinetics models to the data in order to determine the best fitting equations.

Zero order equation: $Q = Q_0 – k_0t$
First order equation: $ln = lnQ – k_1t$
Higuchi equation: $Q = k\ t1/2$
Korsmeyer peppas equation: $Q/Q = ktn$

K_0 to K_2 were release rate constant, Q/Q_0 was fraction of drug released at time t, K was constant n was diffusion constant that indicates general operating release mechanism.

Optimization

The formulations were designed based on factorial design and then evaluated for responses. Three input factors were studied at two levels (2^3) throughout the preparation process to determine their effect on three responses[16], namely drug release at 1hr, 8 hr and encapsulation efficiency. The input factors being selected are the following: pectin concentration (0.1%, 1%) , concentration of Tween (1%, 4%) and speed (10000RPM, 20000RPM). The response values were subjected to multiple regression analysis in order to find out the relationship between the input factors used and the response values obtained. Therefore, the objective of the optimization process was to quantify the effect of the above factors on the polymeric nanoparticles. The multiple regression analysis was done using design expert 11.1.2.0 software, which is specially meant for this optimization process. In the numerical optimization techniques, the desirability approach was used to generate the optimum settings for the formulation. For the optimized formulation, the drug release at 1hour release was kept at targeted , drug release at 8 hr at minimum and the encapsulation efficiency at maximum value. The optimized formulation was prepared according to predicted model and evaluated for responses. The result of the optimized formulation has been further compared with predicted value.

Stability Studies[17]

The stability testing aim is to provide evidence on how the drug substance or product quality varies under the influence of different environmental factors with time (temperature, humidity and light) and to establish drug substance re-test period or a drug product shelf life and recommended storage conditions.

The selected formulation (F9) was stored at 40 C/75% RH in Newtronic Temperature/Humidity Control Chamber QLH- 2004, and at room temperature and humidity for a period of 3 months. Physical stability was analysed by appearance and the chemical stability was analysed by percentage drug content and entrapment efficiency. The samples were withdrawn every month; the drug content and entrapment efficiency were analysed by spectrophotometry at 240 nm.

Table No 1: Formulation of LTZ nanoparticles using 2^3 Factorial Design

Formulation Code	Concentration of Pectin	Concentration of Tween 80	Speed of homogenization (rpm)
F1	0.1	1	10000
F2	1.0	1	10000
F3	0.1	4	10000
F4	1.0	4	10000
F5	0.1	1	20000
F6	1.0	1	20000
F7	0.1	4	20000
F8	1.0	4	20000

Table 2: Mean diameter, entrapment efficiency (EE), and Drug content (DC)

Sample Series	Mean Diameter (in nm)	EE (%W/W)	DC (%W/W)
F1	224.8	68.87	95.05
F2	307.4	79.21	95.14
F3	194	76.77	95.10
F4	284.4	72.6	95.21
F5	286	83.36	95.26
F6	333	88.53	95.30
F7	212.9	92.97	95.33
F8	255.5	95.1	95.24
F9 (Optimized)	218.5	91.12	96.87

Table no 3: Comparison between the predicted and experimented values of the optimized formulation

Optimized formula(F9)	Dependable variables		
	Drug release		Entrapment Efficiency
	At 1hr	At 8hr	
Predicted	22.697	76.850	92.080
Experimental	21.412	74.200	91.123

Table no 4: Physicochemical properties of optimized formulation (After stability)

Time	Drug content (%)	E.E (%)	Appearance
Initial	96.87	92.12	Yellowish
After 3 months	**95.94**	**92.01**	**No change**

Table 5: Summary of ANOVA results

Source	Sum Square	d.f	Mean Square	F value	Prob> F	Comment
Release 1hr						
Model	365.45	3	121.82	100.33	0.003	Significant
Residual	4.86	4	1.21	-	-	-
Total	370.31	7	-	-	-	-
Release 8h						
Model	1253.84	3	417.95	36.85	0.0023	Significant
Residual	45.37	4	11.34	-	-	-
Total	4	11.34	-	-	-	-
Encap. Efficiency						
Model	488.59	1	488.59	20.39	0.0040	Significant
Residual	143.75	6	23.96	-		-
Total	632.35	7	-	-		-

Table 6: Data showing comparison of kinetics for formulations F1-F8

Formulation Code	Zero order	First order	Higuchi model	Korsmeyer- Peppas equation
F1	y = 4.5804x + 23.785 R² = 0.8934	y = -4.5804x + 76.215 R² = 0.8934	y = 18.222x + 7.1316 R² = 0.9402	y = 0.4505x + 1.3703 R² = 0.9363
F2	y = 4.5172x + 5.7345 R² = 0.9862	y = -0.0265x + 1.9838 R² = 0.9944	y = 17.674x - 9.9591 R² = 0.9934	y = 0.7378x + 0.9465 R² = 0.9966
F3	y = 7.6082x + 17.635 R² = 0.9972	y = -0.0763x + 1.9922 R² = 0.9483	y = 29.32x - 7.8868 R² = 0.9744	y = 0.5508x + 1.3706 R² = 0.9834
F4	y = 6.9307x + 3.9845 R² = 0.9986	y = -0.0481x + 2.0145 R² = 0.9881	y = 26.715x - 19.398 R² = 0.9795	y = 0.8283x + 1.0132 R² = 0.9978
F5	y = 5.7515x + 17.619 R² = 0.9927	y = -0.0457x + 1.9452 R² = 0.9833	y = 22.172x - 1.6885 R² = 0.9705	y = 0.4836x + 1.3383 R² = 0.9651
F6	y = 5.6754x + 5.3948 R² = 0.9925	y = -0.037x + 1.9974 R² = 0.972	y = 21.721x - 13.344 R² = 0.9553	y = 0.6966x + 1.0459 R² = 0.9753
F7	y = 7.3526x + 19.797 R² = 0.9942	y = -0.0758x + 1.981 R² = 0.9415	y = 28.191x - 4.5741 R² = 0.9616	y = 0.5018x + 1.4108 R² = 0.9624
F8	y = 7.7541x + 5.6184 R² = 0.9996	y = -0.0601x + 2.0235 R² = 0.9788	y = 29.916x - 20.461 R² = 0.979	y = 0.7823x + 1.1059 R² = 0.9949

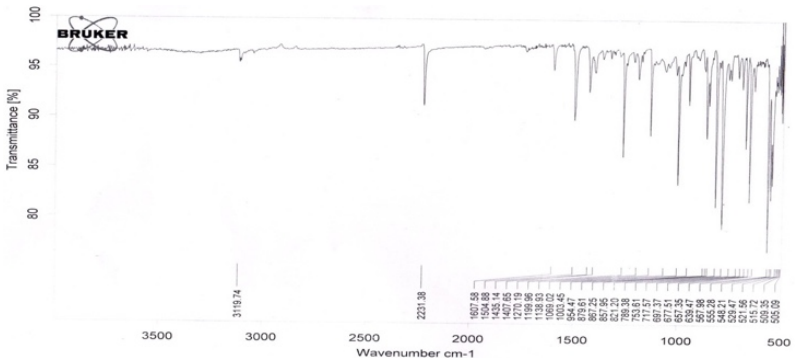

Fig 1. FTIR spectra of Letrozole + Pectin

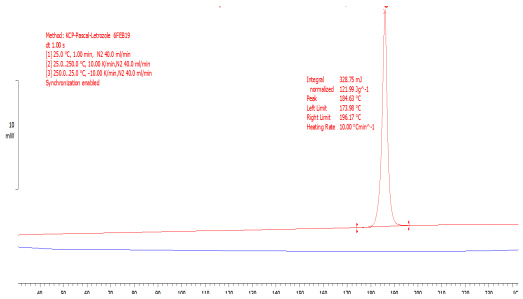

Figure 2. DSC thermogram of Letrozole

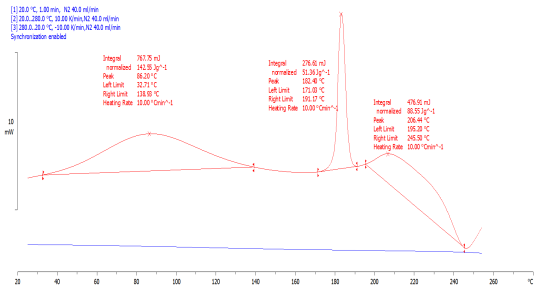

Figure 3. DSC thermogram of Letrozole + Pectin

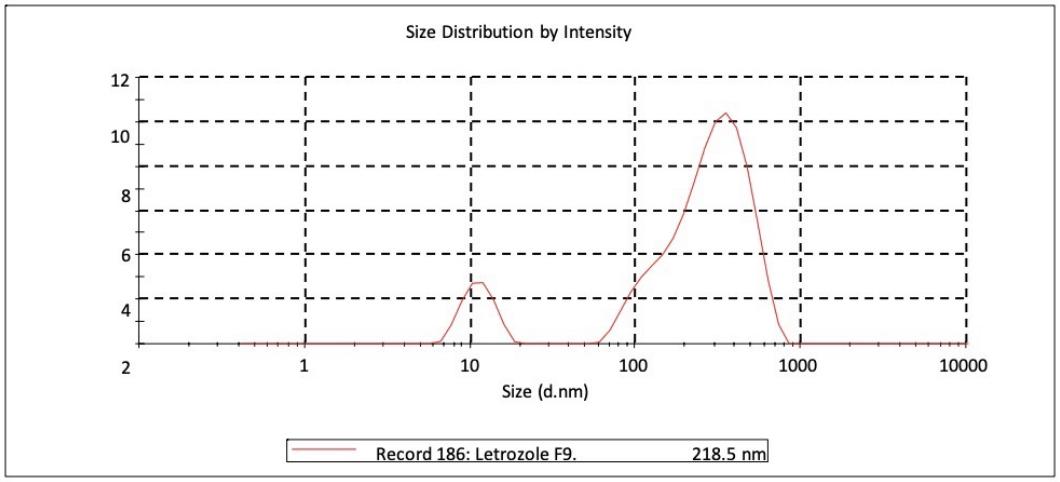

Figure 4. Figure 4. Dynamic light scattering mean particles size of optimized LTZ-PNP

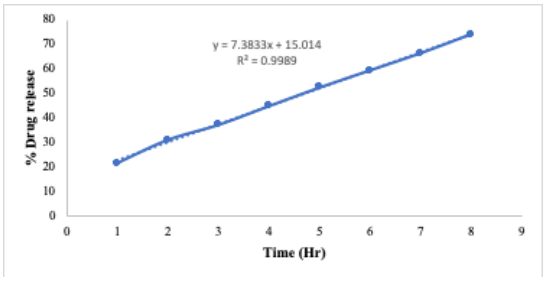

Figure 5. *In-vitro* release of optimized for optimized formulation

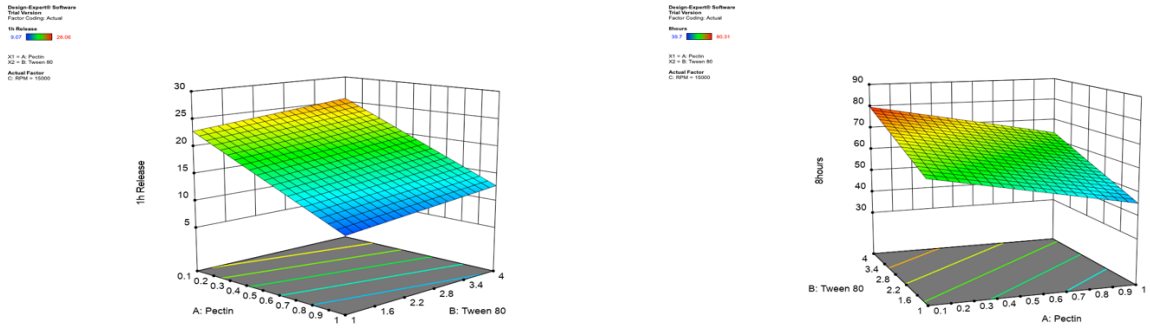

Figure 6: 3-D graph showing effect of input variables on drug release 1 hr and 8hr

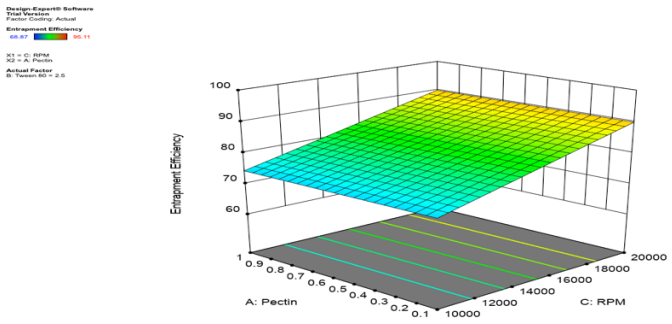

Figure 7: 3-D graph showing effect of RPM on encapsulation efficiency

Statistical analysis

Data were analysed with the help of one-way analysis of variance (ANOVA) test and parameters were significant for the $p< 0.05$.

RESULTS AND DISCUSSION

Preformulation studies

Fourier Transform Infrared (FTIR) studies

The FTIR spectroscopy studies spectrum at wavelength from 4000cm^{-1}- 400cm^{-1} are in **figure 1**. Characteristic peaks in the region of 3314.25, 2231.13, 1606.65 and 867.70 were observed in physical mixture and were identical to that of the pure drug; this confirmed the absence of drug-polymer interactions in the physical mixture. Notably, the IR spectrum display strong absorptions at 1606.98 cm^{-1}, due to the characteristic C=N stretching modes of 1,2,4-trizole groups. There is a strong absorption at 2231.15 cm^{-1}, due to the characteristic C≡N stretching modes of the cyano groups and at 3113.87 cm^{-1} characteristic of =C-H. The absorption at 881.12 cm^{-1} is attributed to the bending of C-H out of the plane.

Differential Scanning Calorimetry (DSC)

Figures 2 and **3** illustrates DSC thermograms for pure Letrozole, pectin and physical mixture of pure drug and polymer in the ratio 1:1. For the pure Letrozole, sharp endothermic peak at 184.40 °C was observed. Bulk Pectin showed melting endotherm at 184.22 °C. Slight shift in peak position for both Letrozole and pectin was observed in physical mixture indicating that there is no interaction between Letrozole and pectin.

Characterization and evaluation of Letrozole polymeric nanoparticles

Particle size

The formulated nanoparticles were analysed for particles size, drug content and entrapment efficiency. The particles diameter of the formulations was found to be between 194 nm and 333 nm (**Table 2**); the optimized prepared formulation showed the particle mean diameter of 218.5 nm (**Figure 4**).

Drug content

The drug content was found to be uniform for all the formulations (**Table 2**). The % of drug content obtained for the optimized formulation was 96.87 %.

Entrapment efficiency

The encapsulation results are shown in **table 2**. The encapsulation efficiencies were found to be between 68.87 % and 95.11 %. Among all formulations, F8 was found to have the highest drug encapsulation efficiency 95.11% and the optimized formulation had 91.12%. The high level of encapsulation has a crucial role in the achievement of desirable prolonged release effect.

***In-vitro* drug release studies and drug release kinetics**

In vitro drug release study was conducted to explore the behaviour of LTZ and the ability of the polymeric NPs to provide controlled drug release. *In vitro* release studies have been used to predict the biodistribution of the drug In-vivo. The In-vitro release of Letrozole from the prepared nanoparticles formulation was studied in 0.1N HCL buffer pH 1.2 and in Phosphate buffer pH 6.8 for 8 hours. Results show that as the concentration of polymer increased, the drug release also decreased proportionally. Formulation F7 showed high percentage of drug release i.e. 80.31 % for a period of 8 hour and F2 showed less % drug release i.e. 39.7% for 8 hours when compared with other formulations. The cumulative drug release for the optimized formulation was about 74.06% at 8hr (**Figure 5)**. In describing the drug dissolution or diffusion profile two characteristics are important for a delivery system: drug release mechanisms and kinetics. It is now well known that qualitative and quantitative changes in the formulation but also in the processing conditions may change the drug release and *in vivo* performances of a pharmaceutical system. The mathematical model methods represent one the rational approaches in the new delivery systems *in vivo* bio-performance assessment and prediction. In this perspective, the drug release data of LTZ-PNP formulations were fitted into different models like zero order, first order, Higuchi equation and Korsmeyer-peppas. The correlation coefficient value (R^2) was used as an indicator to find the best fitting equation for the prepared LTZ-PNPs formulations. The drug release data were fitted into the different models like zero order, first order, Higuchi equation and Korsmeyer-peppas. The results have shown to be above 0.9 R^2 values for zero and first order except formulation one with 0.89 R^2. It can indicate that the drug release is directly proportional to amount of drug remained to be released. The results have also shown to be above 0.9 R^2 values for Higuchi and korsmeyer peppas model. The n values lie between 0.45 and 0.823 which indicates non fickian diffusion and suggested that diffusion is the mechanism of release. The drug release from optimized formulation was found to fit into all the kinetics model. The kinetics release studies results are presented **Table 6.**

Effect of formulation and process variables on drug release

This work aimed to formulate LTZ-PNP with a sustained release in order to optimize its therapeutic outcome but also to reduce side effects. Therefore, the effect of formulation and process variables on the release pattern was studied. The model was found to be significant with an F value of 100.33($p<0.003$), 36.85($p<0.0023$) respectively for 1hr and 8hr release (**Figure 6**).

- **Release at 1 hour:** The amount of Letrozole released from nanoparticles in 1hour ranged from 9.074 % to 28.064%. The 1hour drug release was mainly dependent upon polymer concentration, as the concentration of Pectin increased the drug release decreased.

Design expert equation: 1-hour release = +17.81- 6.43 * A + 1.44 * B+ 1.51 * C (A=Pectin, B=Tween 80, C=RPM).

- **Release at 8 hours:** The amount of Letrozole released from nanoparticles in 8 hr ranged from 39.7% to 80.31%. The concentration of Tween 80 does affect the release at 8hrs. As Tween 80 concentration increases, the release rate increases as well.

Design expert equation: 8-hours release = +62.52 - 7.98 * A + 9.12 * B+ 3.14 * C (A=Pectin, B=Tween 80, C=RPM).

Effect of formulation and process variables on encapsulation efficiency

One of the major challenges in the development of nanocarriers based drug delivery is to engineer a system that can effectively encapsulate drugs at high concentration. The encapsulation offers several advantages such as protection, improvement of intrinsic properties of the encapsulated molecules, targeting properties, and sustained release effect[18]. For this reason, the effect of formulation and process variables on encapsulation efficiency was investigated. The model was found to be significant with an F value of 20.39 ($p < 0.0040$). **Figure 7** represents the effect of RPM on encapsulation efficiency. The 3-D graph shows that the encapsulation efficiency increased with increase RPM. The encapsulation efficiency for formulations was found to be between 68.87 % and 95.11 %.

Design expert equation: Entrapment efficiency = +82.18 + 7.81 * C (C=RPM).

Optimization

The optimized formulation was evaluated and the experimental values were compared to the predicted ones as illustrated in **Table 3.** The summary of ANOVA results is presented in **Table 5**.

Stability studies

The optimized formulation (F9) was subjected to accelerated stability study for a period of 12 weeks. Results are reported in **Table 4**. The formulation was stable for the 3 months period in accelerated stability study. The drug content and entrapment efficiency of the nanoparticle formulation did not vary to a large extent.

CONCLUSION

The present study demonstrated the feasibility of Letrozole Polymeric Nanoparticles preparation using factorial design. The formulation release property and its *in vitro* kinetics model was illustrated. The understanding of the effect of input variables on the selected response has been proven from the 3D graphs. We conclude that the preparation method as well as the optimization technique used for this study are promising for Letrozole polymeric nanoparticles production.

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