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Development And *In-Vivo* Evaluation Of Enzalutamide Loaded Solid Lipid Nanoparticles.

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ABSTRACT

The objective of present study is development and *in vivo* evaluation of the solid lipid nanoparticles(SLNs) of Enzalutamide for the effective drug delivery. Box-Behnken design was used for the study and the results were analysed by using response surface methodology. *In vivo* evaluation of the optimized formulation was performed on rabbits. The influence of formulation variables, Drug to lipid ratio, Concentration of phosphatidylcholine, Concentration of poloxamer 188 was evaluated by regression analysis. The optimized formulation (F3) was found to have the minimum particle size (253 nm) with maximum entrapment efficiency (89.72 %) and drug loading (23.84%). *In vitro* release studies disclosed that maximum cumulative drug release was attained by F3 (99.72 %) in controlled manner. The optimized formulation of Enzalutamide followed zero-order release kinetics with a strong correlation coefficient (R^2= 0.9994). The In vivo bioavailability studies revealed that the results were statistically significant ($p<0.0001$) and indicated that the oral bioavailability of Enzalutamide could be improved after incorporation into SLNs when compared to a pure drug suspension. The obtained results are indicative of SLNs as potential lipid carriers for the effective delivery of Enzalutamide.

Keywords: Enzalutamide, Box-Behnken design, Prostate cancer, SLN, trimyristin, *in vivo* bioavailability studies

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INTRODUCTION

The emerging field of nanotechnology seeks to exploit distinct technological advantages of nanoscience. The booming nanotechnologies are supported by massive state investments in many countries [1]. Solid lipid nanoparticles (SLNs) are an alternative carrier system used to stack the drug for targeting, to improve the bioavailability by the enhancement of drug solubility and protection against pre-systemic metabolism. The circumvention of pre-systemic metabolism is attributed to the nano-metric size range, which makes the drug not to be up taken by the liver from the delivery system for the process of metabolism [2].

Solid lipid nanoparticles are classically spherical with a mean diameter ranging from 1 to 1000 nm. It is a substitutive carrier system to traditional colloidal carriers such as emulsions, liposomes and polymeric micro and nanoparticle [3]. SLN can be defined as colloidal drug delivery system consisting of solid lipid and stabilized with surfactant with particle size ranging from 10 nm to 1000 nm [4].For the preparation of SLN, several factors are to be considered which include effect of lipids and surfactants [5] processing parameters such as homogenization and sonication, formulation parameters such as drug to lipid ratio, lipid type and concentration, surfactant type and concentration [6]. Recently, much emphasis is being laid on the development of multiparticulate dosage forms in comparison to single unit systems because of their potential benefits like increased bioavailability, reduced risk of systemic toxicity, reduced risk of local irritation and predictable gastric emptying [7].

Enzalutamide is an orally active inhibitor of the signalling pathways mediated by the androgen receptor (AR), that was explicitly bring about to overcome castration-resistant prostate cancer (CRPC) harboring AR amplification or over expression. Enzalutamide has demonstrated noteworthy activity in men with metastatic CRPC [8].

In the current work, hot homogenization followed by the ultrasonication method was employed to prepare the Enzalutamide loaded SLNs and the optimization of Enzalutamide loaded SLN was done by Box-Behnken design. The optimized formulation (F3) is subjected to in vitro drug release studies, drug release kinetics, stability studies and pharmacokinetic studies.

MATERIALS AND METHODS

Materials

Tristearin (Dynasan-118), tripalmitin (Dynasan116), and trimyristin (Dynasan-114), were procured from Sigma-Aldrich Chemicals, Hyderabad, India. Enzalutamide was a kind gift sample from Dr. Reddy's labs, India. Poloxamer-188 and Egg Lecithin were gift samples from Aurobindo Labs, India. Solvents and all other chemicals were of analytical grade and were used without further purification.

Design of experiments

According to Box-Behnken design, a total number of 17 experiments conducted. The experiments were conducted as for the design and obtained responses **(Table 1).** The preliminary experiments (one factor at a time approach) were performed to identify the best choice of lipids and surfactants. Based on particle size, polydispersity index (PDI) and Zeta potential (ZP) trimyristin (Dynasan114) was selected as the lipid of choice for further investigations [9]. The Drug-Excipients Compatibility Studies were performed in order to check and interaction between drug and selected excipients.

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Table 1: Box–Behnken experimental design and observed responses

Run	Factor A Drug to lipid ratio	Factor B Conc.of phosphatidylcholine	Factor C Conc.ofpoloxamer 188	Response Y1 Particle size	Response Y2 Entrapment efficiency	Response Y3 Drug loading
1	1:15	75	150	241.82	91.23	20.26
2	1:20	50	150	463.56	93.82	24.42
3	1:15	100	200	400.72	77.46	17.72
4	1:10	100	150	262.62	88.93	23.16
5	1:20	75	100	480.42	91.76	22.36
6	1: 15	50	200	274.62	79.12	19.32
7	1: 15	75	150	242.24	90.86	20.48
8	1:10	75	100	254.32	75.32	15.12
9	1: 15	75	150	241.32	91.36	20.12
10	1:20	100	150	492.56	89.82	20.08
11	1:10	75	200	264.72	79.62	18.98
12	1:20	75	200	491.24	88.36	18.24
13	1: 15	100	100	287.32	87.13	22.26
14	1:10	50	150	240.12	69.36	14.28
15	1: 15	75	150	242.06	89.76	21.12
16	1: 15	75	150	242.96	89.32	20.82
17	1: 15	50	100	357.46	70.72	15.13

On the basis of preliminary studies, the factors like drug to lipid ratio (1:10 - 1:20), concentration of phosphatidylcholine (50-100 mg) and concentration of poloxamer 188 (100-200 mg) were identified as the formulation variables. The response surfaces of the variables within the experimental field were assessed using Stat-Ease Design Expert® software V8.0.1. Subsequently, three additional validation experiments were steered to verify the validity of the statistical experimental strategies.

Preparation of Enzalutamide loaded SLNs

Hot homogenization followed by the ultrasonication method was employed to prepare Enzalutamide loaded SLNs. Enzalutamide (dose 40 mg), lipid and phosphatidylcholine were dissolved in 5 ml of 1:1 mixture of chloroform and methanol. Organic solvents were completely evaporated using a rota evaporator (Heidolph, Schwabach, Germany). Enzalutamide entrenched lipid layer was liquefied by heating to 5°C above melting point of the lipid. Aqueous phase was prepared by dissolving poloxamer 188 in double distilled water and heated to same temperature (based on lipid melting point) of oil phase. Hot aqueous phase was added to the oil phase and homogenization was carried out (at 12000 rpm) using homogenizer (Diax900, Heidolph, Germany) for 4 min. The coarse hot oil in water emulsion so obtained was ultrasonicated using a 12 T probe Sonicator (Vibracell, Sonics, CT, USA) for 20 min. Enzalutamide loaded solid lipid nanoparticles were obtained by allowing hot nanoemulsion to cool to room temperature.

Characterization of Enzalutamide loaded SLNs

Enzalutamide loaded SLNs were characterized by the size, polydispersity index (PDI) and zeta potential (ZP) measured using Zetasizer (Nano ZS90, Malvern, Worcestershire, UK). Characterization of crystallinity carried by powder X-ray diffractometry (Multiflex, M/s. Rigaku, Tokyo, Japan). The morphology of nanoparticles was studied by Scanning Electron Microscope (SEM, Hitachi, Tokyo, Japan).

***In vitro* drug release studies**

In vitro release studies were performed using dialysis bag method. Dialysis membrane (molecular weight cut-off between 12,000 -14,000) was soaked overnight in double distilled water prior to the release studies. Hydrochloric acid (0.1 N) and phosphate buffer pH 6.8 was used as release media. The experimental unit consists of a donor and receptor compartment. Donor compartment consists of a boiling tube which was cut open at one end and tied with dialysis membrane at the other end into which SLN dispersion of 3 ml was taken for release study. Receptor compartment consists of a 250 ml beaker which was filled with 100 ml release medium and the temperature of it was maintained at 37±0.5 °C. At 0.5, 1, 2, 3, 4, 6, 8, 10, 12 and 24 h time points, 3 ml samples each were withdrawn from receiver compartment and replenished with the same volume of release medium. The collected samples were suitably diluted and analyzed by UV-Visible Spectrophotometer at 236 nm.

Drug release kinetics

To elucidate the mode and mechanism of drug release, the data from the *in vitro* release study were fitted into various kinetic models like zero order, first order, Higuchi's and Korsemeyer Peppa's model.

Stability studies

Stability of Enzalutamide nanoparticles suspension in screw-capped glass vials was evaluated over a time period of 90 days. Six samples were divided into two groups and stored at 25 °C and 4 °C. Drug leakage from nanoparticles and mean particle size of the samples were determined at the end of 1, 7, 15, 30, 45, 60 and 90 days.

Pharmacokinetic study design

Animal Preparation

Male Rabbits were (weighing 2-3 kg) selected for this study, all the animals were healthy during the period of the experiment. Animals were maintained at room temperature 25^0C, Relative Humidity 45 % and 12 h alternate light and dark cycle with 100 % fresh air exchange in animal rooms, uninterrupted power and water supply and rabbits were fed with standard diet and water ad libitum. The protocol of animal study was approved by the institutional animal ethics committee with IAEC NO: 439/PO/01/a/CPCSEA.

***In vivo* study design**

The Rabbits were randomly divided into two groups each group contains six animals [10]. The group A was received prepared Enzalutamide optimized solid lipid nanoparticles equivalent 5 mg. Enzalutamide pure drug suspension was administered group B with equivalent dose of animal body weight (5 mg). Blood samples (approximately 0.5ml) were obtained with syringes by marginal ear vein at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 20 and 24 h post dose. During collection, blood sample has been mixed thoroughly with heparin in order to prevent blood clotting. Plasma was separated by centrifugation of the blood at 5000 rpm in cooling centrifuge for 5min to 10 minutes and stored frozen at −20°C until analysis.

HPLC study

Enzalutamide and internal standard (Nilutamide) was separated on the mobile phase consisted of a mixture of ammonium acetate buffer (pH = 4.6, 20 mm) and acetonitrile (60:40, v/v), and was delivered at a flow rate of 1.5 ml/min throughout the 9-min run, an Phenomenex Luna S -C18 column (5 µm, 250 mm X 4.60 mm i.d) with PDA detection at 270 nm. The retention time for nilutamide (internal standard) is 3.9 ± 0.2 min, for Enzalutamide 7.2 ± 0.2 min [11].

Preparation of Plasma Samples for HPLC Analysis

Rabbit plasma (0.5 ml) samples were prepared for chromatography by precipitating proteins with 2.5 ml of ice-cold absolute ethanol for each 0.5 ml of plasma. After centrifugation the ethanol was transferred into

a clean tube. The precipitate was re suspended with 1 ml of Acetonitrile by vortexing for 1 min. After centrifugation (5000 – 6000 rpm for 10 min), the Acetonitrile was added to the ethanol and the organic mixture was taken to near dryness by a steam of nitrogen at room temperature.

Pharmacokinetic studies

The pharmacokinetic parameters, peak plasma concentrations (C_{max}) and time to reach peak concentration (t_{max}) were directly obtained from concentration time data. In this study, AUC_{0-t} refers to the AUC from 0 to 24 h, which was determined by linear trapezoidal rule and $AUC_{0-\alpha}$ refers to the AUC from time at zero hours to infinity. All values are expressed as the mean ±SD. Statistical analysis was performed with Graph Pad InStat software (version 3.00, Graph Pad Software, San Diego, CA, USA) using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison test. Difference with $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Optimization and confirmation experiments

Seventeen experiments were essential for the response surface methodology based on the Box–Behnken design. Data were analyzed using Stat-Ease Design Expert ® software V8.0.1 to obtain analysis of variance (ANOVA), regression coefficients and regression equation (Table 1).

A numerical optimization technique using the desirability approach was employed to prepare enzalutamide nanoparticles with the desired responses. Constraints like minimizing the particle size in addition to maximizing the entrapment efficiency and drug loading were set as goals to locate the optimum settings of independent variables. The optimized levels and predicted values of Y1, Y2 and Y3 are tabulated (Table 2). To verify these values, three batches of nanoparticles were prepared according to the predicted levels of A, B and C. The predicted and observed values tabulated (Table 2). Obtained Y1, Y2 and Y3 values were in a close agreement with the predicted values. This demonstrated the reliability of the optimization procedure in predicting the operating parameters for the preparation of enzalutamide nanoparticles. All the three batches of obtained enzalutamide nanoparticles were subjected to further characterisation.

Table 2: Optimized values obtained by the constraints applies on Y1, Y2 and Y3

Independent variable	Nominal values	Predicted values			Observed values			
		Particle size (Y1)	Entrapment efficiency (Y2)	Drug loading (Y3)	Batch	Particle size (Y1)	Entrapment efficiency (Y2)	Drug loading (Y3)
Drug lipid ratio (A)	1:12.05	243.89	89.198	23.09	F1	248.8	88.24	23.24
					F2	258.3	89.46	22.62
Conc. of phosphatidylcholine(B)	100				F3	253	89.72	23.84
Conc. of poloxomer 188 (C)	115							

All the prepared formulations were analyzed in order to determine their particle size distribution, zeta potential. A particle size, size distribution and zeta potential curve of optimized formulations obtained The mean size of all the formulations was ranging from 248.8 ± 6.3 nm to 258.3 ± 9.8 nm (Table 3). The PDI was ranging from 0.198 to 0.251, indicating the narrow size distribution. The SLN formulations exhibited negative surface charge with the inclusion of enzalutamide which clearly suggested the orientation of enzalutamide in the lipid matrix. The surface charge is a key factor for the stability of colloidal dispersion. In our case, the zeta potential values of SLN formulations were found to be in between -28.7 ± 5.48 mV to -29.3 ± 4.89 mV. ZP is an important factor that affects the stability of colloidal systems. Total entrapment efficiency of the nanoparticles

formulations was determined and found to be ranging from 88.24 ± 0.37 % to 89.72 ± 0.18 %. The precent drug loading of the formulations was found to be in the range from 23.24 ± 0.12 % to 23.84 ± 0.32 %.

Table 3: The mean particle size, PDI, zeta potential, entrapment efficiency and % drug loading of optimized formulations

Batch	MPS ± SD (nm)	PDI	ZP ± SD (mV)	% EE ± SD	% DL ± SD
1	248.8 ± 6.3	0.206	-28.7 ± 5.48	88.24 ± 0.37	23.24 ± 0.12
2	258.3 ± 9.8	0.251	-28.9 ± 7.79	89.46 ± 0.17	22.62 ± 0.42
3	253 ± 4.2	0.198	-29.3 ± 4.89	89.72 ± 0.18	23.84 ± 0.32

***In vitro* Drug release study**

Cumulative release (%) of control (pure drug) Enzalutamide within 24 h was obtained to be 29.56 which indicate a slow release pattern very less drug release for the control (Figure 1).The slow release of enzalutamide can be attributed the limited solubility. The complete and controlled release of the drug from the optimized SLN formulations can be due to the reduced particle size and enhanced effective surface area (Table 4).

Table 4: Dissolution profile of Enzalutamide solid lipid nanoparticles (Optimized batches)

Time (hr)	% CDR			
	Control (Pure drug)	F1	F2	F3
0	0	0	0	0
1	5.72	5.12	5.98	4.88
2	7.16	9.13	10.19	8.96
3	9.36	13.78	13.12	12.67
4	11.12	17.18	16.88	17.06
5	13.34	21.65	21.1	20.68
6	15.12	25.59	25.26	25.42
7	17.78	30.89	30.29	30.66
8	19.04	35.27	34.36	34.83
9	21.13	39.74	39.14	38.72
10	23.34	43.63	42.38	43.04
11	25.12	46.13	45.93	46.02
12	26.23	49.69	50.12	50.26
16	28.13	65.55	66.54	67.12
20	28.72	81.65	82.34	83.08
24	29.56	99.12	99.28	99.72

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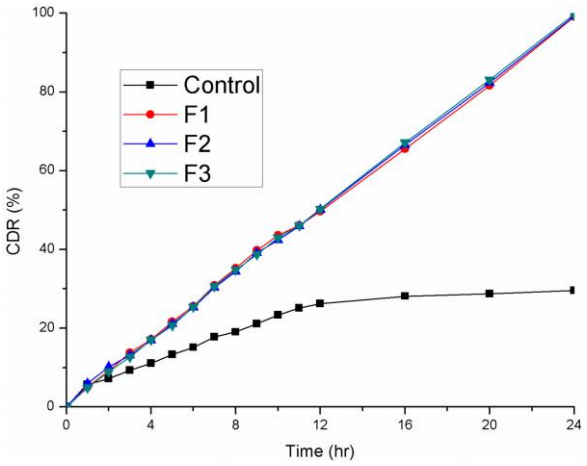

Figure 1: *In vitro* release of Enzalutamide from nanoformulations with pure drug

Drug release kinetics

Release data for optimized formulation (F3) was fitted into various kinetic equations to find out the order and mechanism of drug release. Kinetic analysis of drug release profiles showed that the systems predominantly released Enzalutamide in a zero-order manner with a strong correlation coefficient (R^2= 0.9994).

Stability study

No significant difference ($p < 0.05$) was found in entrapment efficiency and particle size of optimized formulation F3 stored at refrigerated conditions and at room temperature

Pharmacokinetic studies

Mean plasma concentration profiles of prepared Enzalutamide optimized formulation and pure drug are presented in Figure 2. Enzalutamide optimized formulation exhibited higher oral bioavailability in controlled manner when compared with pure drug. All the pharmacokinetics parameters displayed in Table 5. The T_{max} of the Enzalutamide optimized formulation and pure drug was 3.00±0.04 h and 6.00±0.01 h respectively. The C_{max} of test formulation (5.67±1.02ng/ml) was significantly higher when compared with pure drug suspension (3.93±0.73ng/ml). However, the $AUC_{0-\infty}$ values for the optimized (21.12±4.02ng h/ml) was higher when compared with pure drug (14.18±3.12ng h/ml), this suggests that the Enzalutamide contained in the form of SLN was completely absorbed showing more bioavailability when compared with pure drug.

Table 5: Comparison of pharmacokinetic parameters of Enzalutamide Optimized formulation and Enzalutamide Pure drug

Parameters	Enzalutamide optimized formulation	Enzalutamide Pure drug
C_{max} (ng/ml)	5.67±1.02	3.93±0.73
AUC_{0-t} (ng h/ml)	17.12±2.01	12.19±1.51
$AUC_{0-\infty}$ (ng h/ml)	21.12±4.02	14.18±3.12
T_{max} (h)	3.00±0.4	6.00±0.1
$t_{1/2}$ (h)	7.05±1.4	13.12±0.05
K_{el} (h^{-1})	0.0982±0.014	0.052±0.004

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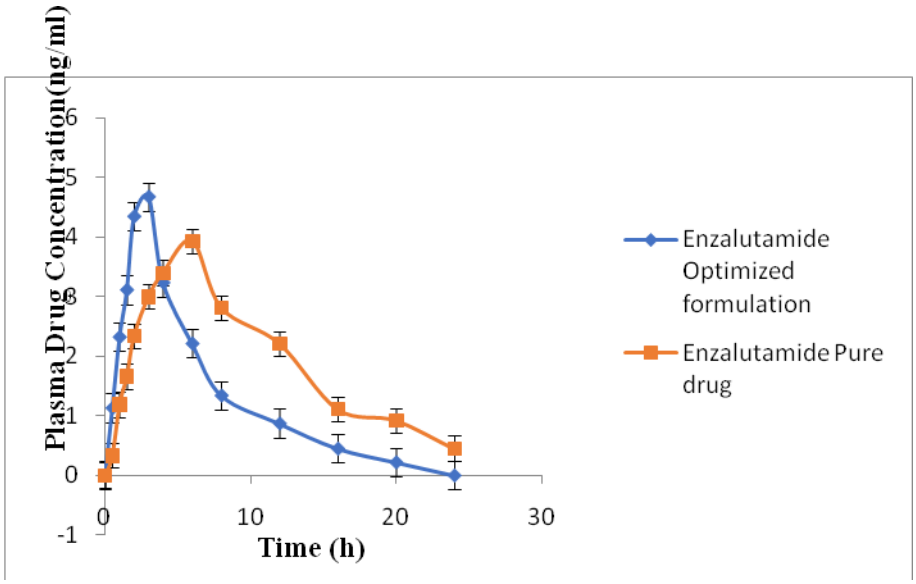

Figure 2: Plasma Concentrations of Enzalutamide optimized formulation (F3) and Enzalutamide Pure drug at different time intervals

CONCLUSION

Enzalutamide is poorly water-soluble drug with poor oral bioavailability due to extensive first pass metabolism. Solid lipid nanoparticles (SLN) of Enzalutamide was produced by hot homogenization followed by an ultrasonication method with the use of a 3-factor, 3-level Box–Behnken design. Trimyristin (Dynasan-114), tripalmitin (Dynasan116) and tristearin (Dynasan-118) were used as lipids and based on the results from the initial studies Trimyristin (Dynasan114) was selected as the lipid for the further studies along with phosphatidylcholine as surfactant and Poloxamer 188 as stabilizer. The optimized formulation (F3) was obtained which have the minimum particle size (253 nm) with maximum entrapment efficiency (89.72 %) and drug loading (23.84 %). The optimised batch(s) were further investigated by XRD, SEM and stability. *In vitro* release studies showed maximum cumulative drug release was obtained for F3 (99.72%) in controlled manner. The optimized formulation Enzalutamide followed zero-order release kinetics with a strong correlation coefficient (R^2= 0.9994). The pharmacokinetic results were statistically significant, indicated that the oral bioavailability of Enzalutamide increased after incorporation into SLNs when compared to a pure drug suspension.

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