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Research Article

Development and Optimization of Enzalutamide Nanosuspension by Design of Experiments for Dissolution Enhancement

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ABSTRACT

Enzalutamide is an anticancer molecule used for prostate cancer. The goal of the study was to develop a nanosuspension of enzalutamide in order to improve its solubility and dissolution properties. High-speed homogenization method was employed to formulate the nanosuspension. Preliminary studies suggested the amount of stabilizer, homogenization time and homogenization speed as critical variables to be taken for the optimization process. Box-Behnken design was employed for the optimization of process and formulation variables. Nanosuspension was evaluated for particle size, PDI, zeta potential, and *in-vitro* drug release at 10 minutes (D10) studies. Regression analysis suggested the influence of independent variables on the responses. The optimized batch obtained from the overlay plot exhibited 198.36 nm of particle size, (-33.27 mV of zeta potential and 80.47% of D10 values). The characterization studies i.e., DSC, and XRD illustrated retention in crystallinity of the drug. The drug and formulation were found to be stable over a 6-month period in accelerated stability testing. Using high speed homogenization method, the particle size of the formulation was reduced to nano-size, which was further responsible for the improvement in dissolution and bioavailability of the drug.

Introduction

Overcoming poor aqueous solubility is one of the most challenging parts of creating novel chemical drug molecules. A large number of new drug candidates have undesirable physicochemical trait. Numerous investigations have been carried out for drugs belonging to BCS class II with low solubility and high permeability.[1-4] Absorption and bioavailability of many drugs are observed to be dissolution rate limited.[5] Thus, dissolution enhancement or improvement techniques are key in ameliorating the absorption rate and bioavailability of poorly soluble drugs.[6,7] Particle size reduction is one of the strategies most frequently employed to enhance drugs biopharmaceutical properties,[8] crystallization,[9] solid dispersion,[10] complexation[11] and other lipid-based drug delivery such as self-emulsifying drug delivery systems.[12] Due to the lack of the need for organic solvents and the ease with which they can be scaled up, wet milling techniques have been successfully employed to develop nanosized drug delivery systems.[13,14] The solubility and dissolving capabilities of drug particles are improved by nanosizing as it increases the surface area of the particles. Pharmaceutical nanosuspensions are aqueous dispersions of insoluble drug particles stabilized by surfactants at the nanoscale.[15,16] However, the nanosuspension liquid form might lead to particle aggregation on long-term storage.[17] Therefore, it seems that solidifying nanosuspensions into nanocrystals is an effective formulation strategy for improving drug stability along with dissolution, solubility, and bioavailability characteristics.[18,19] Solidification of

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nanosuspensions can be achieved by granulation, spray drying, and freeze-drying techniques.[20-22]

Prostate cancer is the second leading cause of cancer-related death among men in both the United States and Europe.[23] Enzalutamide is the first approved androgen receptor (AR) inhibitor recommended for the treatment of prostate cancer. Enzalutamide has a three-stage mechanism of action. At the level of the AR, it is a strong, aggressive androgen binder. The AR is not allowed to go from the cytoplasm to the nucleus as a result. It prevents AR from binding to chromosomal DNA inside the nucleus, which stops the transcription of tumor genes from continuing.[24] Androgen deprivation therapy can lead to resistance in 2 to 3 years due to the accumulation of mutations, including constitutively active mutation, AR overexpression, and changes in AR splicing variants. Enzalutamide was found to be able to decrease cell proliferation and trigger apoptosis in human prostate cancer cell line VCaP in contrast to other antiandrogens.[25] Enzalutamide belongs to BCS class II with poor aqueous solubility and high permeability. Given the observed problem with enzalutamide, only limited solubility and dissolution enhancement work is reported.[26] The reported research approach employed the use of organic solvent/s in the formulation of amorphous preparation of the drug. As a result, there may be a chance that a residual solvent will remain in the final formulation and cause toxicity.

The study aimed to look into the formulation possibilities for enzalutamide nanosuspension employing high-speed homogenization technique without involving organic solvents in the preparation process for dissolution enhancement and better oral bioavailability of the drug. The study was designed to observe the influence of process and formulation variables on mean particle size, zeta potential and drug release. Design of experiment (DoE) - Box Behnken Design was employed to optimize the formulation. Furthermore, the optimized formulation was lyophilized to nanocrystals, which was then characterized for XRD, and DSC studies. *In-vitro* release studies were carried out. Accelerated stability studies for a period of 6 months was conducted on the optimized nanocrystals of enzalutamide.

Materials and Methods

Materials

Enzalutamide was a Aarti Industries Pvt. Ltd., Vapi, India gift sample. Pluronic F68, Pluronic F127 and Soluplus were obtained from BASF Ltd. Mumbai, India. Polyvinyl Pyrolidone-PVP K30 and PVP K90 were purchased from JRS Pharma, Mumbai, India. Tween 80 was purchased from Rajesh Chemicals, Vapi, India. Double distilled water was used throughout the experiment.

Methods

Saturation solubility study

A saturation solubility study of the drug was carried out using an environmental shaker. In 10 mg of drug was added in 25 mL of buffer media, 0.3% w/v CTAB in 0.1N HCl and the suspension was shaken in the shaker at 25°C for 48 hours. The suspension was filtered using 0.2 µ Whatman filter paper and analyzed spectrophotometrically at 238 nm for drug concentration. Analysis was carried out in triplicate and the average result was noted.

Preparation of nanosuspension

Enzalutamide nanosuspension was prepared using a high-speed homogenizer (D-500; High-Speed Homogenizer, Wiggens, Germany). Briefly, 100 mg of drug was added in 50 mL of water in the presence of a hydrophilic stabilizer and 0.1% w/v Tween 80 as a wetting agent. The dispersion was homogenized at specific speed and for specific period of time. Formulated nanosuspension was stored at refrigerated condition until further use.

Screening of process and formulation variables

Various process and formulation variables were assessed for their influence on the performance of the nanosuspension. Process variables included homogenization speed and homogenization time, while formulation variables included type and amount of hydrophilic stabilizer. Initially, the stabilizer was scrutinized by preparing nanosuspension employing various hydrophilic stabilizers such as PVP K30, PVP K 90, Soluplus, Pluronic F68 and Pluronic F127 at a concentration of 1% w/v. The scrutinized stabilizer was screened at various concentrations such as 0.5, 1.0, 1.5 and 2.0% w/v. Homogenization speed and time was kept constant to 10000 rpm and 2 hours, respectively. The homogenization speed was assessed at various levels such as 5000, 7500, 10000, 12500 and 15000 rpm. Lastly, homogenization time was evaluated for 1, 1.5, 2, 2.5, and 3 hours. All the prepared batches were subjected for particle size analysis.

Application of design of experiments

Box-Behnken design is a well-liked technique for improving a system's or process's responsiveness that depends on multiple elements or variables.[27] On the basis of scrutinization trials, homogenization time (X1), amount of stabilizer (X2) and homogenization time (X3) were selected as independent variables. Box-Behnken design was employed for the optimization of enzalutamide nanosuspension using Design Expert software Version 13. Particle size (Y_1), zeta potential (PDI) (Y_2) and drug release at 10 minutes (D10) (Y_3) were selected as dependent variables. Table 1 demonstrates the independent variables with actual values. The responses were evaluated using a statistical model that included interactive and polynomial elements.

$Y=b_0+b_1X_1+b_2X_2+b_3X_3\ b_{11}X_1^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{22}X_2^2 + b_{23}X_2X_3 + b_{33}X_3^2$ (Equation 1)

where Y is response; b_0 is the intercept; b_1, b_2, and b_3 are regression coefficients of the main effects; and b_{12}, b_{23}, b_{13}, and b_{123} are regression coefficients for interaction terms. The coefficients with second-order terms (b_{11}, b_{22}, and b_{33}) indicate the quadratic nature. The main effects of X_1, X_2 and X_3 show the influence of the increase or decrease of either of the factors on the responses. Further, the interaction effects i.e. X_1X_2, X_2X_3 and X_2X_3 show the change in response in relation to the combined effect of independent variables. The optimized formulation was selected from overlay plot employing the desirability function. The optimized batch was lyophilized and then obtained solid nanocrystals were subjected to characterization studies such as XRD, and DSC, followed by 6 month accelerated stability study.

Evaluation Parameters of the Nanosuspension Formulation

Particle size, PDI and zeta potential

Nanosuspensions were evaluated for particle size, PDI and zeta potential employing particle size analyzer (Malvern, UK). The sample was diluted 100 times with water and parameters were analyzed at room temperature.

In-vitro drug release study

Drug release study was performed using 900 mL of 0.3% w/v CTAB in 0.1N HCl as dissolution media at a speed of 50 rpm, temperature 37°C in USP II (Paddle) dissolution apparatus. Sample (5 mL) was collected at specific time-period viz. 5, 10, 15, 30 and 45 minutes fresh media (5 mL) was added to maintain the sink condition. The sample was then filtered, diluted and analyzed spectrophotometrically at 238 nm for drug concentration. The study was carried out in triplicate and average result was noted.

Differential scanning calorimetry

DSC analysis of enzalutamide and lyophilized nanocrystal of drug was carried out using Differential scanning calorimeter (Shimadzu- Model 60, India). The sample of about 5 mg was sealed in the aluminum pan and heated at a rate of 5°C/min, under a nitrogen flow of 25 mL/min from 0 to 300°C.

X-Ray Diffraction studies study

X-ray diffraction study was carried out to study the influence of nanosizing on crystallinity of drug. Pure drug and lyophilized nanocrystal were analyzed using X-ray diffractometer (Shimadzu-Model 6000, Japan). The sample (drug/lyophilized nanocrystals) was placed and scanned from 0 to 50; at 30 mA and 40 kV in a glass sample holder.

Table 1: Factors and levels in the BB design

Independent variable	*Level of Variable*		
	Low (-1)	*Medium (0)*	*High (+1)*
Homogenization time (h) (X_1)	1.5	2	2.5
Amount of stabilizer (%w/v) (X_2)	0.5	1.0	1.5
Homogenization speed (rpm) (X_3)	7500	10000	12500

- *Accelerated stability study*

Lyophilized nanocrystals were subjected for short-term stability studies at accelerated conditions of 40 ± 2°C/75 ± 5% RH for 6 months. The sample was stored in glass bottle and kept in a stability chamber (Remi Instruments, India). Samples were evaluated at intervals of 3 months for particle size, PDI, zeta potential and drug release analysis. Results were compared with the initial data using a student t-test.

Results and Discussion

Saturation Solubility Study

An equilibrium solubility study of enzalutamide was carried out in the dissolution media i.e. 0.3% w/v CTAB in 0.1N HCL buffer. The solubility of drug was observed to be 0.008 ± 0.001 mg/mL.

Scrutinization of Independent Variables

Various nanosuspension batches were prepared to select critical process and product variables for further formulation optimization. Firstly, the hydrophilic stabilizer was scrutinized from the pool of selected stabilizers. Particle size data of the prepared nanosuspension as shown in Table 2 suggested Pluronic F68 as the best hydrophilic stabilizer for the nanosuspension preparation. Further investigation was carried out to check the influence of the amount of hydrophilic stabilizer on the particle size of nanosuspension. Results from Table 2 demonstrated an increase in particle size with an increase in the amount of stabilizer in nanosuspension. Later, impact of different homogenization speed was evaluated on the performance of nanosuspension in terms of particle size. From the results (Table 2), it was observed that as speed was increased, particle size was found to be decreased. However, there was no remarkable difference among the batches S4 and S5. Then, homogenization time's influence was investigated on nanosuspension's particle size. Results (Table 2) exhibited a decrease in particle size with an increase in homogenization time. However, there was no remarkable difference in the particle size after 2.5 hours.

Optimization of Nanosuspension by DoE

The Box Behnken design with three factors and three levels was used to generate parameters for subsequent trial runs based on the estimated optimized set of attributes. Responses of design formulation sets viz. particle size, PDI, zeta potential and D10 are tabulated in Table 3. Outcomes of design batches showed diversity

Table 2: Screening of process and formulation variables

Screening parameter	*Screening levels*	*Particle size (nm)*	*PDI*	*Batch code*
Type of stabilizer	PVPK30	417.25	0.246	P1
	PVP K90	722.46	0.431	P2
	Soluplus	533.81	0.293	P3
	Pluronic F68	231.35	0.254	P4
	Pluronic F127	294.59	0.313	P5
Amount of stabilizer (%w/v)	0.5	228.77	0.152	C1
	1.0	231.35	0.254	C2
	1.5	244.49	0.272	C3
	2.0	268.32	0.418	C4
Homogenization speed (rpm)	5000	265.34	0.524	S1
	7500	242.57	0.373	S2
	10000	231.35	0.254	S3
	12500	225.96	0.278	S4
	15000	224.49	0.259	S5
Homogenization time (h)	1.0	257.35	0.352	M1
	1.5	248.98	0.273	M2
	2.0	231.35	0.254	M3
	2.5	219.65	0.228	M4
	3.0	218.09	0.265	M5

in response values. The data clearly proposed strong effect of designated independent variables on selected dependent variables. A multivariate regression analysis was executed to examine the observations. Response surface plots, including contour plots (Fig. 1a) and 3d surface plots (Fig. 1b) were produced to show interaction effects among variables on the response. The particle size of design batches was found in the 178.85 to 246.78 nm range. The result of regression analysis for particle size showed a negative value for regression coefficients b_1 and b_3, suggesting a decrease in particle size with an increase in homogenization time and speed, whereas a positive sign for regression coefficient b_2 suggested an increase in particle size with an increase in the amount of stabilizer. Further, it was observed that all the model parameters were found to be significant with p value less than 0.05 except for X_1X_3. The narrow size distribution of zotepine-loaded nanosuspension was observed with values ranging from 0.214 to 0.384, exhibiting the absence of the Ostwald ripening phenomenon.[28]

Zeta potential of design batches was found in a range of -43.7 to -16.3 mV. The result of regression analysis for zeta potential showed a positive value for regression coefficient b_2, suggesting an increase in zeta potential with the amount of stabilizer, whereas a negative sign for regression coefficients b_1 and b_3 suggested a decrease in zeta potential with an increase in homogenization speed and time. Further, it was observed that all the model parameters were found to be significant with *p-value* less than 0.05 except for X_1X_3.

All batches of experimental design confirmed notable dissolution enhancement as compared to pure drug dispersion (Fig. 2a). This could be attributed to the nano-size of drug particles in nanosuspension along with a hydrophilic stabilizer assisting in the betterment of drug dissolution.[29] Observed values of D10 in design batches varied from 69.5 to 84.4%. The result of regression analysis for D10 showed positive value for all regression coefficients, i.e., b1, b2 and b3, suggesting an increase in D10 with increases X_1, X_2, and X_3. Further, it was observed that all model parameters were found to be significant with *p-values* less than 0.05.

The results of the multiple regression analysis, as tabulated in Table 4 revealed that factors influenced dependent variables statistically significantly ($p < 0.005$). Two-way ANOVA was used to determine the quantitative role of different amounts of each variable (Table 5). The dependent variable's correlation coefficient (R^2 = 0.9965 (Y_1), 0.9996 (Y_2) and 0.9894 (Y_3)) suggests a satisfactory fit of the mathematical model. The statistical analysis findings demonstrate that the independent variable considerably affects the dependent variable with $p < 0.01$. Hence, reduce model was not generated.

It was empirical that each model fitted well with R^2 value > 0.9. For the response surface design, predicted vs actual plots (Fig. 1c) for all response variables confirmed that investigational results were in harmonization with predicted values. Cook's distance offers vital evidence concerning the extent of regression effect changes if the case is removed. Thus, large values should be recognized,

Table 3: Experimental runs and measured responses of BB Design

Batches	*X_1 (h)*	*X_2 (%w/w)*	*X_3 (rpm)*	*Particle size (nm) Y_1*	*Zeta Potential (mV) Y_2*	*Drug release at 10 min (D10) Y_3*
EN1	1.5	0.5	10000	226.47	-18.8	75.8
EN2	2.5	0.5	10000	178.85	-15.8	76.9
EN3	1.5	1.5	10000	234.33	-41.9	76.5
EN4	2.5	1.5	10000	226.18	-42.3	84.4
EN5	1.5	1	7500	238.35	-33.7	73.2
EN6	2.5	1	7500	208.77	-33.7	81.5
EN7	1.5	1	12500	222.36	-33.1	79.8
EN8	2.5	1	12500	189.63	-31.5	81.2
EN9	2	0.5	7500	234.88	-16.3	72.8
EN10	2	1.5	7500	239.45	-43.7	69.5
EN11	2	0.5	12500	201.15	-16.6	70.9
EN12	2	1.5	12500	246.78	-39.2	82.5
EN13	2	1	10000	231.35	-31.4	72.4

Table 4: Multiple regression analysis

Term	*Coefficient for Y_1*	*p-value*	*Coefficient for Y_2*	*p-value*	*Coefficient for Y_3*	*p-value*
Intercept	291.55	0.0001	-30.1	0.0004	56.725	0.0001
X_1	-14.76	0.0004	-8.0	0.0122	2.34	0.0069
X_2	13.17	0.0006	12.45	0.0001	2.06	0.0098
X_3	-7.69	0.0030	-0.85	0.0103	2.18	0.0084
X_1X_2	9.87	0.0040	0.85	0.0265	1.70	0.0414
X_1X_3	-0.7875	0.5659	0.15	0.5232	-1.72	0.0400
X_2X_3	10.27	0.0036	-1.20	0.0104	3.72	0.0049
X_1^2	-15.34	0.0025	1.15	0.0250	5.50	0.0035
X_2^2	0.4475	0.8002	-2.85	0.0019	0.50	0.5008
X_3^2	-1.23	0.5020	0.40	0.2423	1.02	0.2156
R^2 value	0.9965		0.9996		0.9894	

obtained by recording errors and should be amended. In this study, no big values were found suggesting the data without any errors (Fig. 1d).

Checkpoint Batch Analysis

An optimized batch was prepared employing the obtained values of independent variables from the desirability function plot and overlay plot (Fig. 1e). Experimental results of the optimized batch for particle size (198.36 nm), zeta potential (-33.27mV) and D10 (80.47 %) were found to be in concordance with theoretical values with lower %relative error. PDI value of the optimized batch was found to be 0.261.

Formulation and Evaluation of Enzalutamide-loaded Nanocrystals

Preparation of enzalutamide nanocrystals

Enzalutamide nanocrystals were prepared, subjecting the lyophilization process to the optimized nanosuspension consisting of enzalutamide. The current study exploited three different cryoprotectants (sucrose, mannitol and lactose) for lyophilization at 0.5% w/v concentration.

Table 5: ANOVA

Source of variation	*Degree of Freedom*	*Sum of Squares*	*Mean Square*	*F Value*	*p-value*
Particle size (Y_1)					
Regression	9	5126.04	569.56	95.0	0.0016
Residual	3	17.99	6.0		
Total	12	5144.03			
Zeta potential (Y_2)					
Regression	9	1296.52	144.06	831.10	0.0001
Residual	3	0.52	0.1733		
Total	12	1297.04			
D10 (Y_3)					
Regression	9	275.20	30.58	31.18	0.0083
Residual	32.94		0.9808		
Total	12	278.14			

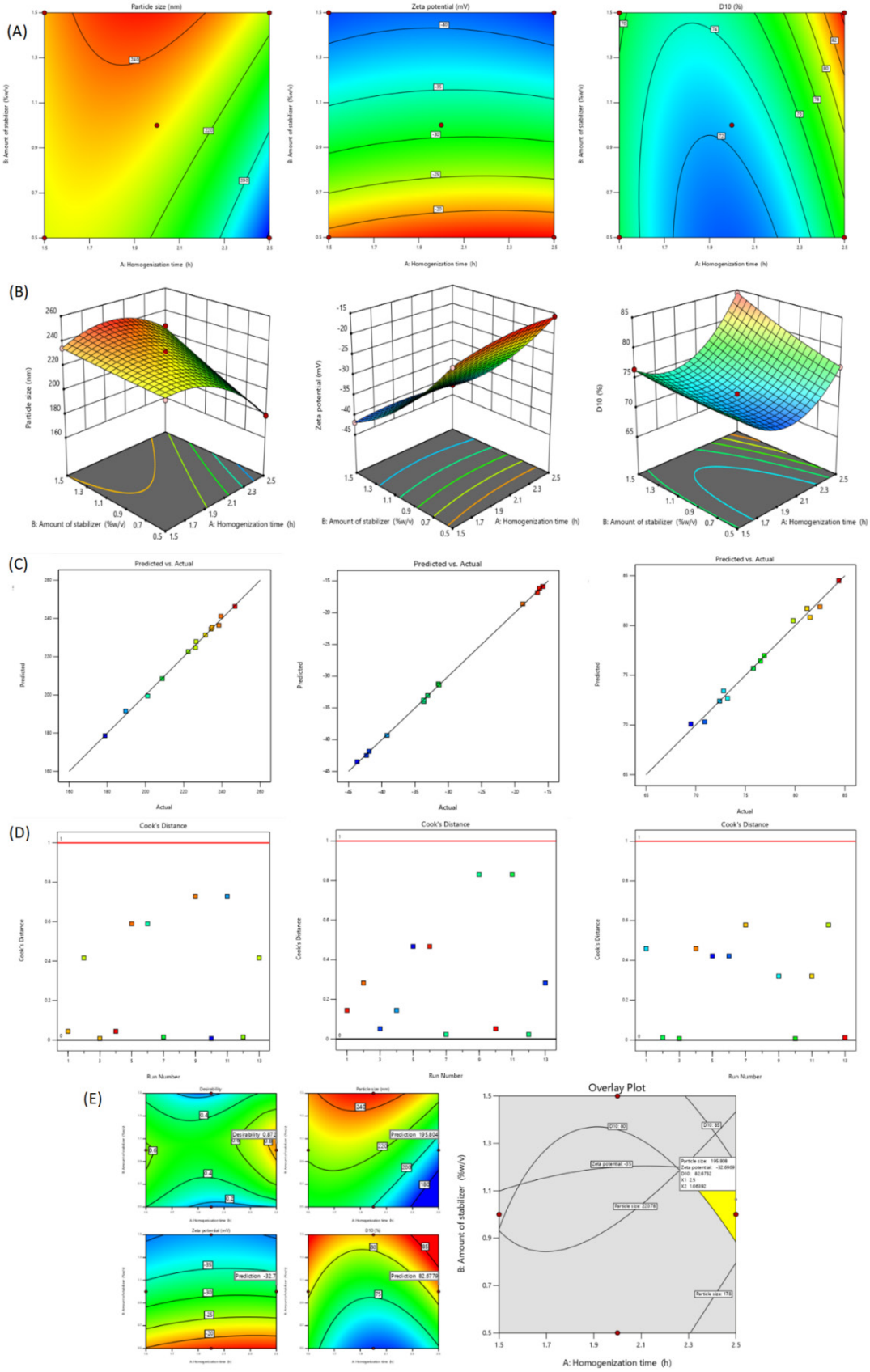

Fig. 1: Graphs showing Response Surface plot: a) 3D Surface plots; b) Contour plots; c) Predicted vs actual; d) Cook's distance and e) Desirability function and overlay plot

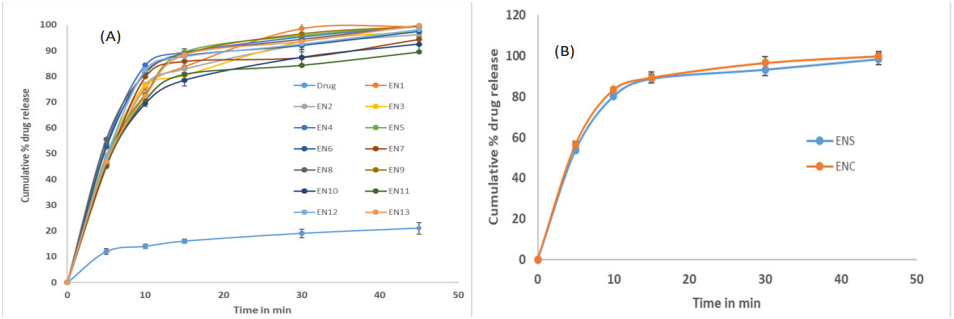

Fig. 2: Dissolution profile of a) design batches and pure drug, b) optimized nanosuspension and lyophilized nanocrystal

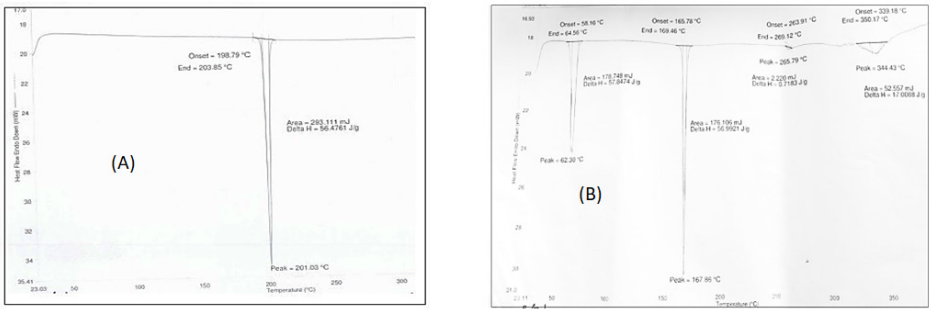

Fig. 3: DSC thermograms (a) Pure drug and (b) Lyophilized nanocrystal

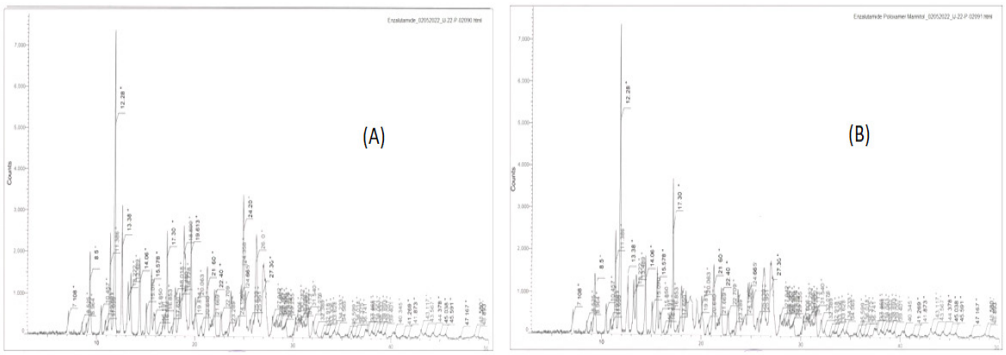

Fig. 4: XRD graphs (a) pure drug and (b) lyophilized nanocrystals

Table 6: Accelerated stability studies

Time (month)	*Particle size (nm)*	*PDI*	*Zeta Potential (mV)*	*Drug release at 60 min (%)*
0	198.36	0.261	-33.27	80.47 ± 2.06
3	198.62	0.310	-34.15	81.27 ± 1.95
6	198.87	0.278	-33.76	80.31 ± 1.88

Ease of dispersibility was selected for the assessment of cryoprotectants. Scores were fixed from 0 to 10 to the assessed criteria, a product with greater score proposed better characteristics. Mannitol was selected out of all the investigated cryoprotectants. Further, mannitol was investigated for influence of concentration. Various concentrations viz. 0.5, 1 and 1.5% w/v were evaluated. The data suggested high average score of 1 % w/v mannitol for all parameters (Data not shown). Besides, the dispersibility of the optimized enzalutamide nanosuspension was observed to be 35 seconds, suggesting its ease of dispersibility. Drug release of an optimized batch of nanocrystals was compared with that of an optimized batch of nanosuspension (Fig. 2b) using similarity (f_2) and dissimilarity factor (f_1). Following equations were employed to calculate f_1 and f_2.

$$f_1 = \{\sum (R_t - T_t)] / \sum R_t \}^* 100 \quad \text{-Equation 2}$$

$$f_2 = 50 * \log \{[1 + (1/n) \sum (R_t - T_t)^2]^{-0.5} * 100\} \quad \text{-Equation 3}$$

where, $\sum R_t$ is the summation of drug release of pure drug from all time points and $\sum T_t$ is the release of nano-micelle-loaded drug from all time points, n is the number of time points. Drug release studies of optimized nanocrystals showed similarity with f_1 value lower than 15 and f_2 value higher than 50.[30]

Characterization of Enzalutamide Nanocrystals

Differential scanning calorimetry

DSC analysis of pure drug and lyophilized nanocrystals were carried out to characterize the thermal transitions of drug. Fig. 3a shows the melting point of pure drug at 201.03°C. DSC thermogram (Fig. 3b) of lyophilized nanocrystal depicted drug peak at 167.86°C. The additional peak was observed at 62.3 and 344.43°C of Pluronic F68 and mannitol, respectively. Thus, DSC demonstrated retention of the crystalline property of drug after milling and lyophilization.

X-ray diffraction

Fig. 4 depicts X-ray diffraction patterns of pure drug and lyophilized nanocrystals of enzalutamide. The figures depicted variations in the crystal structure of the drug. The X-ray patterns of the pure drug exhibited the existence of number of distinct peaks at 12.28°, 13.38°, 17.30°, 19.61° and 24.20°, suggesting the crystalline nature of drug.[31] Nanocrystals showed few peaks with lower intensity, suggesting a decrease in the crystalline nature of the drug, leading to a faster dissolution rate.[32]

Accelerated stability studies

The formulation revealed no significant differences in particle size, PDI, zeta potential, or *in-vitro* drug release results after completing the 6-month accelerated stability trial. There was no substantial change in nanosuspension stability, as observed in Table 6.

Conclusion

Enzalutamide nanosuspension was prepared using a high-speed homogenization method. Various process and formulation variables were screened for further optimization process. Box Behnken design was employed for the optimization of the formulation. The optimized nanosuspension had particle size (198.36 nm), zeta potential (-33.27 mV) and D10 (80.47 %). It was observed that nanosized enzalutamide dissolves at faster rate than its coarser form. Characterization studies exhibited retention of drug crystallinity. Accelerated stability studies were concluded and formulation was found stable for a period of 6 months.

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