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Implementation of Statistical Optimization of Orlistat Loaded Eudragit Nanoparticles

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ABSTRACT

The aim of the present research was implementation of statistical optimization of Orlistat loaded Eudragit nanoparticles. Orlistat loaded Eudragit nanoparticles were prepared by Nanoprecipitation method using 2^3 factorial design. The concentration of Eudragit RS 100 (A), concentration of Poloxamer 188 (B) and concentration of orlistat (C) were chosen as independent variables (Factors) while drug entrapment efficiency and percentage drug release at 48th hour (Responses), were taken as dependent variables. A statistical analysis was performed using Design expert software 11 with respect to effects of factors on responses, regression analysis, ANOVA and model graphs like 3-D plots. From the results of FTIR study, it was confirmed that the principal peaks of Orlistat were retained in the physical mixture denoting compatibility and absence of chemical interactions. Melting point of Orlistat was found to be 45ºC. Statistical analysis showed visual representation of relationship between the experimental responses and the set of independent variables. Regression model equations were validated by a numerical and graphical optimization method. All independent variables were found to significantly influence the entrapment efficiency and percentage of drug release. Additional experimental runs were performed in triplicate and the experimental results were found to be close to the predicted values. Further, the optimized formulation was characterized for particle size, zeta potential, SEM, *in-vitro* drug release and drug release kinetics. Particle size and zeta potential were found to be 493 nm and - 0.1 mV respectively. Drug release was found to be 68.03% at 48 hours and followed 1st order (R^2 = .09615). Thus, using systematic factorial design approach, effect of multiple factors on the responses can be studied with less experimental runs.

INTRODUCTION

Hyperlipidemia

Hyperlipidemia is a broad term which is also known as hyperlipoproteinemia. It is a common disorder in growing and developed countries, and it is the main cause for coronary heart diseases. This results to abnormalities in the lipid metabolism or plasma lipid transport leads to dysfunctioning in the synthesis and degradation of plasma lipids causes proteins. The abnormal changes in the lipid profiles are termed as dyslipidemia by changing the old word termed as hyperlipidemia. Hyperlipidaemia or dyslipidemia means increased fat levels in blood, in these fats includes triglycerides and cholesterols. The cholesterols and triglycerides are essential for our body functioning but increased levels of these two may causes various heart or coronary disorders (Zhang HW, Zhang, 2015).

Definition

Hyperlipidemia is defined as conditions in which the concentration of cholesterol or triglycerides carrying lipoproteins in plasma exceeds a normal limit. The results from abnormalities in lipid metabolism or plasma lipid transport or a disorder in the synthesis and degradation of lipoproteins.

- **Cholesterol**

It is a major component of cell membrane of all tissues and is a precursor of steroidal hormones and bile acids.

- **Triglycerides**

It is found abundantly a lipocytes. These are major components of storage of fats in plants and animal cells. Excess calories, alcohol, sugar in the body gets converted into triglycerides and stored in fat cells throughout the body.

Chemically triglycerides are esters of fatty acids and glycerol.

Normal range - 200 mg/dl

Abnormal range- 500 mg/dl or above leads to development of cardiovascular diseases.

- **Lipoproteins**

Large globular particles that contain an oily cove and non-polymer lipid (cholesteryl esters of triglycerides) surrounded by a polar coat of free phospholipids free cholesterol and apoproteins.

Lipoproteins are of six types:

Chylomicrons: Larger particles and its concentration directly correlated to dietary triglyceride contents.

VLDL: Very low-density lipoproteins are secreted from the liver and carries cholesterol from body. VLDL are formed from of cholesterol.

IDL: VLDL after degradation by lipase enzyme in the capillaries of adipose tissue and muscle give rise to intermediate density lipoprotein.

LDL: Low density lipoproteins are synthesized partly in intestinal chyle and partly after lipolysis of VLDL. It is directly correlated coronary heart diseases.

- **Normal levels for a lipid profile**

Table No. 1: Normal levels of lipids

Type of Lipids	Desirable value (mg/dl)	High risk (mg/dl)
Cholesterol	200	240
Triglycerides	140	200-500
HDL cholesterol	60	Less than 40
LDL cholesterol	60-130	160-190
Cholesterol / HDL ratio	4.0	6.0

Causes

➢ Elevated low-density lipoproteins cholesterol (LDL-C) levels and decreased high-density lipoprotein cholesterol (HDL-C) levels.

➢ Age factor (Males above 45 years, female above 35 years)

- Diabetes mellitus
- Diet rich in saturated fats and cholesterol
- Hypertension
- Smoking and alcohol abuse
- Physical inactivity
- Obesity and overweight

HDL: High-density lipoprotein is referred as good cholesterol and is synthesized in the liver.

Lipoprotein: It is cholesterol rich plasma lipoprotein and density correlated to atherosclerosis high Lp (a) leads to heart disorders.

- Overactive adrenal gland
- Liver and kidney problems
- Drug interactions and usage

Complications of Hyperlipidemia

- **Atherosclerosis**

Formation of fibrous plaques in the arterial linings due to the deposition of fat cholesterol and calcium is called as atherosclerosis. This is the primary and main cause of cardiovascular diseases.

- **Coronary Artery Disease (CAD)**

Narrowing the arteries that supply oxygenated blood to the myocardium and leads to insufficient amount of oxygen to the heart. This narrowing worsens and leads to lack of blood apply to heart and causes heart muscle damage.

- **Myocardial Infraction (MI)**

It is the condition which occurs when blood and oxygen supplies to cardiac arteries are partially or completely blocked, resulting in damage or death of heart cells. This condition is commonly known as heart attack.

- **Ischemic stroke or Cerebrovascular accident (CVA)**

It occurs when the blood circulation in part of brain is blocked or diminished when blood supply, which carries oxygen, glucose and other nutrients is disrupted, brain cells die and become dysfunctional.

Treatment of Hyperlipidemia

Two methods of treatment are available for the management of hyperlipidemia.

I) Therapeutic lifestyle changes

II) Drug therapy

- **Therapeutic lifestyle changes**

  - Diet medications (Low fat diet)
  - Regular physical activity
  - Smoking cessation
  - Weight reduction
  - Less cholesterol intake

- **Drug therapy**

HMG-CoA reductase inhibitors (statins)

- Lovastatin
- Simvastatin
- Pravastatin

- Atorvastatin
- Rosuvastatin
- Fluvastatin
- Pitavastatin

Bile acid (Resins)

- Cholestyramine
- Colestipol

Fibrates

- Clofibrate
- Gemfibrozil

Lipolysis and triglyceride synthesis

- Nicotinic acid

Sterol absorption inhibitor

- Ezetimibe

Nanotechnology

Nanotechnology is a rapidly growing area and incorporating a wide range of research it deals with materials or structures in nanometer typically ranging from sub nanometers to nanometers. There is an increased application of technology in the field of pharmaceutics and drug delivery. The rational for control drug delivery is to alter the pharmacokinetics and pharmacodynamics of drug substance in order to improve the therapeutic efficacy and safety through the use of novel drug delivery system. These systems have been investigated primarily for site specific drug delivery, for controlled drug delivery, and also for the enhancement of dissolution rate/bioavailability of poorly water-soluble drugs.

Definition

Nanoparticles are solid colloidal particles ranging from in size they consist of macromolecular materials in which ingredient (drug or biological active molecule) is dissolved, entrapped or encapsulated or adsorbed.

Types of nanoparticles

1. Nanosuspension
2. Polymeric nanoparticles
3. Polymeric micelles
4. Magnetic nanoparticles
5. Solid lipid nanoparticles (SLN)
6. Nanocrystals
7. Nanotubes
8. Dendrimers
9. Quantum dots (QDS)

Characterization of nanoparticles

Nanoparticles are generally characterized by

1. Fourier transform infrared spectroscopy (FTIR)
2. Particle size
3. Surface charge
4. Scanning electron microscopy (SEM).

DESIGN OF EXPERIMENT

Design of Experiment is a systematic approach to determine the relation between independent process/product variable and their effect on response variable, (Kiran mayee, 2016).

A drug candidate must be chemically, physically stable and manufacturable throughout the product life cycle and manufacturing process. In addition, many quality standards and special requirements must be met to ensure the efficacy and safety of the product. It is always essential to establish the (target product profile) TPP so that the formulation effort will be effective and focused. The TPP usually includes dosage form, route of administration, special-delivery requirement, maximum and minimum doses, and aspects of pharmaceutical elegance (appearance). The TPP guides formulation scientists to establish formulation strategies and keep formulation effort focused and efficient. After the TPP is clearly defined, many studies must be conducted to develop a formulation. DOE is an effective tool for formulation scientists throughout the many stages of the formulation process and can help scientists make intelligent decisions. These steps include optimization of product, drug excipient compatibility, process feasibility studies, formulation, and scale-up, and characterization of manufacturing process (Kiran Mayee, 2016).

Types of Experimental Design

A. Completely randomized designs.

B. Full Factorial designs.

- Two-level full factorial designs.
- Full factorial example.
- Blocking of full factorial designs.

C. Fractional factorial designs.

- A 2^{3-1} half-fraction designs.
- How to construct 2^{3-1} designs.
- Confounding.

- Design resolution.
- Use of fractional factorial designs.
- Screening designs.
- Fractional factorial designs summary tables.

D. Randomized block designs.

- Latin squares.
- Graeco-Latin squares.
- Hyper-Graeco-Latin squares.

E. Plackett-Burman designs.

F. Response surface designs.

- Central composite designs.
- Box-Behnken designs.
- Response surface design comparison.
- Blocking a response surface design.

G. Adding center points.

H. Improving fractional design resolution.

- Mirror-image fold over designs.
- Alternative fold over designs.

I. Three-level full factorial designs.

J. Three-level, mixed level and fractional factorial designs

Application of Experimental Design

- Granulation
- Pre-Tablet Granulation
- Oral-controlled release formulation
- Modelling of properties of powder
- Dissolution testing
- Tablet formulation
- Coating of tablets
- Extrusion-Spheronizatio
- Inhalation formulation

Software used in Experimental Design

- Design expert
- Factop
- Optima
- Xtap
- Omega
- Echip
- Multi-simplex

1. MATERIALS AND METHODS

Formulation of orlistat loaded eudragit nanoparticles

- Nano precipitation method was employed in the preparation of nanoparticles.

➢ Enteric coating polymer like Eudragit RS 100 was used, poloxamer 188 is used as stabilizer and Orlistat used as API.

Method of preparation

Orlistat loaded eudragit nanoparticles were prepared by nanoprecipitation method. The preparation procedure is as follows, weighed amount of Eudragit RS-100 solution was prepared by dissolving Eudragit in 1% w/v acetone using mechanical stirrer at 37^0C. After attaining clear polymer solution, specified quantity of orlistat were dissolved in methanol was added to the polymer mixture and allowed to stir (Remi motor RQ-122) for 1 hour.

Figure No. 1: Eudragit nanoparticles preparation method

To the drug loaded polymer solution poloxamer- 188 1% w/v solution was added dropwise and allowed to stir for 4 hours, then placed in a bath sonicator for 30 minutes. The precipitated mixture was centrifuged (Research Centrifuge R-24) at 10000 rpm for 15min and the sediment was collected, washed with deionized water and dried to get nanoparticles.

❖ **Dependent and independent variables of the study**

The values (Levels) of the variables are +1 (Higher level), -1 (Lower level).

➢ Experimental runs generated based on THREE factor TWO level using 2^3 Factorial design.

➢ Total runs generated with three factor two level, with central points three are 11.

➢ To Levels of the variables are fixed based on the preliminary trails performed.

Table No. 2: Dependent and independent variables

Independent variables (factors)	**Dependent variables**
Eudragit RS 100 (mg)	Percentage entrapment efficiency (%)
Poloxamer 188 (mg)	
Orlistat (mg)	Percentage drug release (%)

❖ **2^3 EXPERIMENTAL RUNS BY DESIGN**

EXPERT SOFTWARE

The fixed levels and their variables are feed in the design expert software with their units and dependent variables experimental runs are given by design.

Table No. 3: Factors and their levels used in 2^3 FD

Factors	**Levels**	
	-1	+1
Eudragit RS100 (mg)	1500	2500
Poloxamer188 (mg)	250	750
Orlistat (mg)	60	120

Table No. 4: Experimental runs given by software

Runs	Factor 1 Eudragit RS 100 (gm)	Factor 2 Poloxamer 188 (gm)	Factor 3 Orlistat (gm)
1	2500	250	60
2	2500	750	60
3	2500	250	120
4	2500	750	120
5	2000	500	90
6	1500	250	20
7	2000	500	90
8	1500	250	60
9	2000	500	90
10	1500	750	120
11	1500	750	60

CHARACTERIZATION AND EVALUATION OF DRUG LOADED NANOPARTICLES

✓ **CHARACTERIZATION STUDIES**

- **Particle size, zeta potential and Polydispersity index**

The particle size and zeta potential of orlistat nanoparticles were measured by using dynamic light scattering (DLS) method. Laser beam operated at scattering angle of 90° and electrode potential maintained at 3.3V was used to determine particle size at 25 °C after appropriate dilution of samples with Zetasizer (HORIBA Scientific S Z-100 model).

- **Surface Morphology by scanning electron microscopy (SEM)**

Surface morphology for prepared nanoparticles is identified by using Scanning Electron Microscopy (SEM) FEI, Netherlands, Quanta 200 F. Using the SEM images, we can determine the surface features of prepared nanoparticles like rough or smooth, spherical or irregular, plane or fractured, freely suspended or agglomerated.

✓ EVALUATION STUDIES

Percentage yield of nanoparticles

The percentage yield of orlistat nanoparticles is obtained by weighing prepared nanoparticles and calculated using the below formula.

$$\text{Percentage yield} = \frac{\text{Weight of nanoparticles}}{\text{Weight of drug and excipients used}} \text{ X } 100$$

Entrapment efficiency

Drug Entrapment efficiency (EE) were determined by ultracentrifugation method. The prepared nanoparticles at solution stage were allowed to centrifuge using an ultracentrifuge at 10000 rpm for 15 min to separate the free drug or unentrapped drug. The free drug present in the supernatant is assayed using UV-Visible spectrophotometer at 203 nm. From the obtained absorbance value the concentration of free drug is known by using standard calibration curve of orlistat.

$$\text{Entrapment efficiency} = \frac{\text{Initial drug} - \text{Free drug}}{\text{Initial drug}} \text{ X } 100$$

In-vitro drug release

In-vitro drug release was evaluated by using dialysis method. Membrane (Himedia, MWCO, Molecular weight of 12,000-14000 Pore size 2.4 nm) was soaked in phosphate buffer solution pH 7.4 for 12 h before using for hydration. The pre-soaked dialysis bag was filled with prepared Orlistat loaded eudragit nanoparticles and 5 ml of phosphate buffer solution pH 7.4, tied at both ends and then immersed in the dissolution medium containing phosphate buffer solution pH 7.4 (900 ml) at 100 rpm and temperature was maintained at 37°C ± 0.5°C.

Figure No. 2: *In-vitro* drug release of orlistat loaded eudragit nanoparticles

Aliquots of 5ml were withdrawn at different time intervals till 48 hrs from dissolution medium and replaced with 5 ml of fresh buffer maintained at same temperature in order to maintain perfect sink conditions. The withdrawn samples were analyzed at 203 nm by using UV-Visible spectroscopy. The % cumulative drug released versus time graphs were plotted.

2. RESULTS AND DISCUSSION

- **Determination of melting point**

The melting point of orlistat drug was measured by melting point apparatus. Orlistat tend to start melt at **40°C**and end at **43°C**, by observing the melting point studies. The orlistat drug was stable.

- **Drug- excipient compatibility study through FTIR**

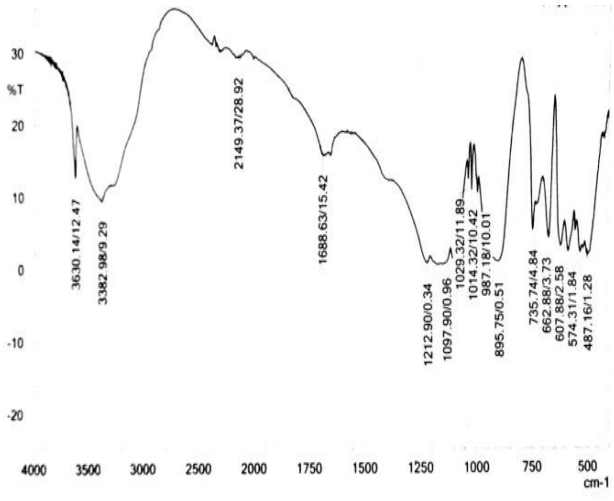

Figure No. 3: FTIR spectrum of orlistat

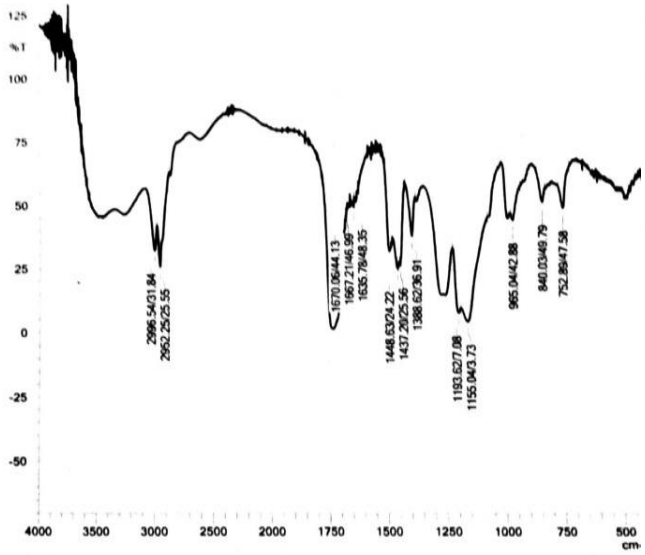

Figure No. 4: FTIR spectrum of physical mixture

The characteristic peak of orlistat with combination of excipients like Eudragit RS 100, poloxamer 188 and drug-mixture show the presence of identification peaks in mixture and individual samples shows compatibility.

The peaks represented the following groups present in orlistat mixture 540.73cm^{-1} of inorganic compounds, 894.32cm^{-1} of C-H bond out of the plane, 1152.90cm^{-1}of aromatic rings, 1752.92cm^{-1}of C=O group, 2877.25cm^{-1}of O-H stretching, 3589.42cm^{-1}of N-H group. The characteristic peaks in figure of orlistat were retained physical mixture, indicating no chemical interaction between the orlistat and excipients.

- **Determination of standard calibration curve for orlistat**

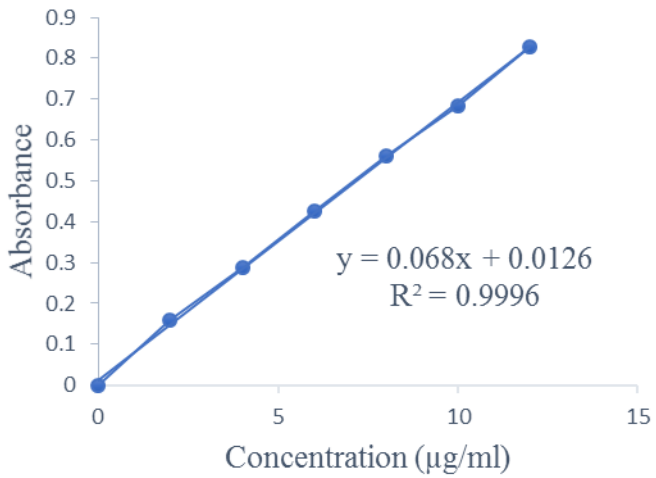

Figure No. 5: Calibration curve of orlistat

The calibration curve of orlistat was performed with methanol and it shows regression coefficient value of 0.9996. As per the limits of IP the calibration graph of orlistat passes regression coefficient and obeys Beers Lambert law.

❖ EVALUATION OF ORLISTAT LOADED EUDRAGIT NANOPARTICLES

The variables, factor and responses are fixed by initial trails of experiments, the data what we fixed are feed in design expert software, we get eleven experimental runs from the software. By performing the eleven experiment runs and their evaluation studies, these are the following responses results are observed.

Table No. 05: Evaluation results compared with factors and responses

RUNS	FACTOR 1 Eudragit RS 100 (mg)	FACTOR 2 Poloxamer 188 (mg)	FACTOR 3 Orlistat (mg)	% EE	%Drug release (48 hours)	Percentage yield
1	2500	250	60	61.84%	49.3%	62.22%
2	2500	750	60	63.74%	50.54%	64.09%
3	2500	250	120	74.66%	61.2%	68.50%
4	2500	750	120	75.58%	62.46%	70.52%
5	2000	500	90	68.12%	56.87%	66.74%
6	1500	250	20	69.55%	56.72%	67.05%
7	2000	500	90	67.89%	56.13%	66.37%
8	1500	250	60	57.02%	44.61%	59.81%
9	2000	500	90	67.1%	55.94%	62.74%
10	1500	750	120	72.42%	60.31%	67.82%
11	1500	750	60	60.76%	48.22%	60.49%

- **Entrapment efficiency**

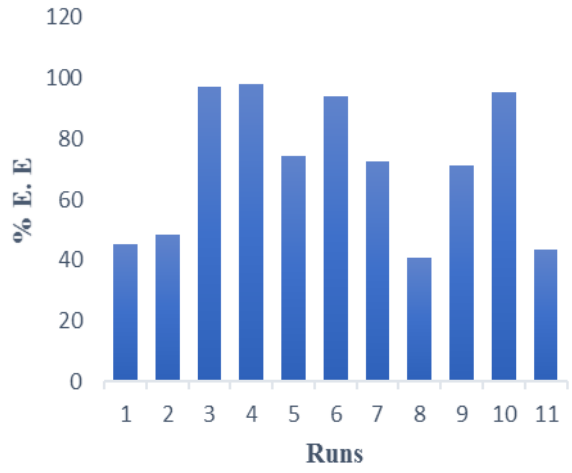

Figure No. 6: Entrapment efficiency of formulation

The entrapment efficiency of 11 formulations was ranged from 57.03 to 75.58 %, where run 4 showed highest entrapment efficiency of 75.58 % and run 8 showed lowest entapment efficiency of 57.02 %. The results are shown in table 05. Due to the maximum amount of eudragir Rs 100, Poloxamer 188 and orlistat are in formulation 4 so it shows higher entraptment efficiency and in formulation 8 the all the three factors are in minimum concentration so it showed low entrapment efficiency. increasing the concentration of orlistat, eudragit RS 100 and Poloxamer188 increasing the entrapment efficiency of the drug was observed.

- **Percentage Yield**

The percenyage yield of 11 formulations was ranged from 59.81 % to 70.52 %, where run 4 showed highest entrapment efficiency of 70.52% and run 8 showed lowest entapment efficiency of 59.81 %. The results are shown in table 05. The percentage of yield were increase while increasing the concentrations of the three factors (Eudragit RS 100, Poloxamer 188 and orlistat). In formulation 4 all three factors are in maximum concentration so it exhibitshigh percentage of yield and formulation 8 shows low percentage of yield due to minimum concentration of the factors.

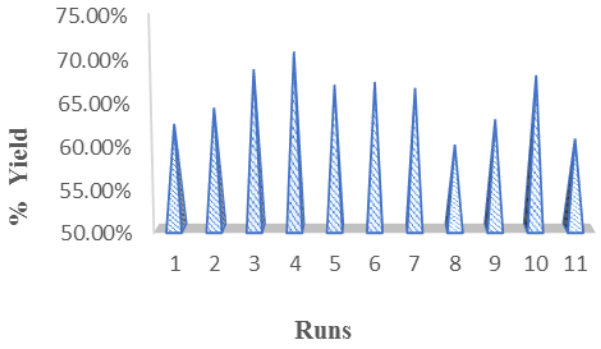

Figure No. 07: Percentage yield of formulation

- ***In-Vitro* Drug release study**

Table 05 and figure 06, 07 showing the drug release study of 11 formulations was ranged from 44.61% to 62.46 %, where run 4 showed highest of 62.46% at the end of 48hour. The brust release of the drug was seen at 1hr with percentage drug release of 29.17 % due to release of the drug which is encapsulated on the surface of the particles. After 1hr brust release slow and sustained release of drug was observed due to the slow release of drug from the eudragit polymer membrane by means of errosion or diffusion mechanisum.

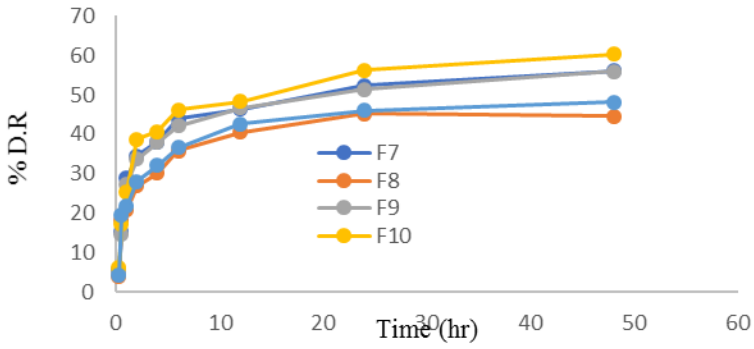

Figure No. 08: Drug release study of F_1 - F_6. Formulations

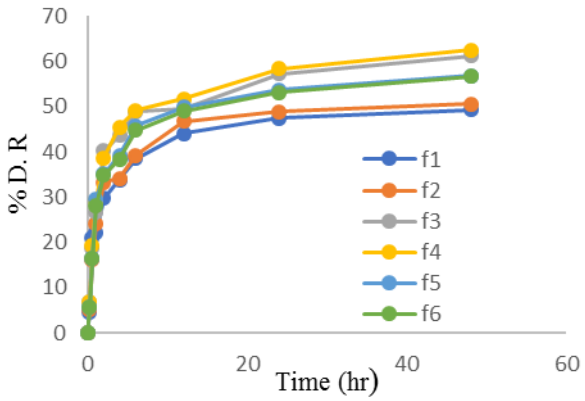

Figure No. 09: Drug release study of F_7 - F_{11} formulations

Effect of formulation parameters by 2^3 factorial Design

The analysis of the responses can proceed once the response data has been entered in the software. If more than one response has been entered, need to analysis the responses separately.

Analysis of two Responses individually they are.

1. Entrapment Efficiency
2. Drug Release

Analyze each response at a time by following these steps:

- Transformation
- Effects
- ANOVA
- Diagnostics
- Model graphs

1. Effect of formulation parameters on entrapment efficiency (EE).

Entrapment efficiency is the most important factor in preparation of nanoparticles. Descriptive statistics of model were represented in below and results demonstrated that entrapment efficiency was increased with increasing concentration of Eudragit, Orlistat and

Poloxamer. Concentration of eudragit and orlistat. were highly influential over %EE. It was noticed that amount of eudragit and orlistat had a huge effect on entrapment efficiency of nanoparticles.

- **Transformation**

Transform screen, Design-Expert notes that the response range is more than two-fold ("Ratio of max to min is 2.4186"). This number falls below the ratio of 3 where "…power transforms have little effect." Therefore, you can leave the transformation at its default: **None**. You will also see diagnostics plot later on (Box-Cox) that will alert you when a transform may help.

- **Effects.**

Half-Normal Plot

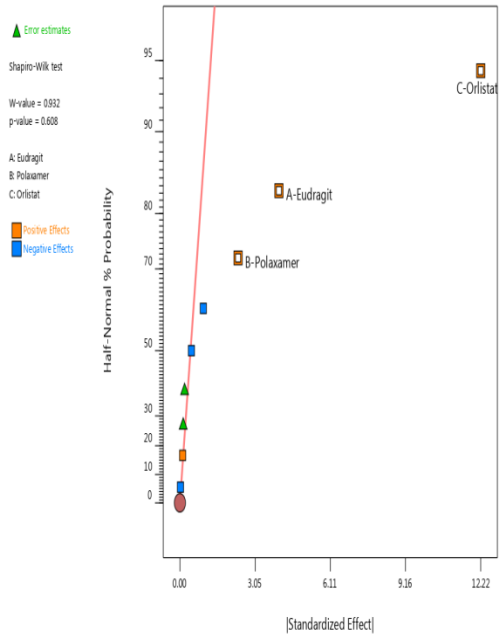

Figure No. 10: Half-Normal plot of EE

The experimental results were analyzed by half normal plot (Figure 10) to determine the significant effects. For building the model equation, large effects were identified in half-normal plot and separated from other repeatable and small effects.

Pareto chart

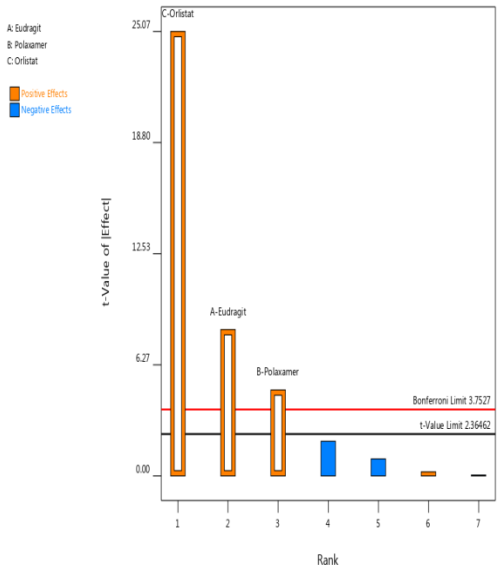

Figure No. 11: Pareto of EE

The higher t-value observed in the Pareto chart (Figure 11)endorse the selection of dominant effects altering entrapment efficiency. The results indicated that concentration of Orlistat exhibited the highest effect in altering the entrapment efficiency with a percentage contribution of 86.23% as compared to other factors and was followed by the concentration of Eudragit and Poloxamer with an individual contribution of 9.34% and 3.22%, respectively.

➢ ANOVA

Statistical Analysis

ANOVA analysis depicted that developed linear model was highly significant, as was evident from very low probability value <0.0001. The goodness of fit was checked by regression coefficient (R^2). Here, the value of regression coefficient (R^2 =0.9917) indicated that only 0.82% of the total variations was not explained by the adopted regression model. Besides, the difference between R_a^2 and R_p^2 was less than 0.2, which assures the reliability of model to interpolate. Furthermore, a good deal of reliability and high degree of precision of conducted experiments was indicated by low value of coefficient of variation (CV=1.03%). The adequate precision measures the signal-to-noise ratio, and a ratio greater than 4 is desirable to navigate in design space. In this case, the adequate precision was found to be 40.0317, which indicates the best fitness of developed model.

Table No. 06: ANOVA of EE

Statistical term	Value
Model P-value	<0.0001
Eudragit P-value	0.0002
Poloxamer P-value	0.0029
Orlistat P-value	<0.0001
Regression coefficient (R^2)	0.9917
Predicted coefficient (R_p^2)	0.9698
Adjusted coefficient (R_a^2)	0.9876
Coefficient of variance (% CV)	1.03
Adequate precision	40.0317

This can also be witnessed in Pareto chart and also from low p-value, p <0.0001 observed in the model. The effect of TPP concentration was little yet significant with a p-value of 0.0036. However, the slight increase in entrapment efficiency was observed as the amount of TPP was increased.

- **Diagnostics**

Predicted versus actual values

Model diagnostic plots like predicted value versus experimental value graph (Figure 12) helped in depicting the relationship between the experimental and predicted values and in assessing the model sufficiency. It is prerequisite to ensure if fitted linear model provides a broad approximation of the actual values and ignores small and misleading effects for optimization. In the graph drawn between the predicted versus actual values, the data points were found to be adjacently dispersed, which indicates the minimum deviation and efficacy accord between the predicted and actual values Residual versus run plot.

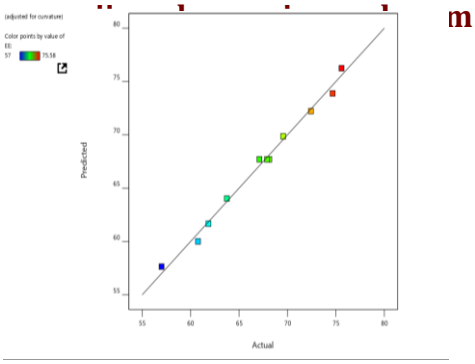

Figure No. 12: Predicted Vs Actual of EE

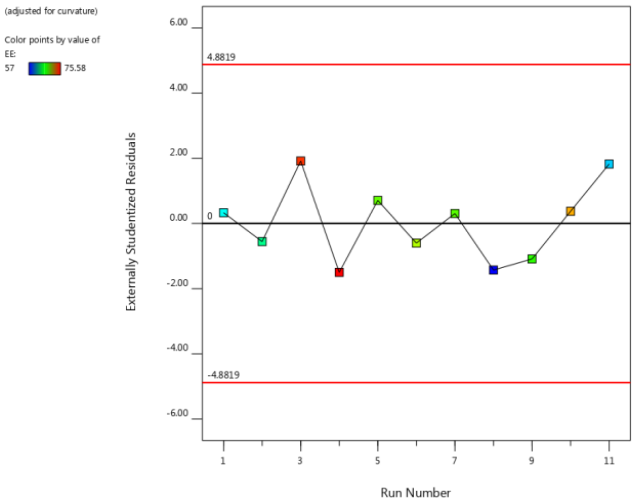

Figure No. 13: Residual versus run plot

An internally studentized residual versus experimental runs plot (Figure 13) was constructed to ensure the satisfactory fit of the developed model. A random trend was observed in residual vs run plot, and all the data points fell within the range of control limits, indicating the experiments were carried out in a random manner, thereby eliminating chance of errors and ensuring adequate fit.

- **Model graphs.**

3-Dimensional plot of EE

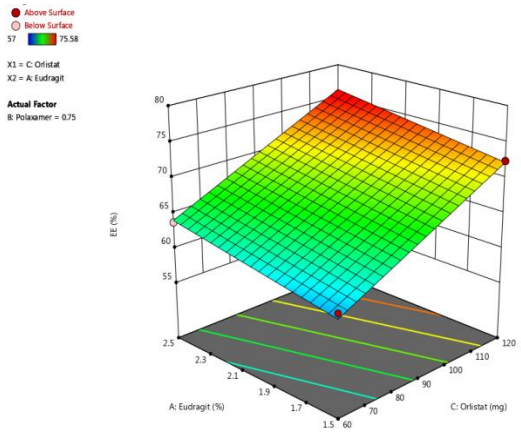

Figure No. 14: 3D-Surface response of EE

Intending to visualize the relationship between entrapment efficiency and formulation variables, model graphs, namely, perturbation and 3D response surface were generated, to assess the individual and interactive effects on the response. From the perturbation chart, it was evident that increasing the concentration of variables will increase the entrapment efficiency of the formulation. The planar 3D surface diagram and linear curves observed that its contour region also assures the same. Moreover, the 3D plot also indicated an absence of curvature effect in the explored design space.

2. Effect of formulation parameters on drug release (DR)

Drug release is the most important factor in preparation of nanoparticles. Descriptive statistics of model were represented in below and results demonstrated that drug release was increased with increasing concentration of eudragit and orlistat. Concentration of, orlistat and poloxamer were highly influential over % DR. It was noticed that amount of orlistat had a huge effect on drug release of nanoparticles.

➢ **Transformation**

Transform screen, Design-Expert notes that the response range is more than two-fold ("Ratio of max to min is 2.4186"). This number falls below the ratio of 3 where "…power transforms have little effect." Therefore, you can leave the transformation at its default: **None**. You will also see diagnostics plot later on (Box-Cox) that will alert you when a transform may help.

➢ Effects

Half-Normal Plot

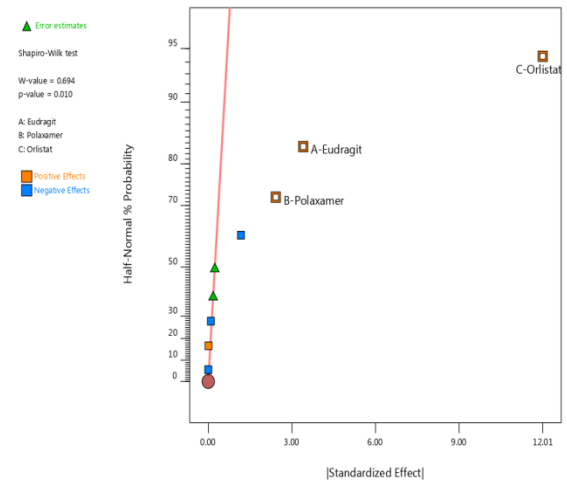

Figure No. 15: Half-Normal plot of DR

The experimental results were analyzed by half normal plot (Figure 15)to determine the significant effects. For building the model equation, large effects were identified in half-normal plot and separated from other repeatable and small effects.

Pareto chart

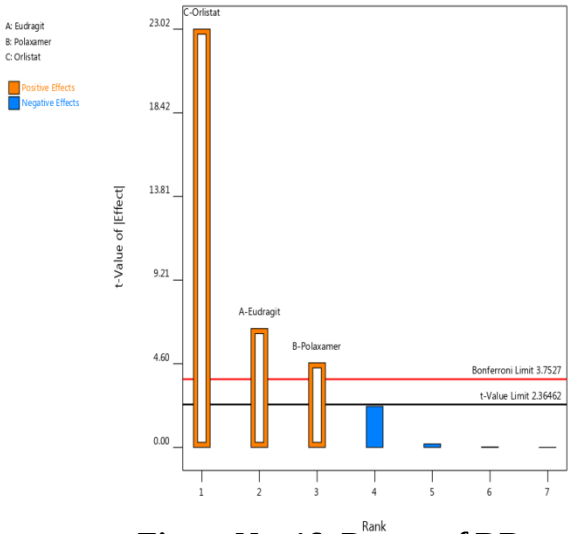

Figure No. 16: Pareto of DR

The higher t-value observed in the Pareto chart (Figure 16) endorse the selection of dominant effects altering drug release. The results indicated that concentration of Orlistat exhibited the highest effect in altering the drug release with a percentage contribution of 85.19% as

compared to other factors and was followed by the concentration of eudragit and poloxamer with an individual contribution of 3.49% and 85.64%, respectively.

➢ **ANOVA**

Statistical Analysis

ANOVA analysis depicted that developed linear model was highly significant, as was evident from very low probability value <0.0001. The goodness of fit was checked by regression coefficient (R^2). Here, the value of regression coefficient (R^2 =0.9900) indicated that only 0.82% of the total variations was not explained by the adopted regression model. Besides, the difference between R_a^2 and R_p^2 was less than 0.2, which assures the reliability of model to interpolate. Furthermore, a good deal of reliability and high degree of precision of conducted experiments was indicated by low value of coefficient of variation (CV=1.35%). The adequate precision measures the signal-to-noise ratio, and a ratio greater than 4 is desirable to navigate in design space. In this case, the adequate precision was found to be 35.88, which indicates the best fitness of developed model.

Table No. 7: ANOVA of DR

Statistical term	Value
Model P-value	<0.0001
Eudragit P-value	0.0002
Poloxamer P-value	0.0029
Orlistat P-value	<0.0001
Regression coefficient (R^2)	0.9917
Predicted coefficient (R_p^2)	0.9626
Adjusted coefficient (R_a^2)	0.9876
Coefficient of variance (% CV)	1.25
Adequate precision	35.8852

Diagnostics

Predicted versus actual values

Model diagnostic plots like predicted value versus experimental value (Figure 17) graph helped in depicting the relationship between the experimental and predicted values and in assessing the model sufficiency. It is prerequisite to ensure if fitted linear model provides a broad approximation of the actual values and ignores small and misleading effects for optimization. In the graph drawn between the predicted versus actual values, the data points were found to be adjacently dispersed, which indicates the minimum deviation and efficacy accord between the predicted and actual values.

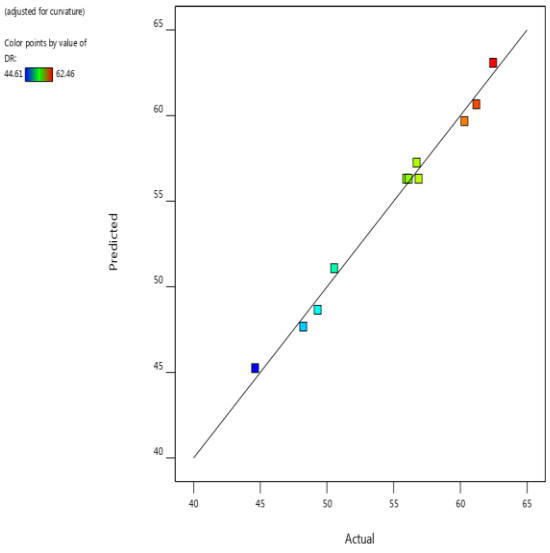

Figure No. 17: Predicted versus actual values of DR

Residual versus run plot

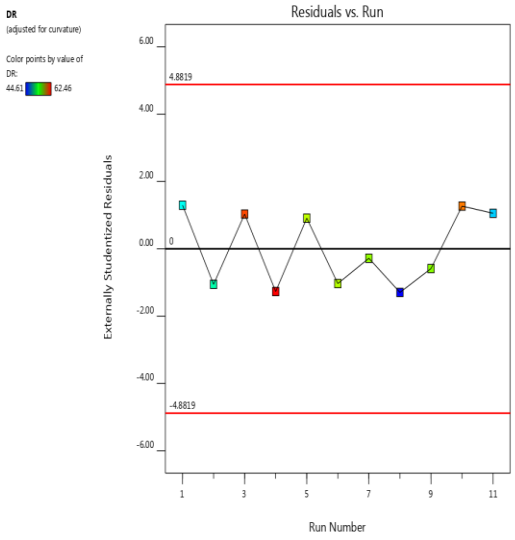

Figure No. 18: Residual versus run plot of DR

An internally studentized residual versus experimental runs plot (Figure 18) was constructed to ensure the satisfactory fit of the developed model. A random trend was observed in residual vs run plot, and all the data points fell within the range of control limits, indicating the experiments were carried out in a random manner, thereby eliminating chance of errors and ensuring adequate fit.

- **Model graphs**

3-Dimensional plot

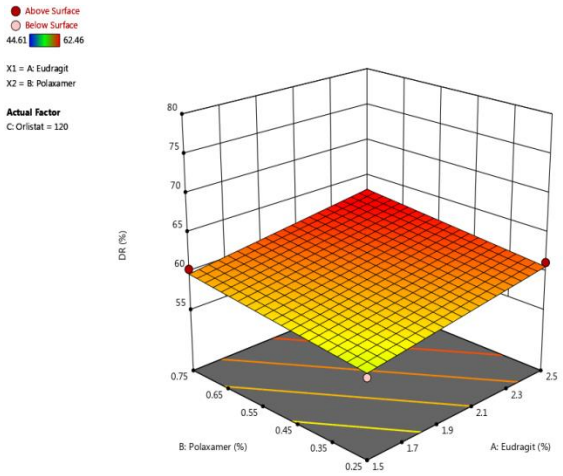

Figure No. 19: 3D Response of % DR

Intending to visualize the relationship between drug release and formulation variables, model graphs, namely, perturbation chart, contour plots, and 3D response surface, were generated, to assess the individual and interactive effects on the response. From the perturbation chart, it was evident that increasing the concentration of variables will increase the drug release of the formulation. The planar 3D surface diagram and linear curves observed that its contour region also assures the same. Moreover, the 3D plot also indicated an absence of curvature effect in the explored design space.

OPTIMIZATION AND VALIDATION OF OPTIMIZED CONDITIONS.

- **Entrapment efficiency (%EE)**

Regression model developed in this study was used to identify out the optimal conditions to prepare nanoparticles with entrapment efficiency of 75.58%. Furthermore, Derringer

desirability served the purpose to decide on picking an appropriate combination of formulation variables. An algebraic solution for the preparing desired nanocarriers was presented by software in the coded form and was found to be X_1(Eudragit RS 100) =1.61, X_2(Poloxamer 188) =1.18 and X_3(Orlistat) = 5.33. The corresponding experimental parameters for X_1, X_2, X_3were 2500 mg, 750 mg, and120 mg, respectively. Under these optimal conditions, the predicted entrapment efficiency was 76.245 %. To compare predicted results with experimental values, additional triplicate experiments were performed.

- **Drug release (%DR)**

Regression model developed in this study was used to identify out the optimal conditions to prepare nanoparticles with drug release of 62.46%. Furthermore, Derringer desirability served the purpose to decide on picking an appropriate combination of formulation variables. An algebraic solution for the preparing desired nanocarriers was presented by software in the coded form and was found to beX_1(Eudragit RS 100) = 1.71, X_2(Poloxamer 188) = 1.21 and X_3(Orlistat) = 6.0. The corresponding experimental parameters for X_1, X_2, X_3 were 2500 mg, 750 mg, and 120 mg, respectively. Under these optimal conditions, the drug release was 62.46% for 48 hours. To compare predicted results with experimental values, additional triplicate experiments were performed and the drug release was done for 48 hours.

Table No. 08: optimized formula

Response	X_1	X_2	X_3	Predicted	Experimental	Error (%)
EE % (Y_1)	2500 mg	750 mg	120mg	76.24	75.58	0.68
DR % (Y_2)	2500 mg	750 mg	120mg	63.69	62.46	0.73

Characterization of optimized orlistat loaded eudragit nanoparticles

- **Particle size**

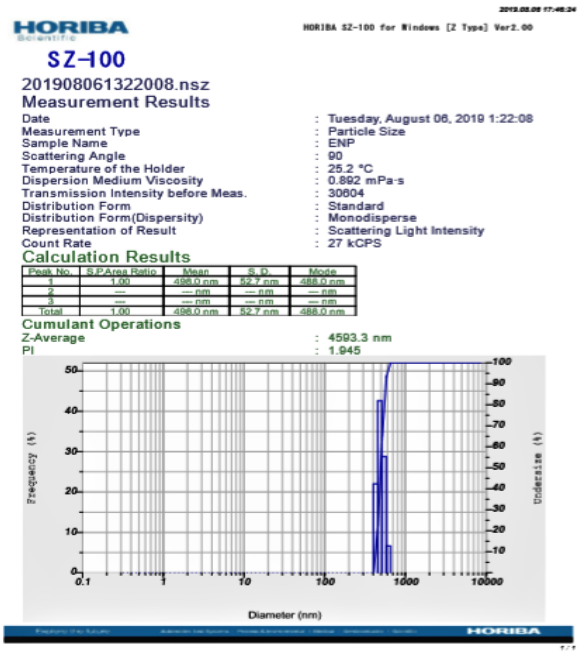

Figure No. 20: Particle size analysis of ENP

The Figure 20 particle size has direct effect on stability, drug release and biodistribution. The mean particle size of the prepared nanoparticles were found to be 496.0nm. The nanoparticles which range from 100-500nm are easily adhere to intestinal epithelium.

- **Zeta potential**

The zeta potential of prepared optimized formulation was found to be -0.1mv. it indicates the nanoparticle have a little anionic charge due to the presence of eudragit that would help in the better interaction of nanoparticle with cationic intestinal lining. Due to lower negative value of zeta potential, results in high attractive force leads to aggregation of nanoparticles as shown in figure 21.

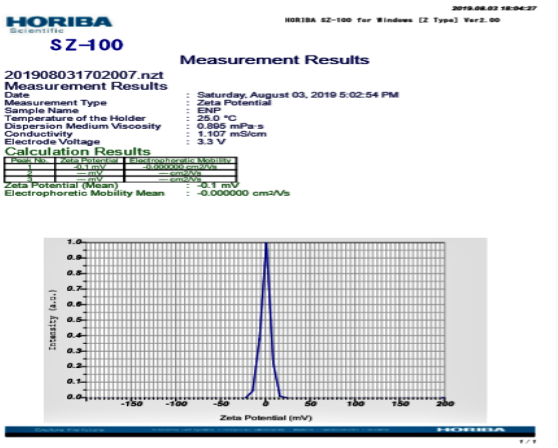

Figure No. 21: Zeta Potential analysis of ENP

- **Morphology by SEM**

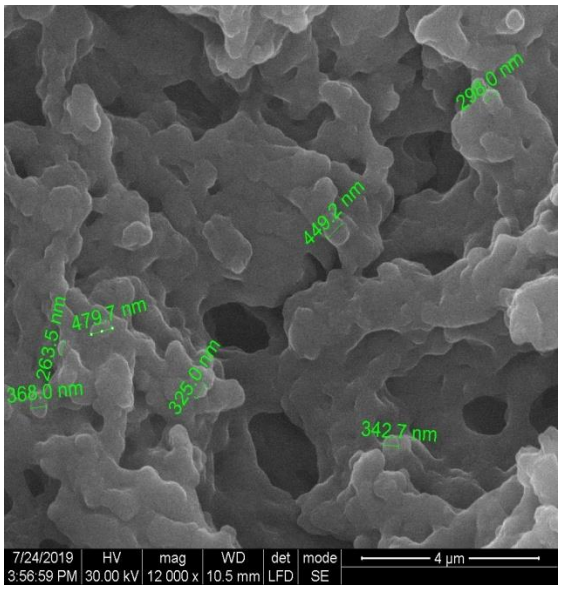

Figure No. 22: SEM image of optimized ENP nanoparticle

Scanning electron microscopy, a potential method to study surface characterization of nanoparticles. The optimized formulations are examined under high voltage and the microphotograph was represented in fig no-22. Particles are formed successfully with smooth topography in spherical or ovate shape with aggregation of nanoparticles. Aggregation of particles due to overlapping of nanoparticles one on other, during centrifuge or spreading. Bright coloured shading was observed due to application of high voltage.

➢ ***In-Vitro*** **Drug release**

Table No. 09: Drug release of optimized formula

S. NO.	TIME (hr)	% DRUG RELEASE
1	0.25	16.29
2	0.5	19.11
3	1	29.17
4	2	41.69
5	4	46.63
6	6	52.23
7	12	63.62
8	24	65.33
9	48	68.03

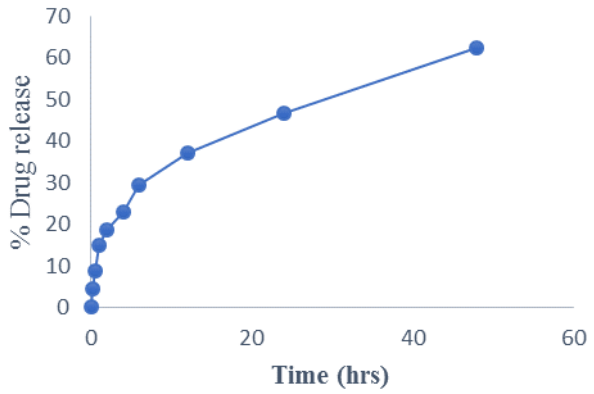

Figure No. 23: *In-vitro* drug release study of optimized nanoparticles

The drug release rate from the prepared nanoparticles was influenced by drug-polymer concentration. The in-vitro drug release profile of prepared orlistat loaded eudragit nanoparticles shows slow release of drug from the particles. The initial brust released was observed at 1hr(29.17%), followed by slow sustained release of drug 68.03% at end of the 48 hrs.

➢ **Drug Release Kinetics**

Table No. 10: Drug release kinetic model fit

Model Fitting (Average)-

	R	K
Zero order	0.9129	1.3253
T-test	6.324	(Passes)
1st order	0.9615	-0.0188
T-test	9.892	(Passes)
Matrix	0.8539	7.0467
T-test	4.641	(Passes)
Hix.Crow.	0.9486	-0.0055
T-test	8.476	(Passes)

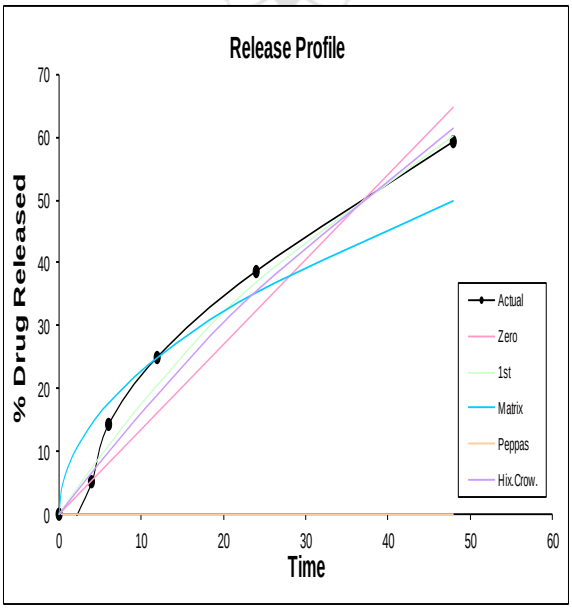

Figure No. 24: Drug release

kinetics

The release constant was calculated from the slop of appropriate plots and the regression coefficient (R^2) was determined after the comparative evaluation of R^2 values the kinetics of drug release from the optimized orlistat loaded eudragit nanoparticles was found to follow 1st

order kinetics as the plots of cumulative percentage drug release verses time was linear (R^2=0.9615).

3. CONCLUSION

Statistical optimization of Orlistat loaded Eudragit nanoparticles were fabricated by using nanoprecipitation method because of less economic, less laborious and reproducibility technique which involves only a few steps, in preparation. 2^3 factorial design was successfully applied as a design of experiment (DoE) to understand the effects of independent variables (Eudragit RS 100, poloxamer 188, orlistat) on responses like entrapment efficiency, drug release. All the formulations were prepared randomly as per the design and characterized for entrapment efficiency and drug release. The results were analyzed and validated using the Design expert 11 software. The concentration of orlistat showed dominant effect on the entrapment efficiency and drug release, whereas concentration of Eudragit and poloxamer had small and significant effect on entrapment efficiency and drug release.

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