Ind. J. Pharm. Edu. Res., 2025; 59(2s):s494-s507.
https://www.ijper.org

Original Article

Box-Behnken Design: Unleashing the Potential of Desirability Function for Enhanced Permeability of Antidiabetic Nanoparticles

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ABSTRACT

Aim: The present study is focused to develop and optimize nanoparticulate delivery system for two antidiabetic drugs; Sitagliptin Phosphate (SP) and Empagliflozin (EMP) of BCS class III drugs as a single dosage form using Box Behnken Design (BBD) and desirability function in lieu of superior permeability. **Materials and Methods:** Nanoparticles (NPs) were prepared using natural polymer Chitosan (CS) by ion gelation technique. BBD was employed to attain optimized formulation via responses (particle size and % entrapment efficiency). The optimized formulation was assessed for various parameters viz. and evaluated for particle size, entrapment efficiency, *in vitro* release kinetics and morphology through Scanning Electron Microscopy (SEM). **Results:** The responses showed a noteworthy influence of the formulation composition with a significant effect ($p<0.05$). Leveraging the power of desirability function, the most favourable formulation was handpicked and thereafter subjected to reformulation. The findings demonstrated chitosan-based nanoparticles with remarkable stability, boasting a zeta potential registering at +30.5 mV, featuring petite particles at 89.43 nm and an impressive entrapment efficiency of 80.99%. The optimized preparation exhibited uniformity, stability and consistency between the observed and predicted responses. **Conclusion:** The efficacy of SP-EMP-CS nanoparticles in enhancing the bioavailability of poorly permeable drugs, combined with the utilization of BBD and desirability function, has provided a valuable framework for optimizing SP-EMP-CS nanoparticulate formulation. This approach has allowed for a comprehensive understanding of the impact of various independent variables on formulating an optimized SP-EMP-CS nanoparticulate system with superior responses. In summary, using Box-Behnken design and desirability function, the research successfully developed a Nanoparticulate delivery system for SP and EMP, providing a promising approach to increase drug bioavailability and potentially improve diabetic therapy outcomes like improved glycemic control, enhanced patient adherence etc.

Keywords: Box-Behnken design, Desirability function, Optimization, Chitosan-based nanoparticles, Sitagliptin phosphate, Empagliflozin.

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Received: 20-10-2023;
Revised: 13-07-2024;
Accepted: 15-01-2025.

INTRODUCTION

In the realm of diabetes, India emerges as a country where around 77 million populations, constituting 1 out of every 11 Indians, have been formally acknowledged with this metabolic challenge. Such staggering figures firmly establish India as the world's second most afflicted nation, trailing only behind China.[1]

Type-2 Diabetes Mellitus (T2DM) is an advanced, multiple factors related to hereditary, metabolic and cardiovascular disease pertaining several pathophysiologic anomalies.[2] Impaired or altered insulin secretion and altered insulin action are two chief pathological flaws of T2DM, which are results of pancreatic β-cell dysfunctioning and resistance to insulin respectively.[3] In conditions of predominant insulin resistance, β-cells collectively undergo a transformation leading to increase in insulin amount and reimbursement for the extreme and abnormal need of insulin. In such a case, both fasting and meal plasma insulin concentrations are augmented classically. The plasma insulin concentration is inadequate to continue normal glucose homeostasis in comparison to the harshness of insulin resistance. Hence the ultimate consequence is insulin resistance and hyper insulinemia leading to impaired glucose tolerance.[4] Several other additional factors or pathological conditions are considered equally responsible for causing the disease - excessive alpha glucagon production, irregular incretin outcome, improved hepatic glucose production, augmented lipid breakdown,

DOI: 10.5530/ijper.20255506

neurotransmitter dysfunction, abnormal renal management of hyperglycemia.[5]

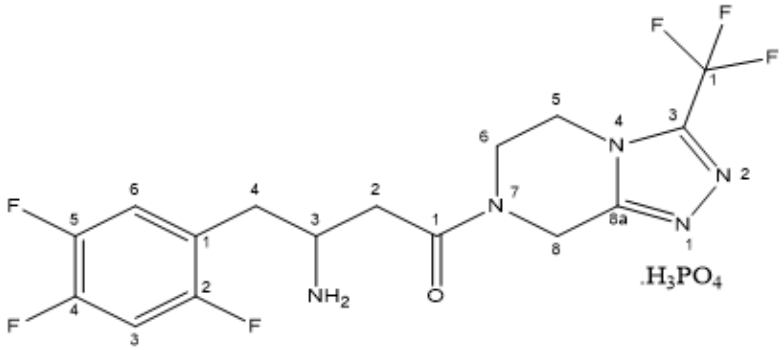
Nearly half of diabetic adult patients are unable to attain the endorsed glucose regulation, hence demanding treatment modification (i.e., up-titration of dose or additional medications). Research reveals a striking connection between polypharmacy in diabetes and suboptimal treatment obedience and tenacity. Subpar obedience has been linked to the erosion of glycemic control, heightened susceptibility to hospitalization and death and the amplification of healthcare expenses in individuals diagnosed with type 2 diabetes mellitus.[6] All these pros have encouraged the preliminary use of combination therapy along with added hostile initial therapy as they retain the A1C from intensifying much above the target range.[7] The early initiation of combination remedy is suggested by many scientists for T2DM, which results in sustained decrease in glycated hemoglobin HbA1c levels by correcting the allied pathophysiologic anomalies. The use of low-dose combined therapy results in greater glycemic control with fewer side effects as compared to the high dosed of the monotherapy.[8] Combination therapy stands as a strategic approach to enhance compliance among individuals with T2DM. Its purpose is to alleviate the load of pills, simplify treatment regimens and cut down on healthcare expenses.[9]

Preferably, a combination of hypoglycemic agents must discourse multiple pathophysiologic trails through complementary mechanisms without any integral risk of weight gain, hypoglycemia and cardiovascular complications. It must be safe to use at all stages of diabetes, imparting adequate efficacy and well tolerability and should also offer patient compliance and enhanced dosage adherence in terms of oral, single-dose pill with once-a-day dosing frequency.[10]

Sitagliptin Phosphate[11-13] (SP) [(3R)-3-amino 1-[3-(trifluoromethyl)-5,6-dihydro [1,2,4] triazolo[4,3-α] pyrazin-7(8H)-yl]-4-(2,4,5-trifluorophenyl) butan-1-one phosphate monohydrate] is an orally existing, competitive, beta-amino acid derived inhibitor of DPP-IV (dipeptidyl peptidase-IV) employed in the management of T2DM. The suppression of DPP-IV induces the augmented levels of GIP (glucose dependent insulinotropic polypeptide) and GLP-1 (Glucagon like peptide-1) incretin hormones which ultimately reduces the blood glucose levels. Figure 1a depicts the Sitagliptin Phosphate's chemical structure. Empagliflozin (EMP)[13-15] [(1S)-1,5-anhydro-1-(4-chloro-3-{4-[(3S)-tetrahydrofuran-3-yloxy]benzyl} phenyl)-D-glucitol, also known as D-Glucitol,1,5-anhydro-1-C-[4-chloro-3-[[4-[[(3S)-tetrahydro-3 furanyl] oxy] phenyl] methyl] phenyl]-(1S).], is an obstructer of sodium glucose co-transporter-2 (SGLT-2), through which renal reabsorption of glucose is carried out. It is used clinically as an assistant to food régime and workout, frequently in blend alongside alternative drug therapies in the management of T2DM. Among other commercially available gliflozins, empagliflozin has the maximum discernment for SGLT-2 (2500-fold) as compared to SGLT-1. The structure is presented in Figure 1b.

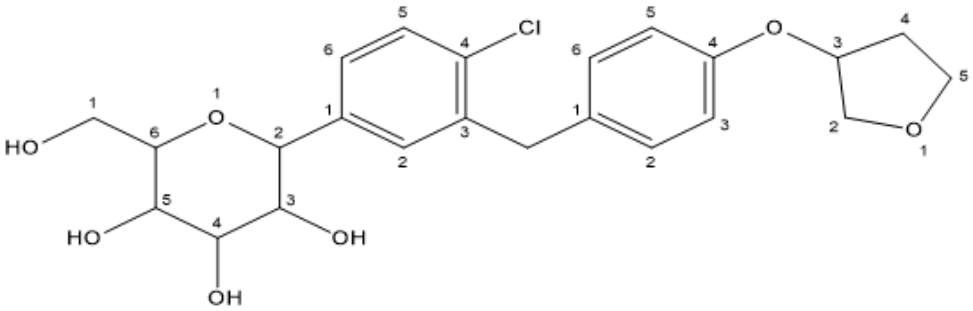
The intrinsic physicochemical characteristics of SP and EMP suggest a harmonious fit within the realm of Nanoparticles (NPs). These systems have garnered recognition for their ability to elevate drug solubility, permeability and bioavailability upon oral administration.[16]

The increasing incidence of diabetes worldwide highlights the pressing need for novel treatment strategies. With millions of people impacted globally, there is an urgent need for more focused and efficient treatment approaches. In this context, new technologies provide viable ways to tackle the challenges associated with managing diabetes. Among them, drug administration via nanoparticulate delivery methods is unique and has the potential to completely transform therapeutic approaches. Such systems provide precise control over drug release kinetics and biodistribution, so maximizing therapeutic efficacy while reducing systemic negative effects. A breakthrough



3-amino-1-(3-(trifluoromethyl)-5,6-dihydro-[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl)-4-(2,4,5-trifluorophenyl)butan-1-one phosphate monohydrate

Figure 1a: Structure of SP.



2-(4-chloro-3-(4-((tetrahydrofuran-3-yl)oxy)benzyl)phenyl)-6-(hydroxymethyl)tetrahydro-2*H*-pyran-3,4,5-triol

Figure 1b: Structure of EMP.

in diabetes remedies will be brought in by encapsulation of antidiabetic drugs in nanosized carriers, which will be essential to enhancing patient outcomes and reducing the economic burden of the disease.

Hence the present research endeavors to adopt a synergistic approach employing the BBD and the desirability function to meticulously craft and optimize NPs formulation by utilizing CS[17] in the ion gelation technique.[18] This SP-EMP-CS based NPs formulation aimed to encapsulate SP and EMP, BCS class III drugs possessing low permeability with the goal of enhancing its permeability. Furthermore, the influence of concentration of CS and Tri Polyphosphate (TPP).[19] volume-ratio of CS and TPP and agitation haste on the particle size and entrapment efficiency was meticulously assessed.

MATERIALS AND METHODS

Materials

SP and EMP were furnished by Intas pharmaceutical Ltd., (Ahmedabad, Gujarat, India) as gift samples. CS and TPP were acquired from Loba chemical Pvt. Ltd., Mumbai, India. All other chemicals and reagents were of analytical grade.

Methods

In adherence to the methodology initially proposed by Calvo *et al*. (CS NPs were formed spontaneously upon addition of 2 mL of TPP solution (0.1%) to 5% of CS acidic solution under magnetic stirring), the preparation of NPs was carried out by utilizing the ion gelation method.[20] CS and TPP were chosen in NP creation, because of their biocompatibility, biodegradability and low toxicity, enabling stable NP synthesis via ionotropic gelation. TPP serves as a cross-linking agent while CS provides mucoadhesive qualities appropriate for targeting mucosal surfaces in oral medication administration. This combination provides prolonged release kinetics, excellent drug encapsulation and regulated particle size, which makes it a useful platform for improving the treatment of diabetes.

To encapsulate the process concisely, CS was skillfully blended into a 1% (v/v) acetic acid solution yielding 0.1%, 0.2% and 0.3% w/v of CS concentrations for preventing particle aggregation, Tween 80 (0.5% v/v) was introduced as a resuspending agent into the CS solution. SP and EMP were added dropwise in prepared CS solution in a 5:1 ratio (this ratio was selected based on empirical studies to maximize the complementary advantages of both drugs to improve glucose regulations in diabetes) The CS solution pH was meticulously fine-tuned to 4.5-4.8 using 0.1 M NaOH (in order to promote CS solubility through protonation of its amino groups at this pH). Simultaneously, TPP solutions with concentrations of 0.25%, 0.50% and 0.75% w/v were obtained by solubilizing it in distilled water. All solutions were meticulously filtered using a 0.2 µ filter. At room temperature TPP solutions were subjected to magnetic agitation and subsequently, the prepared CS solutions were introduced drop by drop into it, maintaining CS: TP 1:1 (v/v). This process facilitated the spontaneous creation of SP-EMP-CS NPs through the TPP-stimulated ion gelation technique. Lastly the NPs were harvested through centrifugal separation at 20,000 rpm for 20 min at 20°C, employing a cooling centrifuge. The supernatant was analyzed for the determination of free drugs using UV simultaneous estimation method. The schematic presentation of the method is in Figure 2.

Box-Behnken Design (BBD)

To facilitate the exploration of constituents and factors influencing the outcome variables, Design Expert software (version 12) was employed to craft BBD. In present study, BBD, a statistical screening comprising 3-factors (CS concentration, Tri polyphosphate (TPP) concentration and stirring speed) and 3-levels encoded as high (+1), medium (0) and low (-1), which are depicted in Table 1, was employed. The study then analyzed the primary effects and interaction effects of formulation factors on the measured outcomes (particle size (R1) and drug %Entrapment Efficiency %EE (R2)) of the prepared NPs.[21] The observations of these effects are depicted in Table 2. The study also sought to evaluate the desirability function's suitability for formulation optimization. Five key points and a total of 17 experimental runs were required by BBD to evaluate the design's

meticulousness and measure variability. The formidable outcome of the BBD takes shape in the form of a non-linear quadratic model.

After conducting statistical analysis with ANOVA, the responses were assessed to identify the optimum formulation. The selection process involved using a numerical optimization procedure based on the desirability function. To visualize the relationships between the factors, 3D response surface images were generated. An intensive grid search was conducted across the entire experimental region to ensure thorough exploration. The actual experimental values were then quantitatively compared to the predicted values to assess the accuracy of the model.

Evaluation of SP-EMP-CS NPs

Determination of Particle size (R1)

The particle size (R1) analysis of all the prepared SP-EMP-CS NPs was conducted utilizing the laser light scattering technique with a Malvern Zetasizer. To prevent multi-scattering events and achieve the desired particle concentration, a 1ml sample SP-EMP-CS NPs was appropriately diluted with double distilled water[22-24] and then stirred in the sample unit. The data analysis was conducted at a constant temperature of 25°C, using a fixed light incidence angle of 90° to ensure consistent and accurate measurements.

Determination of % Entrapment Efficiency (%EE)/ (R2)

For all prepared SP-EMP-CS NPs, the %EE was determined through a direct method. Each nanoparticle formulation was accurately measured and immersed in 50 mL of double distilled water for approximately half an hour. The aqueous medium was thoroughly processed via centrifugal force utilizing centrifuge (Remi CM-12 Plus) at 25000 rpm for half an hour to obtain the isolated NPs containing the free drug. Subsequently, the supernatant was appropriately diluted with appropriate solvent and subjected to analysis using a UV spectrophotometer (Schimadzu 1800) at a wavelength of 267 nm for SP and 275 nm for EMP, to quantify the amount of unentrapped drugs present. The %EE was calculated utilizing the ensuing formula:

$$\%EE = [\text{Entrapped drug (mg)}/\text{Total drug (mg)}]X100$$

$$\%EE = [\{\text{Total drug (mg)}-\text{Unentrapped drug (mg)}\}/\text{ Total drug (mg)}]X100$$

Utilizing the Desirability Function for optimization

To optimize both responses simultaneously, a statistical optimization method known as the Desirability Function (DF) approach[25,26] was employed in the study. This approach involved setting specific target goals for each response as depicted in Table 1. For each response, a partial DF was defined, where values ranging from 0 to 1 represented the degree of acceptability. An undesired or unacceptable response was assigned a value of 0, while values closer to 1 indicated a more favourable proximity

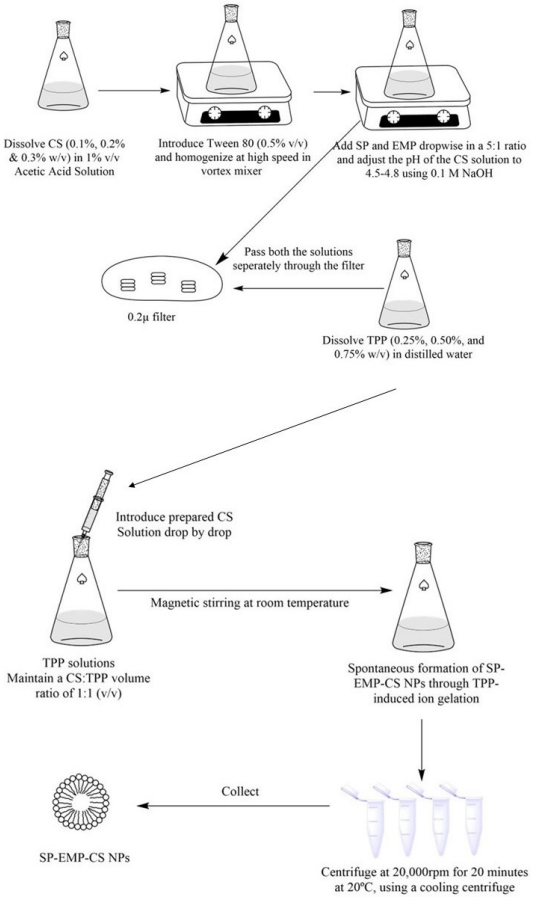


Figure 2: Schematic representation of CS-NPs preparation via Ion-gelation technique.

Table 1: Variables and Constrains in BBD.

Factor	Levels used, actual coded			
Independent Variables	**Low (-1)**	**Medium (0)**	**High (+1)**	**Constrains**
A=CS conc. (%w/v)	0.1	0.2	0.3	In range
B=TPP conc. (%w/v)	0.25	0.50	0.75	In range
C=Agitation speed (rpm)	1000	1500	2000	In range
Dependent Variables				
Particle size (nm)				Minimize
%Entrapment Efficiency (%EE)				Maximize

Table 2: Experimental array and recorded responses obtained from randomized runs in BBD.

					Dependent Variables			
		Independent Variables			Particle size (nm)		%EE	
Formulation Code	Runs	A (%w/v)	B (%w/v)	C (rpm)	Actual	Predicted	Actual	Predicted
					Particle (nm)		%EE (%)	
F1	3	0/1	0.25	1500	110.12	110.2	81.28	81.25
F 2	4	0.3	0.25	1500	115.09	115.23	71.22	71.89
F 3	10	0.1	0.75	1500	100.34	100.50	79.54	80.23
F 4	2	0.3	0.75	1500	99.01	99.10	78.62	78.40
F 5	11	0.1	0.5	1000	98.87	98.20	77.32	77.10
F 6	6	0.3	0.5	1000	130.12	130.26	65.73	65.20
F 7	14	0.1	0.5	2000	120.65	119.80	73.56	73.21
F 8	17	0.3	0.5	2000	127.76	126.32	69.96	69.37
F 9	5	0.2	0.25	1000	145.89	145.29	69.17	69.70
F 10	8	0.2	0.75	1000	107.71	107.32	85.62	85.43
F 11	13	0.2	0.25	2000	150.27	150.10	72.21	72.36
F 12	7	0.2	0.75	2000	178.22	177.57	81.31	81.49
F 13	15	0.2	0.5	1500	102.75	102.20	78.55	78.59
F 14	1	0.2	0.5	1500	107.11	106.10	72.48	72.36
F 15	9	0.2	0.5	1500	109.52	109,94	81.67	80.10
F 16	12	0.2	0.5	1500	119.34	119.46	77.45	77.62
F 17	16	0.2	0.5	1500	121.29	120.89	75.38	75.42

A=CS conc. (%w/v), B= TPP conc. (%w/v), C= Agitation speed (rpm).

to the target value, making it more desirable. By utilizing the DF, the supreme favourable and suitable point was identified within the layout domain that achieved the predefined aims for the responses. DoE was employed to determine the maximum DF, allowing assessing the optimal combination of factors to achieve the desired outcomes effectively. Using the DF, the Final Optimized Formulation (FOF) was selected and prepared. Afterward, FOF underwent an evaluation of various parameters and response variables including particle size, %EE (both as aforementioned procedure), zeta potential, Polydispersibility Index (PDI), FTIR spectra, superficial morphology by Scanning Electron Microscopy (SEM), *in vitro* drug release study, release kinetics, accelerated stability studies, *in vivo* antidiabetic activity.

Zeta potential and PDI analysis

FOF was assessed for zeta potential and PDI measurement using the Zetasizer. Prior to the measurements, a diluted sample from FOF was prepared by mixing it with distilled water in a proportion of 1:100 and smoothly agitating to verify even dispersal in the hydrous medium. PDI was evaluated as a measure of homogeneity, with the optimal range having between 0.1 and 0.3. Lower PDI values signify a more uniform and homogeneous population, whereas higher values indicate a higher degree of heterogeneity.[27]

FTIR spectra

In order to assess the potential of drug-component compatibility in the FOF, Infrared (IR) spectroscopy was conducted. The ingredients namely Chitosan (CS), SP, EMP and the SP-EMP-CS NPs (FOF) were subjected to IR spectroscopy after compressing each sample into a disc with Potassium Bromide (KBr) using FTIR (Agilent 4500 Series Portable FTIR) with a detection range from 4000 cm^{-1} to 400 cm^{-1} for each compressed sample.[28,29]

Surface Morphology by SEM

The captivating exploration of FOF and surface morphology unfolded through the marvels of SEM using the Scanning Electron Microscope. To enhance the visual clarity, the samples were adorned with a delicate layer of gold through sputter-coating, allowing for meticulous observation of their intricate morphology. This awe-inspiring exploration took place under an acceleration voltage of 10 kV, while reaching the pinnacle of magnification at an astonishing 50X, unveiling a world of intricate details with unparalleled clarity and precision.[30]

In vitro drug release study and release kinetics

The *in vitro* release behavior of the FOF was meticulously investigated using a dialysis membrane (Himedia, India) with a

pore size of 2.4 nm. The study was accomplished using Phosphate Buffer Saline (PBS) pH 7.4, at a controlled temperature of 37±2°C. To ensure optimal conditions, the dialysis membrane was immersed in the twofold distilled water overnight prior to the release experiments. For the drug release study, the FOF was placed inside a dialysis bag and perched in a container filled with PBS, which was subjected to continuous magnetic agitation to uphold an optimal sink state. At specific time intervals, aliquot specimens were extracted and promptly substituted with equivalent volume of new dissolution fluid. The released drug's concentration was then assessed spectrophotometrically at appropriate wavelength. To facilitate a comparative analysis, the drug release analysis was likewise conducted for the commercially available formulations of SP and EMP (Januvia and Jardiance) utilizing a USP paddle type dissolution apparatus.[31,27]

The kinetic profile of FOF was analyzed and investigated using various mathematical models (zero order, first-order, Higuchi, Korsmeyer-Peppas, Hixson-Crowell). Each of these models was utilized to gain comprehensive insights into the release behavior of FOF from the NPs and to provide a holistic understanding of the release kinetic.[32]

Table 3: Model statistics.

Model Statistics	R1(PS)	R2 (%EE)
Model	Quadratic	Linear
R^2	0.8812	0.4466
Adjusted R^2	0.7284	0.3189
Predicted R^2	-0.3757	-0.0494
SD	11.08	4.41
Mean	119.88	75.47
CV%	9.24	5.84
F Value	5.77	3.50
p Value	0.0154	0.0468
Lack of fit F value	3.02	2.04
Lack of fit *p* value	0.1567	2.568

Accelerated stability studies

As per the guidelines laid out by the International Conference on Harmonization (ICH) Q1A (R2), the FOF underwent a comprehensive stability testing for 3 months span. Freshly prepared NPs were carefully relocated into 5 mL glass vials, taped up securely with plastic caps and entrusted to a stability chamber. The stability chamber was diligently maintained at a constant temperature of 25±2°C and RH of 60±5% throughout the 3-month duration. Within the controlled environment, the formulation underwent meticulous monitoring to assess any change in particle size and %EE. This extensive stability testing was undertaken to ensure the robustness and reliability of the FOF, offering valuable insights into their long-term performance and potential for practical applications.[32]

RESULTS

Fitting data to the model and validation

The ANOVA results indicated the significance of the experimental outcomes, with F values, R^2, predicted R^2 and adjusted R^2 values provided in Table 3. Graphs were utilized to illustrate the relationship between each factor and the different levels, aligning well with the BBD.

Effect of formulation factors on Particle size (R1)

The particle size represents a crucial parameter for the nano formulation and in this research, the size of the developed NPs ranged between 98.87 to 178.22 nm.

Effect of formulation variables on %EE (R2)

The central objective of this research was to optimize the %EE of the formulated NPs. To delve into the influence of formulation variables (CS %w/v, TPP %w/v and agitation speed) on the %EE

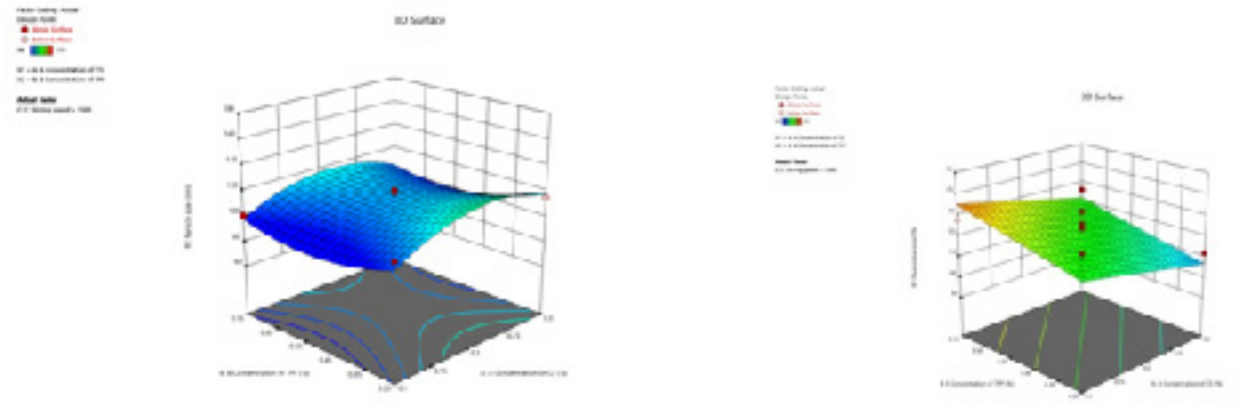


Figure 3: (a):Effect of formulation variables on Particle size (R1). (b): Effect of formulation variables on %EE (R2).

of the prepared NPs, a comprehensive MLRA was conducted employing a linear equation as the foundation of this investigation:

$$\%EE\ (R2)=+75.47-3.38A+3.75B-0.1250C$$

The study revealed (Figure 3b) a notable increase in the %EE with the rise in the concentration of CS and TPP. Identification and evaluation of Optimized formulation using Desirability Function (DF).

The present study employed the DF approach, utilizing DoE 10 trial version, to identify and evaluate the optimum formulation. Constrains were set for all responses, with R1 aimed being minimized and R2 was targeted for maximization. Uniform importance was given to all responses in the software, indicated by +++.

The predicted values of the FOF for R1 and R2 were 86.69 nm and 82.72% respectively. To affirm the efficacy of the optimization, the optimized formulation was replicated 3 times, diligently assessing the outcomes for all the iterations. The recorded and predicted values were compared as shown in Table 4.

The percent biased range was found to be between +2.09% and -3.16%, indicating good agreement between the observed and predicted values.

Zeta potential (ZP) and PDI

The ZP value was found to be +30.5 mV (depicted in Figure 4a) and PDI value was found to be 0.2 as depicted in Figure 4b.

In Figure 5 (a, b, c, d, e) the FTIR spectra of SP, EMP, SP+EMP, CS, SP-EMP-CS NPs (FOF) are presented.

Surface morphology by Scanning Electron Microscopy (SEM)

The surface morphology of FOF was examined using SEM and the corresponding images are displayed in Figure 6. The image indicated that the synthesized NPs exhibited solid and compact structure, displaying nearly spherical shapes with well-defined morphology. Notably, there was no evidence of particle aggregation or agglomeration observed.

In vitro drug release study

In vitro drug release studies were conducted for SP-EMP-CS NPs (FOF) and the marketed formulations of SP and EMP (Januvia and Jardiance) in PBS pH 7.4 at 37±2°C (Figure 7).

Release kinetics

The release mechanism of FOF was investigated by aligning the *in vitro* release data to different kinetic analyses (Figure 8). The model expressions were correlated with the experimental data to obtain the respective R^2 values, based on which the best fit model was determined. The korsmeyer-Peppas model showed the highest R^2=0.9872 for SP and 0.9906 for EMP, subsequently, the first order kinetics R^2=0.9795 and 0.9874). The corresponding values for the tested models are presented in Table 5.

Table 4: Comparison of Predicted and observed experimental values of FOF.

Response	Predicted Value	Observed value	% Bias
Particle size (R1)	86.69 nm	89.43±1.134	-3.16
%EE (R2)	82.72%	80.99±0.859	2.09

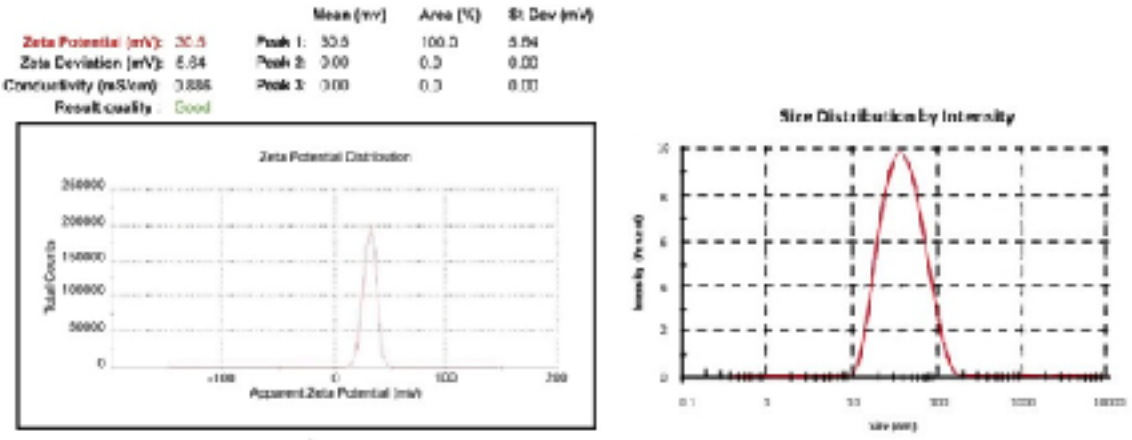


Figure 4: (a): ZP of SP-EMP-CS NPs (FOF). (b): Poly Dispersity Index (PDI) of FOF. Fourier Transform Infra-Red (FTIR) spectra

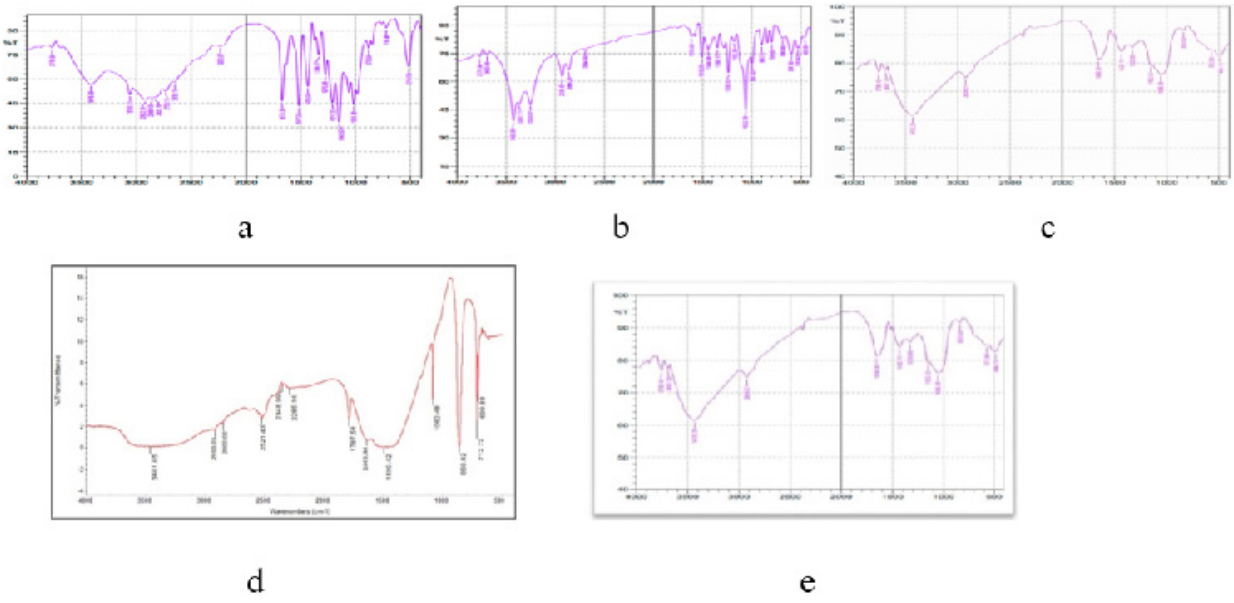


Figure 5: FTIR spectra of- a. SP, b. EMP, c. SP+EMP, d. CS, e. FOF.

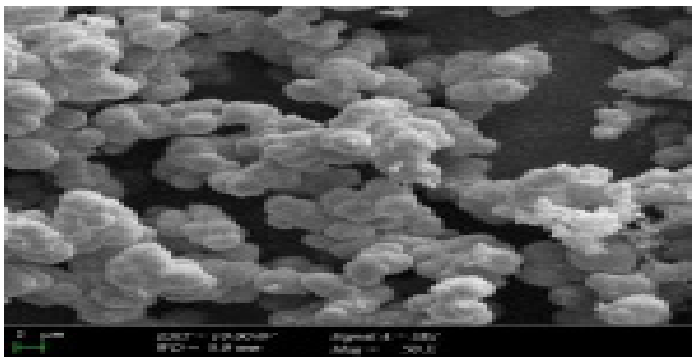


Figure 6: SEM image of SP-EMP-CS NPs (FOF).

Drug release kinetic plots

Accelerated stability studies

Stability studies were performed in triplicate for FOF over a period of 3 months and variations in particle size, ZP and %EE were observed (Table 6).

DISCUSSION

Fitting data to the model and validation

In this research, SP-EMP-CS NPs were prepared using the ion gelation method with CS as the polymer, TPP as the cross-linking agent and tween 80 as the resuspending agent. A BBD was employed to estimate the optimal conditions that had the most significant influence on the response variables. The ANOVA results indicated the significance of the experimental outcomes, with F values, R^2, predicted R^2 and adjusted R^2 values provided in Table 3. Graphs were utilized to illustrate the relationship between each factor and the different levels, aligning well with the BBD.

Effect of formulation factors on Particle size (R1)

One of the primary objectives of this research was to meticulously fine tune NPs dimensions, since their size directly influences the tempo at which drug liberation upholds. To investigate the impact of the formulation factors (CS %w/v, TPP %w/v and stirring speed) on the size of the prepared NPs, Multiple Linear Regression Analysis (MLRA) was performed employing the expression:

$$\text{Particle size (R1)} = +111.60+5.25A-4.37B+11.88C-1.75AB-6.25AC+16.50BC-15.80A^2+10.45B^2+22.95C^2$$

The manipulation of CS concentration (from 0.1-0.3%) had an intriguing effect on particle size (Figure 3a). The subtle increase observed can be attributed to the rise in viscosity within the CS fluid, which consequently limited the stirrer's shear competence, ultimately influencing the size of the particles. Similarly, the study revealed that the increasing concentration of crosslinking agent TPP directed to a reduction in particle size. This phenomenon can be accredited to the significant positive charge (amine group) entanglement in CS and the anionic charge resulting from PO4- ions in TPP. Consequently, at higher TPP concentrations, the polymer layer tends to shrink, contributing to the observed decrease in particle size. Conversely, when the stirring speed was altered at different levels, a remarkable decrease in particle size occurred.

Effect of formulation variables on %EE (R2)

The central objective of this research was to optimize the %EE of the formulated NPs. To delve into the influence of formulation variables (CS %w/v, TPP %w/v and agitation speed) on the %EE of the prepared NPs, a comprehensive MLRA was conducted employing a linear equation as the foundation of this investigation:

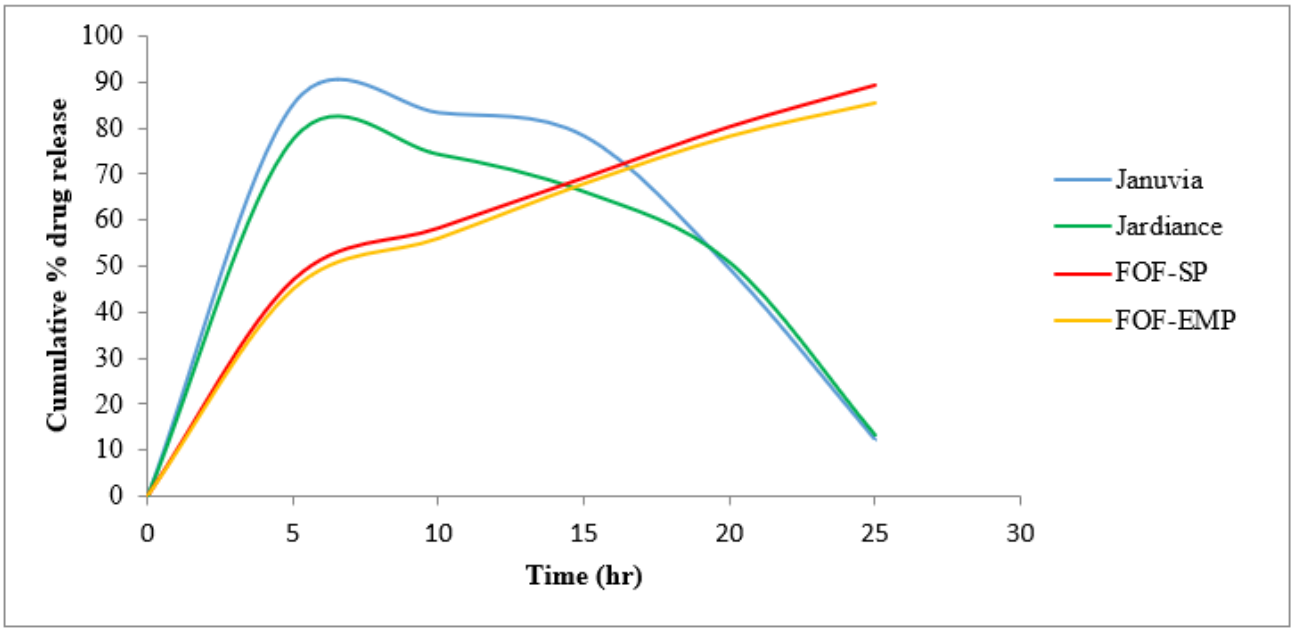


Figure 7: Comparative *in vitro* drug release data of FOF and marketed formulations.

Table 5: Comparative analysis of release profiling of FOF through different mathematical models.

Kinetic data	Optimized Formulation (FOF)	
R^2	SP	EMP
Zero order	0.8732	0.8718
First order	0.9795	0.9874
Higuchi	0.8732	0.8718
Korsmeyer-Peppas	0.9872	0.9906
Hixon-Crowell	0.9708	0.9664
n-value	0.4005	0.4058

%EE (R2)=+75.47-3.38A+3.75B-0.1250C

The study revealed (Figure 3b) a notable increase in the %EE with the rise in the concentration of CS and TPP. This observation is likely attributed to the formation of a robust and rigid structure within the prepared NPs, resulting from the crosslinking interaction between the CS and TPP.

Figure 9 illustrates the standardized effect of various factors on responses.

Identification and evaluation of Optimized formulation using Desirability Function (DF)

The FOF with the best DF, meeting the maximum requirements of response variables was chosen. The FOF consisted of A at 0.1% w/v, B at 0.75% w/v and C at 1000 rpm with an overall desirability of 0.903. The predicted values of the FOF for R1 and R2 were 86.69 nm and 82.72% respectively. To affirm the efficacy of the optimization, the optimized formulation was replicated 3 times, diligently assessing the outcomes for all the iterations. The percent biased range was found to be between +2.09% and -3.16%, indicating good agreement between the observed and predicted values.

Zeta Potential (ZP) and PDI

ZP plays a crucial role in determining particle stability and mucoadhesivity. The cationic CS is accountable for high +ZP value resultant of the acetic acid-catalyzed addition of the proton of amino groups. +ZP facilitates strong mucoadhesion with mucin (containing anionic sialic acid and sulfonic acid residues), which in turn affects the drug release kinetics by improving the entrapment efficiency and prolonged release profiles. Consequently, the obtained ZP value +30.5 mV deemed advantageous in preserving the physical stability of the FOF through possessing strong positive charge which creates electrostatic repulsion between particles and prevents aggregation. Higher ZP values indicate increased stability by preventing particle aggregation. The obtained ZP value is within the standard range of +20 mV to +40 mV reported in the literatures related to CS-based NPs utilized in drug delivery systems. Hence the obtained ZP value is in alignment with the existing literature.

PDI a dimensionless parameter assesses the diversity of particle sizes within the NPs. The achieved value of PDI (0.2) suggests a relatively narrow size distribution of the particles.[33] The FOF demonstrated a relatively narrow particle size distribution, evident from the low PDI value of 0.2. This low PDI value further signifies the homogeneous nature of the formulation, further contributing to their stability and uniformity.

Fourier Transform Infra-Red (FTIR) spectra

The IR spectrum of SP displays Figure 5(a) characteristic peaks at 3415.90 cm^{-1} (indicative of the amine functional group), 2922.16 cm^{-1}, 3059.10 cm^{-1} (associated with C-H stretching), 1670.35 cm^{-1} (related to the C=O group as ketone), 1211.30 cm^{-1} (signifying C-F stretching) and 1149.57 cm^{-1} (C-N stretching of aromatic amine).

In Figure 5 (b), the IR spectra of EMP reveals distinctive absorption bands at various wavelengths 3425.58 cm^{-1} (attributed to O-H stretching), 2929.87 cm^{-1} (related to C-H stretching), 1238.30 cm^{-1} (indicative of C-O stretching for alkyl aryl ether), 1062.78 cm^{-1} (attributed to C-O stretching) and 686.86 cm^{-1}, 796.60 cm^{-1} (indicative of C-Cl stretching).

The Figure 5 (c) depicts FTIR spectra of SP+EMP, showcasing all the characteristics peaks of both SP and EMP, suggesting that there is no noteworthy interaction between SP and EMP, indicating their compatibility in the formulation.

The Figure 5 (d) represents the FTIR spectra of CS, which exhibits distinct peaks at 3461.65 cm^{-1} (attributed to intermolecular hydrogen bond stretching involving -OH groups and N-H

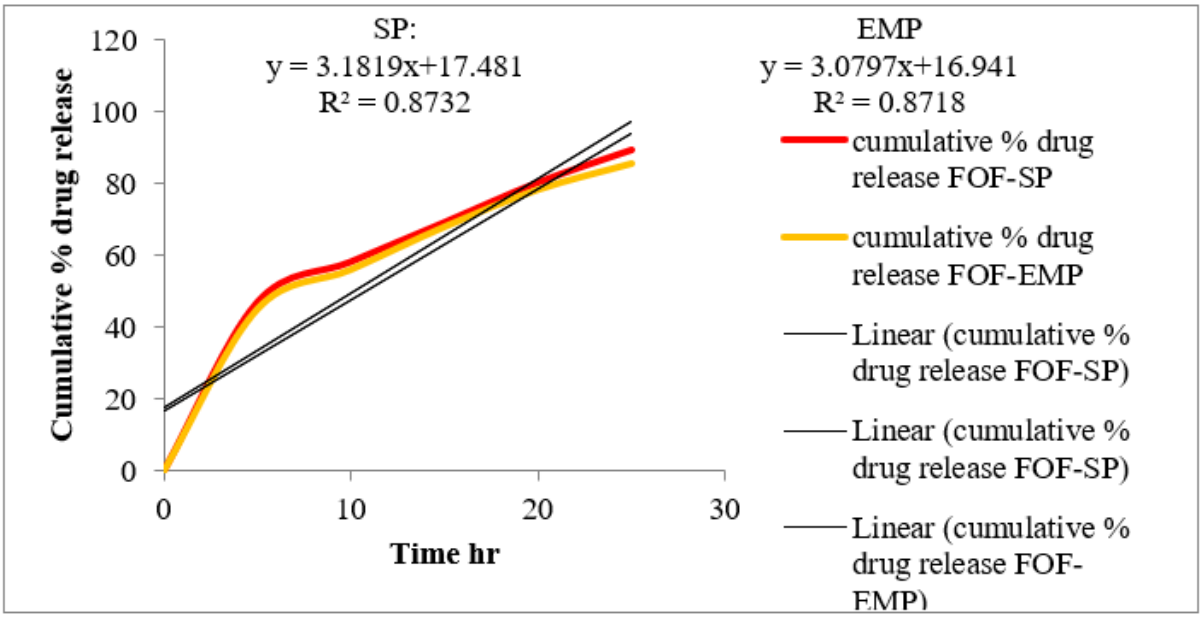


Figure 8 (a): Zero Order release kinetics.

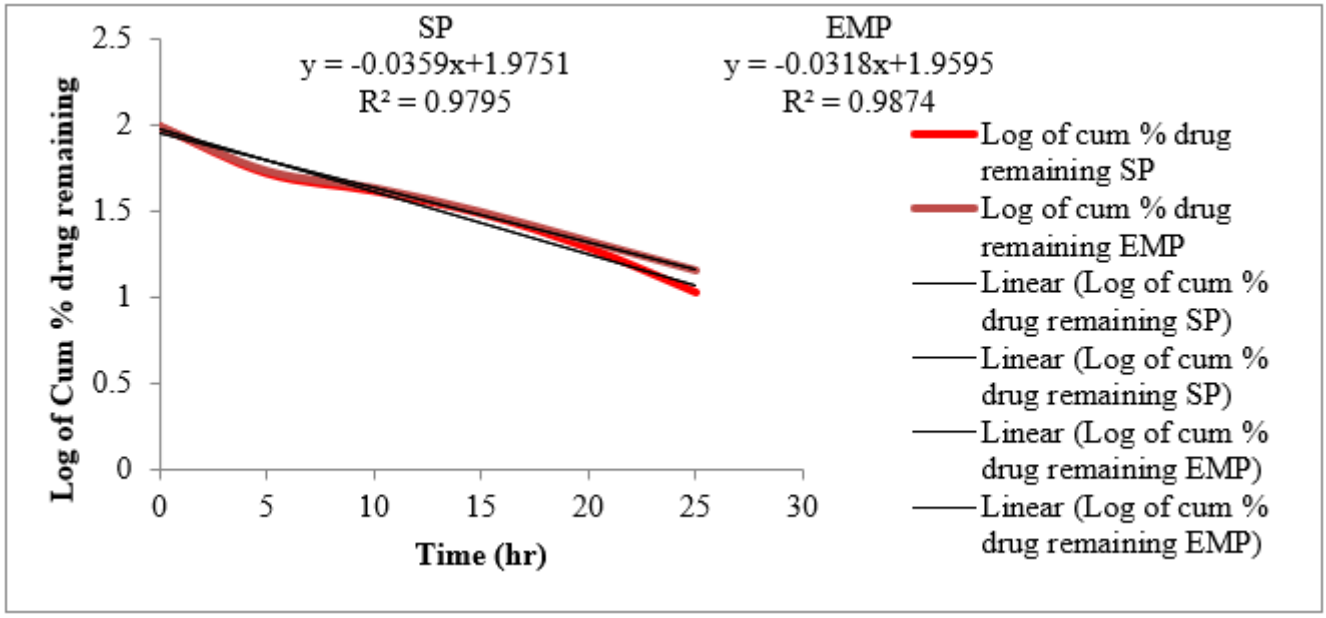


Figure 8 (b): First Order release kinetics.

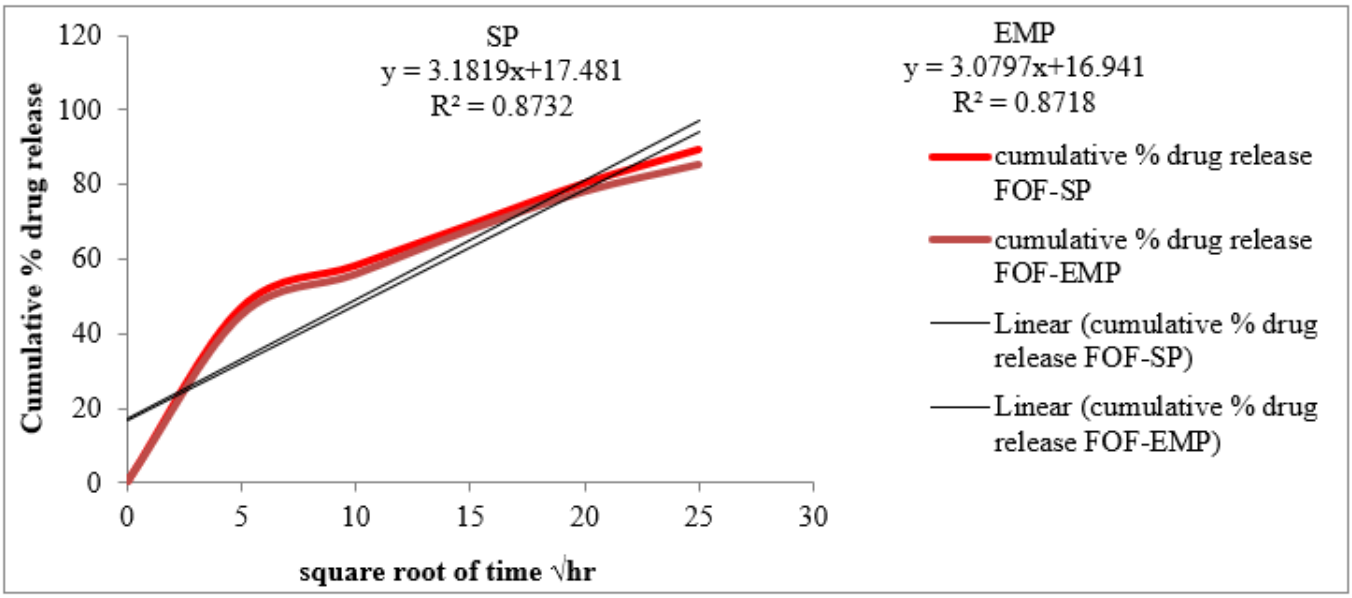


Figure 8 (c): Higuchi release kinetics.

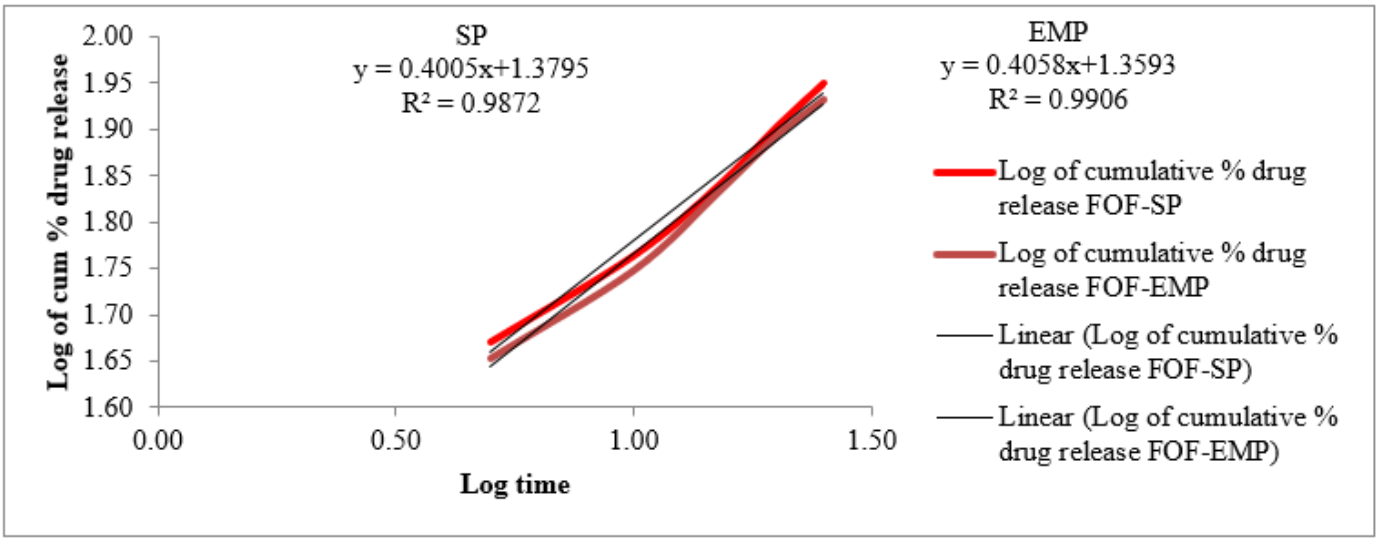


Figure 8 (d): Korsmeyer-Peppas kinetics

vibrations), 1643.44 cm^{-1} (related to C=O), 2910.01 cm^{-1}, 2800.02 cm^{-1} (ascribed to CH_2 stretching vibrations), 1082.48 cm^{-1} (associated with C-N stretching).

The Figure 5 (e) represents the FTIR spectra of SP-EMP-CS NPs (FOF), which exhibits all the characteristics peak of SP, EMP and CS indicating absence of noteworthy interaction between drugs and polymer.

Surface morphology by Scanning Electron Microscopy (SEM)

The SEM image indicated that the synthesized NPs exhibited solid and compact structure, displaying nearly spherical shapes with well-defined morphology. Notably, there was no evidence of particle aggregation or agglomeration observed.

In vitro drug release study

The drug release profile of FOF exhibited a biphasic pattern, with an initial burst release observed with approximately 47% of the drug released during the first 4 hr (ascribed to the dissolution and diffusion of inadequately entrapped drug molecules within the system) and around 89.21% (SP) and 85.47% (EMP) release at 24 hr (attributed to slow and continuous release resulted from drug diffusion from the polymer matrix). In contrast, Januvia demonstrated 85.1% cumulative drug release while Jardiance exhibited 77.62% cumulative drug release within the first 2 hr. The findings endorsed the use of the NP formulation as a method

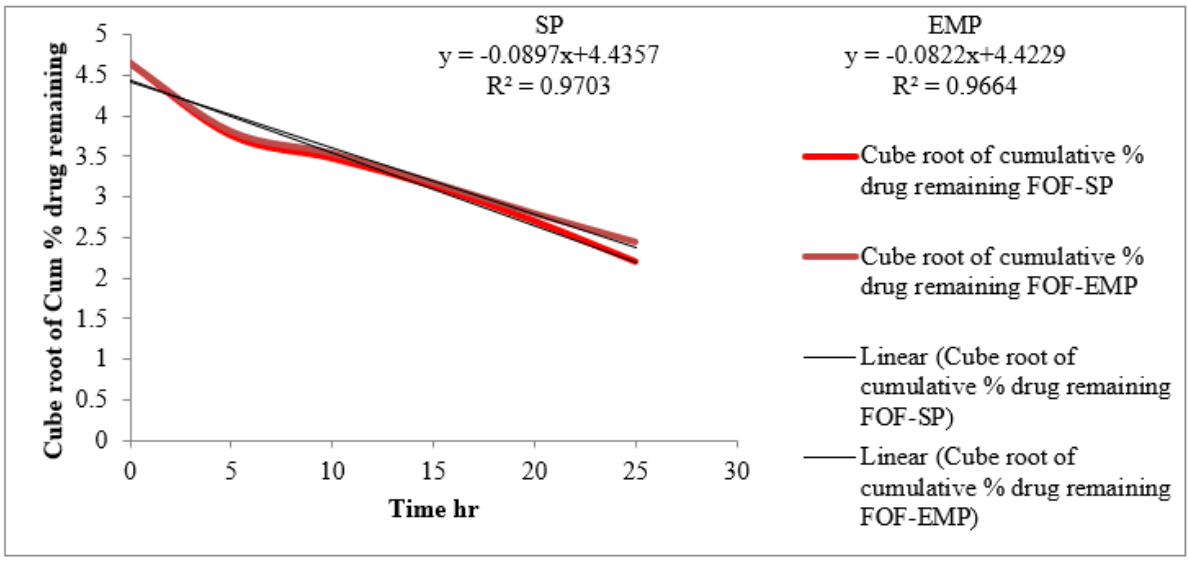


Figure 8 (e): Hixon-Crowell Plot.

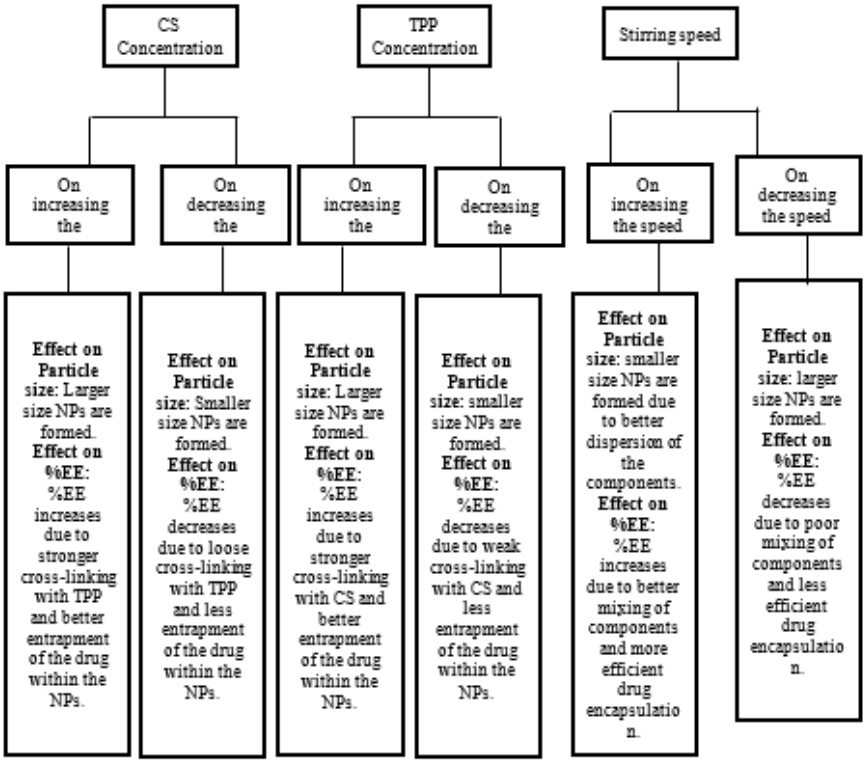


Figure 9: Standardized effect of various factors on Responses.

Table 6: Accelerated stability study data.

Stability parameter	Assessment Interval (in month)			
	0	1	2	3
Particle Size (nm)	89.43±1.13	91.95±0.75	94.68±0.56	98.66±0.54
ZP (mV)	30.5±0.2	29.8±0.15	28.2±0.20	27.5±0.40
%EE (%)	80.99±0.86	77.11±0.97	74.2±0.79	72.9±0.85

of controlled drug delivery with enhanced bioavailability for the given issue.

Release kinetics

In the context of the developed FOF, the derived values of n 0.4005 (for SP) and 0.4058 (for EMP), eloquently indicate a profound adherence to Fickian diffusion as the governing mechanism for the release behavior.

Accelerated stability studies

The particle size exhibited a slight increase from 89.43±1.13 to 98.66±0.54 nm during the stability studies, which was attributed to the aggregation of small NPs leading to an enlargement of their size. Despite this slight change in particle size, the ZP of the optimized batch remained consistent after 3 months, with values changing from +30.5±0.2 mV to +27.5±0.40 mV. This advocated that the drug was successfully recollected within the NPs during the stability period. Furthermore, there was a marginal diminution in %EE from 80.99±0.86 to 72.9±0.85 possibly due to drug expulsion from the polymer matrix during storage. Comprehensively, the derived outcomes demonstrated no noteworthy alterations in particle size, ZP and %EE throughout the quarter year of preservation, warranting the stability of the NPs.

CONCLUSION

The primary challenge in formulating new chemical entities or existing drug molecules lies in their poor aqueous solubility followed by poor permeability. Conventional approaches often struggle to address these issues, leading to various pharmacological or therapeutic performance concerns. However, NPs bid an encouraging solution for augmenting the solubility and oral bioavailability of poor soluble and permeable drugs.

It is worth noting that BBD coupled with the DF emerges as a promising and noteworthy avenue to delve into the intricate interplay of formulation variables, ingeniously orchestrating their optimization to craft and exceptionally efficient and finely tuned formulation.

In this investigation established NPs were successfully formulated employing the ionic gelation technique. The FOF (SP-EMP-CS NPs) demonstrated ideal parameters, featuring minimal particle size and maximum %EE. Through *in vitro* studies it was observed that the FOF exhibited superior *in vitro* characteristics when compared to the conventional marketed formulations,

In summation, the strategic development of CS nanoparticles through ion gelation method serves as a compelling and efficacious maneuver, propelling the amelioration of oral bioavailability for SP and EMP.

This research emphasizes how poorly soluble and poorly permeabilized antidiabetic medications (SP and EMP), may be managed by using nanoparticulate drug delivery systems. By using BBD to successfully optimize formulation factors, SP-EMP-CS NPs with improved *in-vitro* properties have been developed over traditional formulations. But it's important to recognize that more study is required to completely utilize SP-EMP-CS NPs in therapeutic environments. For these formulations to be successful in translation, challenges including scalability, long-term stability, biocompatibility and *in-vivo* performance must be resolved. Furthermore, confirming the therapeutic advantages and safety of the formulation would require investigating their pharmacokinetic and pharmacodynamic characteristics in animal models and subsequently in human trials. Despite these difficulties the results provide an enormous advancement in the management of diabetes, opening the door to increased therapeutic efficacy and better patient outcomes.

ACKNOWLEDGEMENT

The authors are grateful for the opportunity to conduct a portion of their research at Acropolis Institute of Pharmaceutical Education and Research, Indore, M.P., India.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

SP: Sitagliptin Phosphate; **EMP:** Empagliflozin; **BBD:** Box Behnken design; **CS**: Chitosan; **NPs:** Nanoparticles; **T2DM:** Type-2 diabetes mellitus; **TPP:** Tripolyphosphate; **DF:** Desirability Function; **FOF:** Final Optimized Formulation; **ANOVA:** Analysis of Variance; **ZP**: Zeta Potential; **PDI:** Poly Dispersity Index; **SEM:** Scanning Electron Microscopy.

SUMMARY

The present study efficaciously dealt with the challenges belonging to the BCS class III drugs (SP and EMP) regarding formulating them as Nanoparticulate system. Precisely optimized chitosan-based nanoparticles of SP and EMP were prepared utilizing the BBD and DF, which are having remarkable stability, positive ZP (+30.5 mV), prominent particle size (89.43 nm) and entrapment efficiency (80.99%). The uniformity and consistency of the optimized nanoparticles were confirmed by SEM and *in vitro* assessment parameters. Broadly the developed FOF not only augments the bioavailability of poorly permeable SP and EMP but also institutes a valuable context for future optimization accomplishments. The incorporation of BBD and the DF has been recognized to be an influential tactic, proposing acumens into the complex interaction of independent variables and enabling the formation of a vigorous, optimized formulation with superior responses. This research contributes to the advancement of Nanoparticulate drug delivery systems and holds promise for improving the therapeutics efficacy of BCS class III drugs.

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Cite this article: Dwivedi A, Sharma R. Box-Behnken Design: Unleashing the Potential of Desirability Function for Enhanced Permeability of Antidiabetic Nanoparticles. Indian J of Pharmaceutical Education and Research. 2025;59(2s):s494-s507.