Journal of Pharmaceutical Research International

33(46B): 273-286, 2021; Article no.JPRI.75270
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

Optimization of Site-Specific Drug Delivery System of Tyrosine Kinase Inhibitor Using Response Surface Methodology

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Authors' contributions

This work was carried out in collaboration among all authors. Author SPK designed the study, wrote the protocol and supervised the entire work. Author CMR performed the study and carried out analysis of the study. Author CBR managed the literature searches and prepared manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i46B32941
Editor(s):
(1) Dr. Prem K. Ramasamy, Brandeis University, USA.
Reviewers:
(1) D. J. Sen, Techno India University, India.
(2) Sateesh Kumar Vemula, Lovely Professional University, India.
Complete Peer review History: https://www.sdiarticle4.com/review-history/75270

Original Research Article

Received 10 August 2021
Accepted 14 October 2021
Published 21 October 2021

ABSTRACT

Aims: The aim of present study was to develop a stomach specific formulation of Imatinibmesylate to increase the fraction of drug absorbed in stomach.
Study Design: Development and Optimization of Microspheres for site specific delivery..
Place and Duration of Study: The study was carried out in Department of Pharmacy, Annamalai University, between October 2020 and July 2021.
Methodology: Ionotropic gelation method with Sodium alginate and Chitosan were used to formulate the mucoadhesive microspheres with calcium chloride. The formulation was optimized using Box – Behnken design to study the effect of independent variables, Amount of Sodium Alginate (X1), Amount of Chitosan (X2) and concentration of Calcium Chloride (X3) on dependent variables Particle Size (Y1), Entrapment Efficiency (Y2) and In-vitro drug release (Y3).
Results: Particle size of prepared microspheres varied from 458.25 to 810.75 µm, entrapment efficiency from 64.87 to 82.63% and *in-vitro* release from 69.22 to 83.50%. The optimized formulation was found using point prediction, and formulation showed optimum results. The drug release was controlled for more than 12 h.

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Conclusion: Stomach specific formulation of Imatinibmesylate was successfully optimized by a three-factor, three level Box – Behnken design.

Keywords: Stomach specific; Ionotropic gelation; Response Surface Methodology; Box – Behnken design.

1. INTRODUCTION

Response surface methodology (RSM) is an approach to produce and process optimization work [1]. RSM was introduced by Box and Wilson in 1951, and later popularized by Montgomery. As per the introducer of the idea, response surface methodology can be defined as an empirical statistical technique employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariate equations simultaneously. The graphical representations of these equations are called response surfaces, which can be used to describe the individual and cumulative effect of the test variables on the response and to determine the mutual interactions between the test variables and their subsequent effect on the response [2]. It consists of a combination of statistical experimental design fundamentals, regression modelling techniques, and optimization methods. RSM uses design of experiments techniques (DOE), such as Box – Behnken design (BBD), central composite design (CCD), full and fractional factorial designs, as well as regression analysis methods. DOE techniques are employed before, during and after the regression analysis to evaluate the accuracy of the model. Design of experiments (DOE) is a statistical technique that can be used for optimizing such multivariable systems. In recent years, the pharmaceutical industry has used experimental designs more for the optimization of pharmaceutical agents; however, only a few are reported in the literature for the development of dosage forms [3,4].

By applying RSM method in the optimization process, only a short period of time is required to test all of the variables pertaining to the consumer evaluation, making the laboratory test stage more efficient. In addition, parameters estimation can identify the variables that are largely affecting the model which then helps researcher to focus on those particular variables that contribute to the product acceptance.

It is often desirable to use the smallest number of factor levels in an experimental design. One common class of such designs is the Box – Behnken designs. These are formed by combining two factorials with balanced incomplete block designs, which reduces the number of experiments considerably. As an example, for a three factor, three-level study, only 15 experiments are required with this design, whereas the full factorial design would require 27 experiments. The design consists of replicated centre points and the set of points lying at the midpoints of each edge of the multidimensional cube that defines the region of interest. Besides, Box – Behnken design is suitable for the exploration of quadratic response surfaces and construction of a second-order polynomial model.

Dosage forms that can precisely control the release rates and target drugs to a specific body site have created enormous impact in formulation and development of novel drug delivery systems. Microspheres form an important part of the novel drug delivery systems. They have varied applications and are prepared using various polymers.

Microsphere carrier systems made from the naturally occurring biodegradable polymers have attracted considerable attention for several years in sustained drug delivery [5]. However, the success of these microspheres is limited due to their short residence time at the site of absorption. It would, therefore be advantageous to have means for providing an intimate contact of the drug delivery system with the absorbing membranes [6,7]. This can be achieved by coupling bioadhesion characteristics to microspheres and developing bioadhesive microspheres.

Bioadhesive microspheres have advantages like efficient absorption and enhanced bioavailability of the drugs due to a high surface to volume ratio, a much more intimate contact with the mucus layer and specific targeting of drugs to the absorption site [8,9,10]. Chitosan was selected as a polymer in the production of bioadhesive microspheres due to its mucoadhesive and biodegradable properties. Chitosan (obtained by deacetylation of chitin,) is a cationic polymer that has been proposed for use in microsphere systems by various authors [11,12].

ImatinibMesylate is an anti-cancer agent which is used to treat chronic myelogenous leukemia (CML), gastrointestinal stromal tumors (GISTs) and a number of other malignancies. It is the first member of a new class of agents that act by inhibiting particular tyrosine kinase enzymes, instead of non-specifically inhibiting rapidly dividing the cells. In the present study, we have developed a site-specific mucoadhesive multiunit system to increase the bioavailability using process optimization software. A three-factor, three-level Box – Behnken design was applied to the formulation for designing and selecting the optimum formulation. The formulations were prepared using ionotropic gelation method, and evaluated for size, entrapment efficiency and *in-vitro* drug release.

2. MATERIALS AND METHODS

2.1 Materials

Imatinib Mesylate was a kind gift sample from Hetero Drugs, Hyderabad, whereas Sodium alginate and Calcium chloride were from Thomas Baker chemicals, Mumbai. Chitosan from Cochin Fisheries Department, Cochin. All other reagents were of analytical grade.

2.2 Formulation Development

Mucoadhesive alginate microspheres were prepared by emulsification ionic gelation technique. Sodium alginate and copolymer Chitosan was dispersed in deionised water separately with continuous stirring to form homogenous polymer dispersion and both the dispersions were added. Imatinibmesylate was added to polymer dispersion and mixed thoroughly to form a viscous suspension. The dispersions were sonicated for 30 mins to remove any air bubbles. The stream of smooth viscous suspension was added to light liquid paraffin in the form of a thin stream. Stirring of the above mixture was done in a beaker placed on mechanical stirrer. Then Calcium Chloride solution was added slowly and stirring was continued for 15 minutes. The mixture was allowed to settle and product was separated. Obtained microspheres were washed several times with Petroleum ether to remove the adhering paraffin and dried in room temperature [13]. The formulations were prepared using Box – Behnken experimental design, and optimized formulation was generated using statistical screening. Seventeen runs of the experiment were evaluated for particle size, drug entrapment efficiency and in-vitro drug release.

2.3 Experimental Design

A three-factor, three-level design is suitable for exploring quadratic response surfaces and for constructing second order polynomial models with Design Expert. The independent and dependent variables are listed in Table 1 along with their low, medium and high levels. The polynomial equation generated by this experimental design is given as-

$$\mathrm{Yo} = b_0 + b_1A + b_2B + b_3C + b_{12}AB + b_{13}AC + b_{23}BC + b_{11}A^2 + b_{22}B^2 + b_{33}C^2$$

Where Yo is the dependent variable, corresponding to either particle size (Y1) or drug entrapment efficiency (Y2) or in-vitro drug release (Y3), and A, B and C are the independent variables representing amount of sodium alginate, Chitosan and concentration of Calcium chloride respectively. b0 is a constant; b1, b2 and b3 are the coefficients translating the linear weight of A, B and C, respectively; b12, b13 and b23 are the coefficients translating the interactions between the variables; and b11, b22 and b33 of the coefficients translating the quadratic influence of A, B and C. Linear and second-order polynomials were fitted to the experimental data to obtain the regression equations, and their observed and predicted responses.

Table 1. Process parameters for Experimental design

Process Parameters	**Levels**		
Independent Variables	**(-1)**	**(0)**	**(+1)**
(A) Sodium alginate (mg)	500	750	1000
(B) Chitosan (mg)	500	750	1000
(C) Calcium chloride (%)	2.5	5	7.5
Dependent Variables			
(Y1) Particle size			
(Y2) Drug entrapment efficiency			
(Y3) In-vitro drug release			

2.4 Evaluation of Formulations

2.4.1 Particle size analysis

Many methods are available for determining the particle size, such as optical microscopy, sieving, sedimentation and particle volume measurement. Optical microscopy is most commonly used for particle size determination. The optical microscope is fitted with an ocular micrometer and stage micrometer. The eyepiece micrometer was calibrated. The particle diameters of more than 200 microspheres were measured randomly by optical microscope [14].

The average particle size is determined by using Edmondson's equation:

$$\boldsymbol{D_{mean}} = \frac{\sum \mathrm{nd}}{\sum \mathrm{n}}$$

Where,

n - Number of microspheres observed.
d - Mean size range.

2.4.2 Drug entrapment efficiency

To determine the amount of drug encapsulated in microspheres, a weighed quantity of microspheres was crushed in a glass mortar and pestle and the powdered microspheres were suspended in 100 ml of 0.1 N HCl. After 24 hours the solution was filtered and 1 ml of filtrate was pipetted out and diluted to 25 ml and analyzed for the drug content using UV-Specrophotometer at 255 nm. The drug entrapment efficiency was calculated using the following formula:

$$Drugentrapmentefficiency(\%) = \frac{Estimateddrugcontent}{Theoriticaldrugcontent} \times 100$$

Theoretical drug content was determined by calculation assuming that the entire Imatinib present in the polymer solution used gets entrapped in Imatinibmesylae microspheres, and no loss occurs at any stage of preparation of Imatinibmesylate microspheres [15,16,17].

2.4.3 *In-vitro* drug release studies

Dissolution studies were carried out by using USP type - I dissolution assembly in stimulated gastric fluid pH 1.2. A weighed amount of microspheres equivalent to 400 mg drug were dispersed in 900 ml of 0.1 N HCl (pH 1.2) maintained at 37 ± 0.5°C and stirred at 100 rpm. Five ml of aliquots were withdrawn at 60 minutes intervals and filtered. The required dilutions were made with 0.1 N HCl and the solutions were analyzed for the drug content by UV spectrophotometer against suitable blank at 255nm. From this the percentage of drug released was calculated and plotted against function of time [13].

2.4.4 Kinetic characteristics of the drug release

To know the mechanism of the drug release from the microspheres, the results obtained from the *In-vitro* dissolution process were fitted into different kinetic equations as follows [18,19,20].

1. Zero order drug release: Cumulative % drug release Vs Time.
2. First order drug release: Log cumulative % drug retained Vs Time.
3. Higuchi's classical diffusion equation: Cumulative % drug release Vs Square root of time.
4. Peppa"s Korsemeyer Exponential equation: Cumulative % drug release Vs Log time.

"n" values can be used to characterize diffusion release mechanism.

2.5 Statistical Analysis

Results were determined and expressed as mean ± S.D of three determinations. Response surface methodology (RSM) using Box – Behnken Design was used to carry out statistical analysis using Design Expert software. The components of microspheres were taken as process variables, and their effect on Particle size, Entrapment efficiency and Drug release statistically was analyzed using ANOVA. The differences were considered significant at a level of $p < 0.05$.

2.6 Optimization of Formulation through Response Analysis

Polynomial equation produced by optimization software was validated by using ANOVA application. A total of seventeen runs (F01 – F17) were evaluated in terms of statistically significant coefficients and R squared values. The composition of optimized formulation was found by validating the results over the entire

experimental region. One optimum formulation was selected to validate the chosen experimental design and polynomial equations. The predicted optimum formulation was formulated and checked for various responses. The observed values were compared with predicted values, and linear regression plots between actual and predicted values of the responses were generated by optimization software.

3. RESULTS AND DISCUSSION

An experimental design of seventeen runs was generated for three factors at three levels to identify the optimum levels of different independent process parameter according to Box – Behnken design. Table 2 shows the observed responses along with the predicted values for designed formulations. The observed values for particle size, entrapment efficiency, and drug release range from 458.25 to 810.75 µm, 64.87 to 82.63%, and 69.22 to 83.50%, respectively. The responses were simultaneously fitted to linear, two-factor interaction (2FI), cubic and quadratic models using Design Expert software. The values of R-squared, Adj -squared, Pred R-squared, SD and % CV are shown in Table 3 along with the regression equation. Since the cubic model was aliased due to insufficient design points to estimate the coefficients, the quadratic model was chosen for its larger adjusted R-squared value. The ANOVA values for different responses are represented in Table 4, and all statistically significant ($p<0.05$) coefficients are included in the equations. As per the optimization design, a positive value shows favorable optimization, whereas a negative value shows an inverse relationship between the factor and the response. It is evident that all the three independent variables, namely the amount of sodium alginate (A), Chitosan (B), concentration of Calcium chloride (C), have interactive effects on the three estimated responses, for example, particle size (Y1), drug entrapment efficiency (Y2) and drug release (Y3).

3.1 Effect on Particle Size (Y1)

The model proposes the following equation for particle size;

$$ParticleSize: 732.95 + 72.3125A + 18.6563B + 3.59375C - 17.3125AB + 54.6875AC - 26.25BC - 66.85A^2 - 102.788B^2 + 29.9625C^2.$$

Where A is the Amount of Sodium alginate; B is the Amount of Chitosan, and C is the concentration of Calcium chloride. The Model F-value of 39.26 implies the model is significant. There is only a 0.01% chance that an F-value could occur due to noise. P-values less than 0.0500 indicate model terms A, B, AC, BC, A^2, B^2, C^2 are significant. The Lack of Fit F-value of 3.47 implies the Lack of Fit is not significant. The Predicted R^2 of 0.7670 is in reasonable agreement with the Adjusted R^2 of 0.9556. Adequate Precision value of 25.362 indicates an adequate signal. This model can be used to navigate the design space. Figs. 1, 2 and 3 are the response surface plot showing the effect of different independent variables on the particle size of microspheres. The results showed that an increase in polymers concentration resulted in an increase in the particle size of microspheres. In our study, formulation F17 showed maximum particle size, that is, 810.75 µm (at Sodium alginate (+1), Chitosan (0) and Calcium chloride (+1)). This could be due to higher concentration of sodium alginate and calcium chloride. Sodium alginate increases the droplet size, and the increase in concentration of cross-linking agent causes formation of larger mesh work.

3.2 Effect on Entrapment Efficiency (Y2)

The model proposes the following equation for drug entrapment efficiency:

$$EntrapmentEfficiency = 79.332 + 1.7075A - 0.64B + 3.9075C - 0.27AB - 1.025AC - 0.73BC - 8.6785A^2 - 2.7485B^2 + 0.6265C^2.$$

Where A is the Amount of Sodium alginate; B is the Amount of Chitosan, and C is the concentration of Calcium chloride. The Model F-value of 26.88 implies the model is significant. There is only a 0.01% chance that an F-value could occur due to noise. P-values less than 0.0500 indicate model terms A, C, A^2, B^2 are significant. The Lack of Fit F-value of 0.53 implies the Lack of Fit is not significant. The Predicted R^2 of 0.8401 is in reasonable agreement with the Adjusted R^2 of 0.9357. Adequate Precision value of 15.93 indicates an adequate signal. This model can be used to navigate the design space. Figs. 4,5 and 6 ares the response surface plot showing the effect of different independent variables on percentage drug entrapment. Formulation F16 showed maximum entrapment efficiency, that is, 82.34 % (Sodium algiante at 0 level, Chitosan at -1 level, and Calcium chloride at +1 level), while F3

showed minimum entrapment efficiency (Sodium algiante at -1 level, Chitosan at +1 level, and Calcium chloride at 0 level). This implies that at lower concentration of Sodium alginate, lesser volume of cross-linked network is present which has a negative effect on entrapment efficiency.

Table 2. Observed and predicted values of responses of Box – Behnken design

Formulation Code	Run	Independent Variables			Dependent variables					
					Actual			Predicted		
		(A)	(B)	(C)	(Y1)	(Y2)	(Y3)	(Y1)	(Y2)	(Y3)
F01	1	0	+1	-1	702.50	73.54	76.87	701.44	73.39	76.90
F02	2	0	-1	-1	592.50	72.58	75.91	611.63	73.21	76.00
F03	3	0	0	0	754.25	79.52	77.52	732.95	79.33	77.47
F04	4	-1	0	-1	690.75	65.45	74.28	674.84	64.64	74.41
F05	5	-1	-1	0	458.25	66.39	80.38	455.03	66.57	80.16
F06	6	0	0	0	720.75	78.87	78.33	732.95	79.33	77.47
F07	7	+1	-1	0	651.25	71.48	74.26	634.28	70.52	74.42
F08	8	+1	0	-1	712.25	69.78	73.77	710.09	70.11	73.51
F09	9	-1	0	+1	570.50	74.83	82.68	572.66	74.50	82.94
F10	10	0	0	0	723.25	76.88	78.88	732.95	79.33	77.47
F11	11	0	0	0	735.75	81.28	75.23	732.95	79.33	77.47
F12	12	+1	+1	0	633.75	68.88	69.22	636.97	68.70	69.44
F13	13	0	+1	+1	675.25	80.38	74.81	656.13	79.75	74.72
F14	14	0	0	0	730.75	80.11	77.38	732.95	79.33	77.47
F15	15	-1	+1	0	510.00	64.87	77.43	526.97	65.83	77.27
F16	16	0	-1	+1	670.25	82.34	83.50	671.31	82.49	83.47
F17	17	+1	0	+1	810.75	75.06	70.40	826.66	75.87	70.27

Table 3. Analysis of variance results of calculated model

ANOVA Results	Particle Size	Entrapment Efficiency	In-vitro Drug release
Regression			
Sum of Squares	129000	515.88	218.40
Degrees of freedom	9	9	9
Mean Square	14328.53	57.32	24.27
F-value	39.26	26.88	20.99
p-value	< 0.0001	0.0001	0.0003
Residual			
Sum of Squares	2555.00	14.92	8.09
Degrees of freedom	7	7	7
Mean Square	365.00	2.13	1.16
Lack of Fit			
Sum of Squares	1845.70	4.26	0.3362
Degrees of freedom	3	3	3
Mean Square	615.23	1.42	0.1121
F-value	3.47	0.5332	0.0578
p-value	0.1303	0.6837	0.9794
Correlation Co-efficent (R^2)	0.9806	0.9719	0.9643
Correlation of variation (%CV)	2.86	1.97	1.41

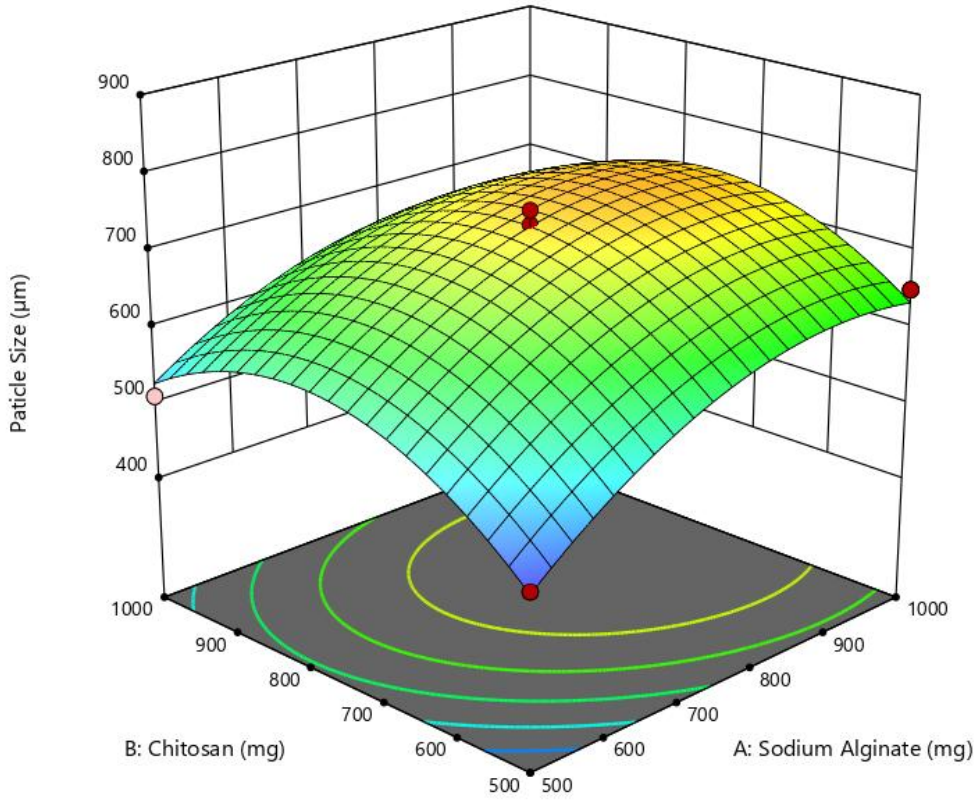

Fig. 1. Effect of Sodium alginate and Chitosan on Particle Size

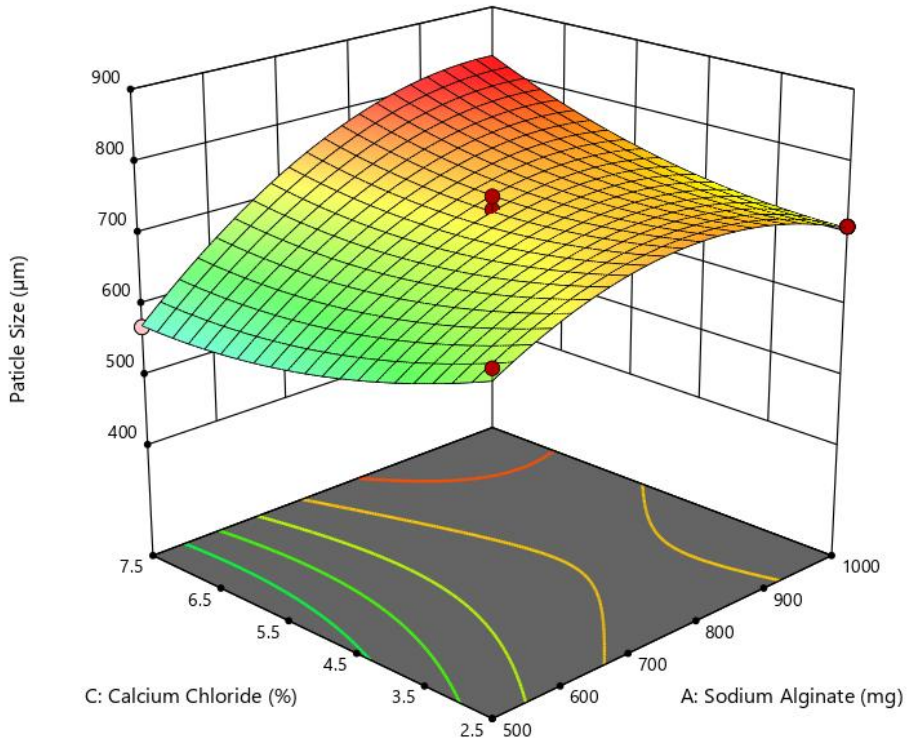

Fig. 2. Effect of sodium alginate and calcium chloride on particle size

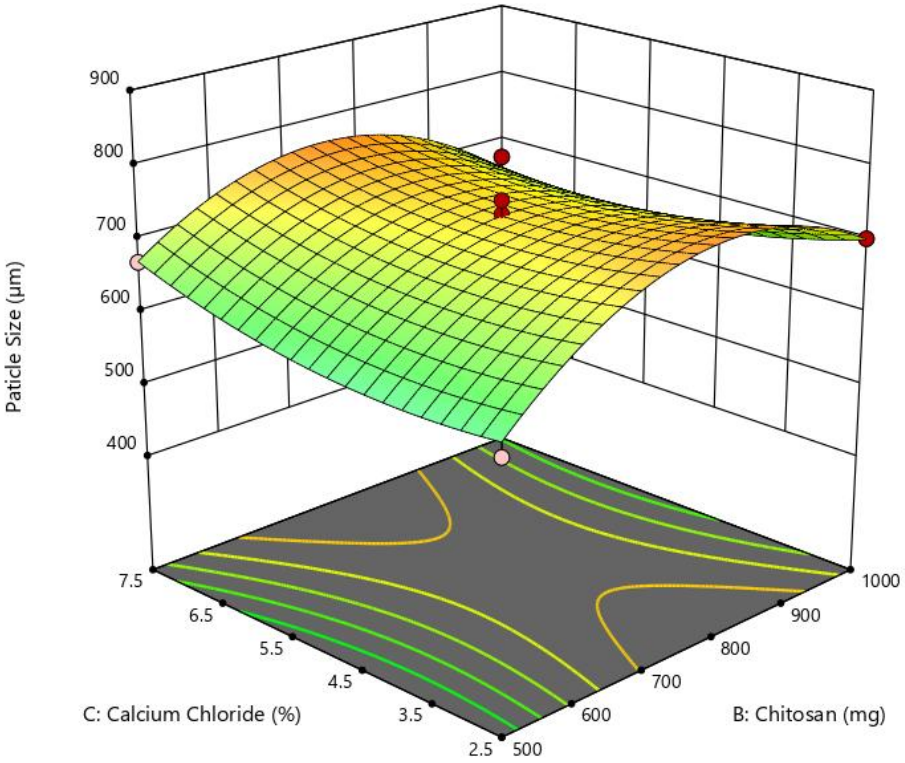

Fig. 3. Effect of Calcium chloride and Chitosan on Particle Size

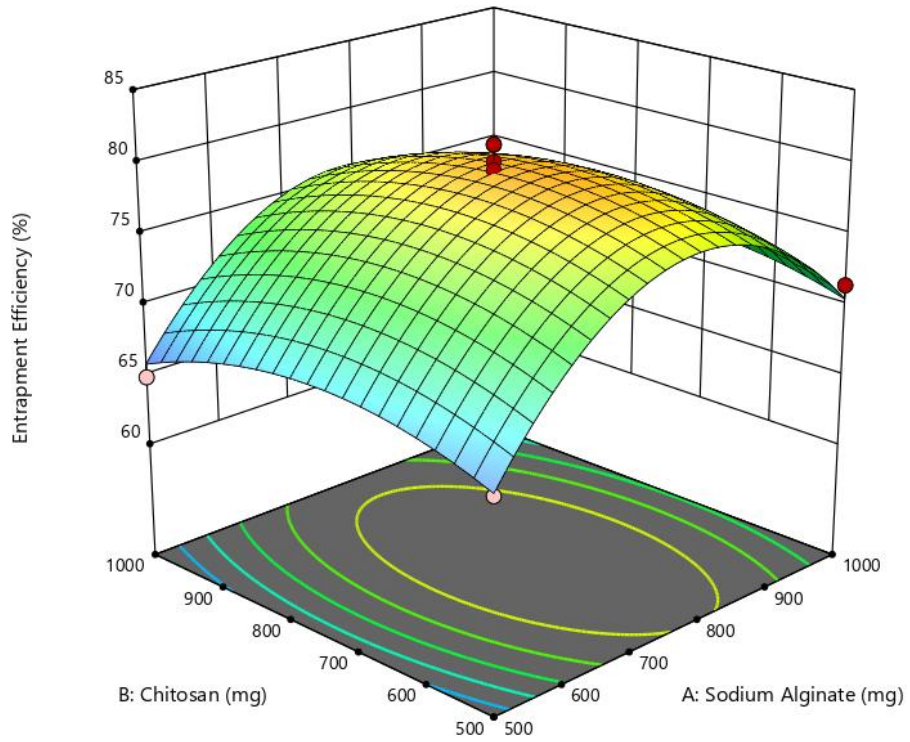

Fig. 4. Effect of Sodium alginate and Chitosan on Entrapment efficiency

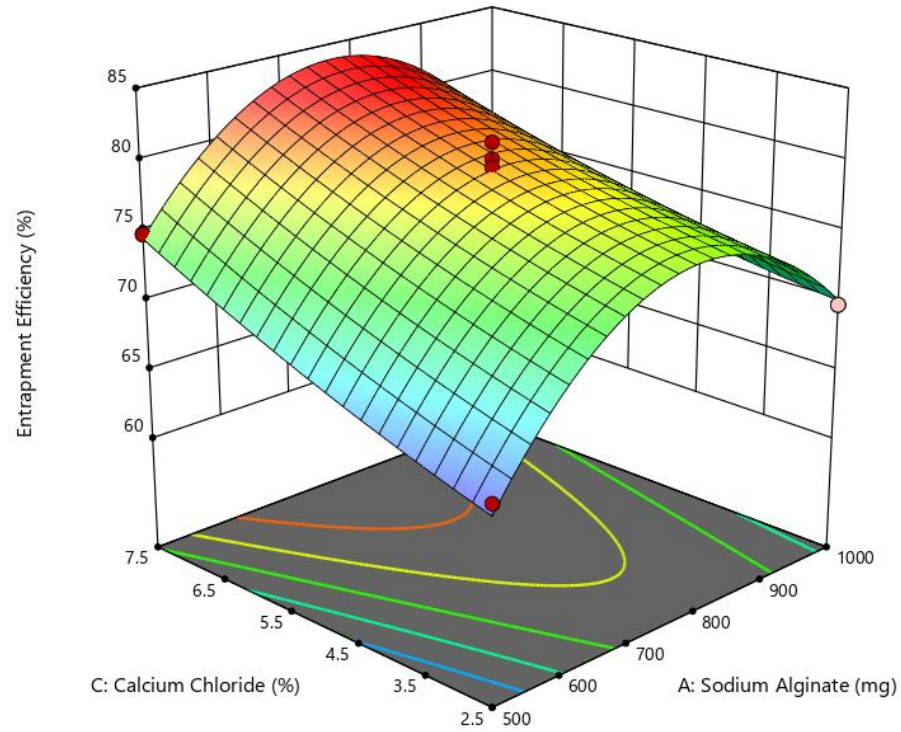

Fig. 5. Effect of Sodium alginate and Calcium chloride on Entrapment efficiency

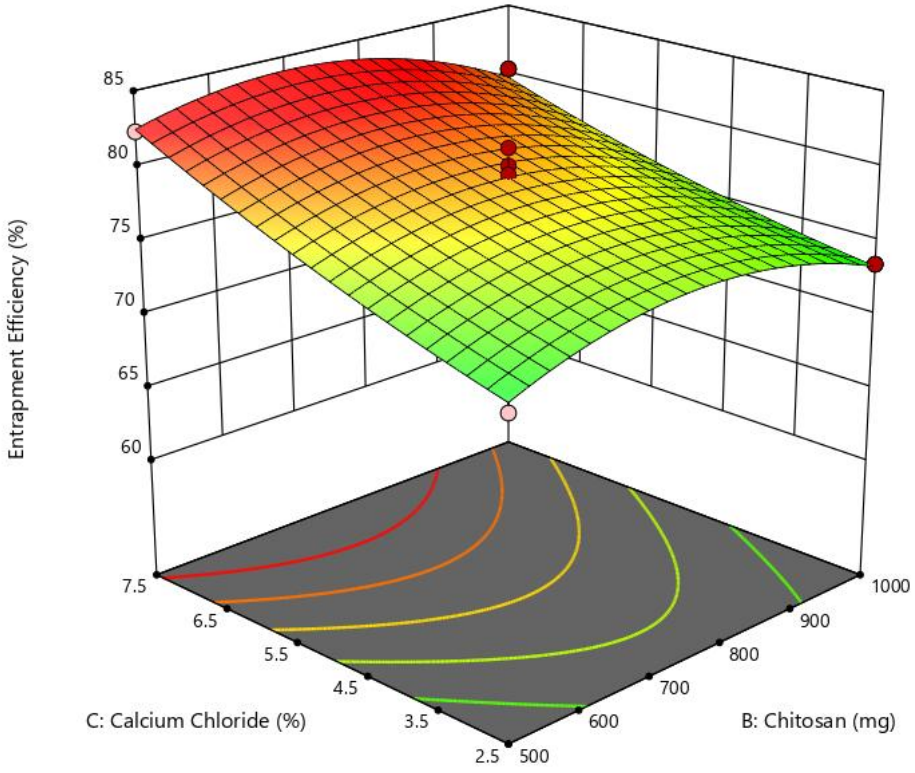

Fig. 6 Effect of Chitosan and Calcium chloride on Entrapment efficiency

3.3 Effect on In-vitro Drug Release (Y3)

The model proposes the following equation for in-vitro drug release;

$$In-vitrodrugrelease = 77.468 - 3.39A - 1.965B + 1.32C - 0.5225AB - 2.9425AC - 2.4125BC - 2.31775A^2 + 0.17225B^2 + 0.13225C^2.$$

Where A is the Amount of Sodium alginate; B is the Amount of Chitosan, and C is the concentration of Calcium chloride. The Model F-value of 20.99 implies the model is significant. There is only a 0.03% chance that an F-value could occur due to noise. P-values less than 0.0500 indicate model terms A, B, AC, BC, A^2 are significant. The Lack of Fit F-value of 0.06 implies the Lack of Fit is not significant. The Predicted R^2 of 0.9227 is in reasonable agreement with the Adjusted R^2 of 0.9183. Adequate Precision value of 17.00 indicates an adequate signal. This model can be used to navigate the design space. Figs. 7,8 and 9 are the response surface plot showing the effect of different independent variables on in vitro drug release. Results indicated that in-vitro drug release was affected by concentration of both the polymer and the cross-linking agent significantly.

3.4 Selection of Optimized Formulations Using Point Prediction Method

The optimum formulation was selected to achieve the optimum values of each response that is to minimize the particle size (Y1), maximize the Entrapment efficiency (Y2) and maximize the % in-vitro drug release (Y3). Based on the prediction, three formulations were prepared and the responses of particle size, entrapment efficiency and % cumulative drug release were evaluated. The validation for RSM involving all the three formulations was found to be within limits. The composition of optimum check point formulation, their predicted and observed values for all the responses and the percentage error are shown in Table 4. The percentage prediction error was found to be varying between -1.94% and 1.80%.

$$\text{Error } (\%) = \frac{\text{Predicted value} - \text{Observed value}}{\text{Predicted value}} \times 100$$

Point prediction of the design expert software predicted the optimized responses to be 551.45 µm particle size, 78.31% drug entrapment efficiency and 86.24% cumulative in-vitro drug release at polymer concentration, Sodium alginate 589.8 mg, Chitosan 500.0 mg and Calcium chloride 7.49 (%w/v) as a cross-linking agent.

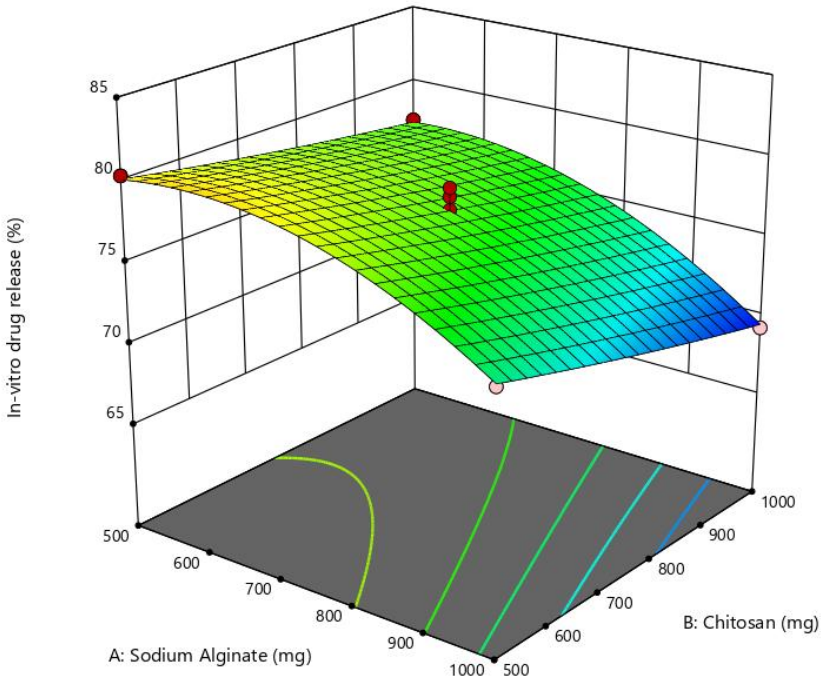

Fig. 7. Effect of Sodium alginate and Chitosan on in-vitro drug release

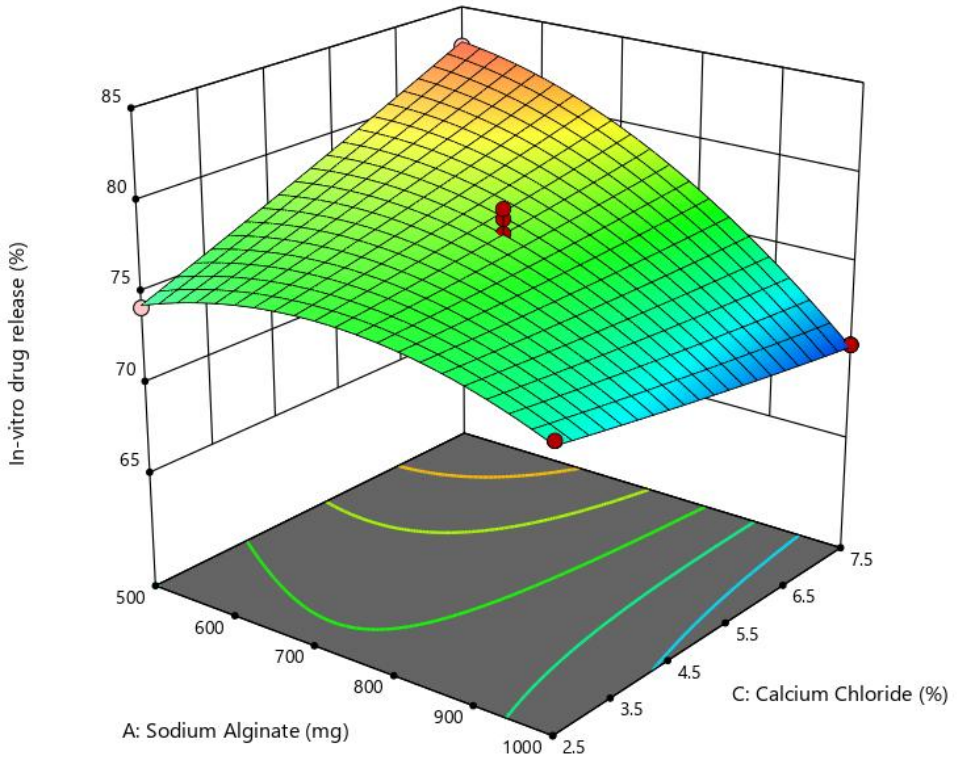

Fig. 8. Effect of Sodium alginate and Calcium chloride on in-vitro drug release

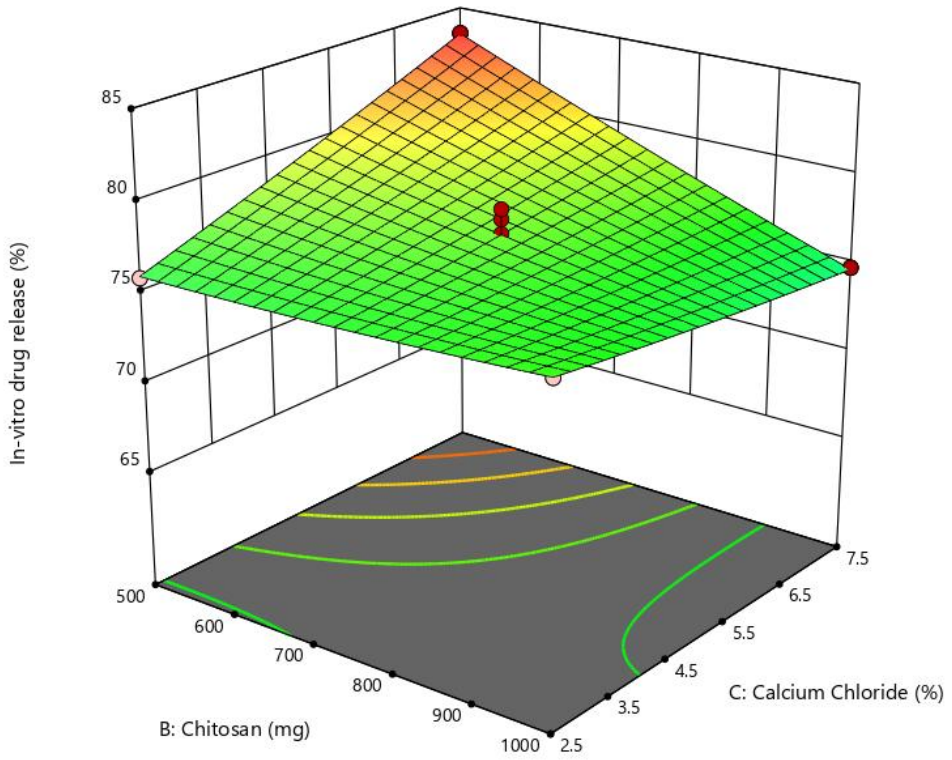

Fig. 9. Effect of Chitosan and Calcium chloride on in-vitro drug release

Table 4. Composition of checkpoint formulation with predicted and observed values

Response Variables	Predicted Value	Observed Value	% Error
Paticle Size (Y1)	551.45	561.58	+ 1.80
Entrapment Efficiency (Y2)	78.31	76.82	- 1.94
In-vitro drug release (Y3)	86.24	86.93	+ 0.79

Table 5. Data for analysis of drug release mechanism

Formulation Code	Zero order	First order	Matrix	Peppas		Best Fit Model
	R	R	R	R	n	
F01	0.9977	0.9589	0.8362	0.9986	0.960	Peppas
F02	0.9750	0.9579	0.8323	0.9788	0.919	Peppas
F03	0.9916	0.9238	0.7834	0.9960	1.104	Peppas
F04	0.9869	0.9227	0.7647	0.9957	1.149	Peppas
F05	0.9970	0.9570	0.8387	0.9983	0.952	Peppas
F06	0.9985	0.9562	0.8352	0.9992	0.963	Peppas
F07	0.9952	0.9633	0.8350	0.9964	0.955	Peppas
F08	0.9942	0.9652	0.8399	0.9959	0.944	Peppas
F09	0.9491	0.9704	0.9120	0.9941	0.764	Peppas
F10	0.9951	0.9579	0.8479	0.9979	0.929	Peppas
F11	0.9961	0.9535	0.8310	0.9965	0.970	Peppas
F12	0.9887	0.9329	0.7747	0.9949	1.123	Peppas
F13	0.9702	0.9641	0.8759	0.9879	0.839	Peppas
F14	0.9739	0.9679	0.8825	0.9930	0.833	Peppas
F15	0.9444	0.9888	0.9183	0.9960	0.752	Peppas
F16	0.9784	0.9643	0.8848	0.9968	0.836	Peppas
F17	0.9002	0.9566	0.9255	0.9812	0.703	Peppas

3.5 Kinetics Study

From the drug release profile of formulations, the R values of Korsmeyer peppas model were close to 1 as in Table 5. The diffusion coefficients (n) values ranged from 0.764 to 1.149. The observed diffusion coefficient values were indicative of the fact that the drug release from the formulation follows non-Fickian transport mechanism.

4. CONCLUSION

The mucoadhesive microspheres of Imatinib mesylate were formulated and optimized using Box – Behnken process optimization software. The quantitative responses of particle size, entrapment efficiency and in-vitro drug release for different combinations of independent variables, Sodium alginate as release retarding polymer, Chitosan as mucoadhesive polymer and Calcium chloride as cross-linking agent were obtained experimentally, and the results were found to fit the design model. The quantitative effect of these factors at different levels on the responses could be predicted using polynomial equations, and high linearity was observed between predicted and actual values of response variables. The results for the present study revealed that the content of polymers and cross-linking agent affected the responses, particle size, entrapment efficiency and *in vitro* drug release in a significant and interactive manner. The drug release kinetics followed non-Fickian transport mechanism. The optimum formulation predicted by point prediction of the design expert software. Percentage error between the observed and predicted results of the quantitative responses of particle size, entrapment efficiency and *in-vitro* drug release of optimum formulation were found relatively less. Therefore, it can be concluded that a mucoadhesive microsphere for Imatinib mesylate was developed and optimized using a three-factor, three-level Box – Behnken design.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not

intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

We conducted our research after obtaining proper IEC approval.

ACKNOWLEDGEMENTS

The authors are thankful to staff Department of Pharmacy of Annamalai University for their support during the research work. That authors declare that no funding has received for the research or in the preparation of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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