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DEVELOPMENT AND OPTIMIZATION OF CHITOSAN-CA-PECTINATE BEADS OF AMORPHOUS CELECOXIB BY RESPONSE SURFACE METHODOLOGY

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Keywords:

Celecoxib, Polyvinyl Pyrrolidone, hydroxypropyl-β-cyclodextrin, design of experiment, pectin, chitosan

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ABSTRACT: The aim of the present work was to develop a new microbeads system for colon-targeted delivery of celecoxib. The ternary system were develop using PVP (polyvinylpyrrolidone) and hydroxypropyl-β-cyclodextrin (HPB) to increase solubility of celecoxib. DSC and XRD results confirmed formation of amorphous celecoxib complex with HPB. Chitosan and pectin were used for the development of microbeads of new amorphous celecoxib. Calcium chloride was use as cross linking agent for electrolyte complexation between polymers. Eudragit RS100 was used to embed into microbeads in order to avoid premature delivery of a drug in acidic environment of stomach. Statistical design of experiment was employed to investigate the combined effect of three formulation variables, i.e., % of chitosan, pectin, and $CaCl_2$, on responses like drug release pattern in acidic dissolution medium (CDR in UGIT), colonic dissolution medium with fungus culture of *Aspergillus niger* (CDR in LGIT) and drug entrapment efficiency (EE%). Response surface methodology was used to analyze multivariate approach for understanding the multifactorial relationships among formulation parameters. Full central composite design was employed to define a design space. Desirability was used to attain simultaneous optimization of responses. Optimized formulation were evaluated and it was then found that obtained experimental values very close to predicted values. Eudragit RS 100 was successfully embedded in mocrobeads because there was negligible drug loss in acidic environment and maximum drug was released in colonic medium.

INTRODUCTION: Celecoxib (CLX), a fluorinated benzene sulfonamide derivative, is a nonsteroidal anti-inflammatory drug (NSAID) with highly selective cyclooxygenase-2 (COX-2) inhibitory action. It possesses anti-inflammatory, analgesic, and antipyretic activities due to the inhibition of prostaglandin synthesis catalyzed by COX-2.

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Recently, CLX was frequently investigated for its anticancer activity using in vitro and in vivo models [1-4]. Preclinical studies on CLX reported prominent anticancer activity against head and neck squamous cell carcinoma, colon cancer, breast cancer, and lung cancer [1-2].

To develop a promising drug delivery system for the treatment of colon cancer and colon polyps there are challenges like it should release drug at colonic site. It is difficult to protect drug molecule through upper gastrointestinal tract (GIT) because of highly versatile environment. However there are some promising approaches to deliver drug molecule at colon, like pH sensitive systems, time release systems, bioadhesive systems, pressure dependent release system, osmotically controlled system, microbial triggered system, etc.

Natural polysaccharides can be used to formulate the microbial triggered system [5-8]. Chitosan is a cationic polysaccharide obtained from alkaline *N*-deacetylation of chitin, the second most abundant natural polysaccharide on earth and its polymer chains consist of N-acetyl glucosamine and glucosamine residues [9, 12]. Pectin (pectin) is an anionic polysaccharide able to provide colon specific delivery for several drugs[10-12]. Gelation of droplets in the presence of calcium ion provides a promising approach to the formation of multiparticulate system for colon delivery [13]. The pectin particle can form polyelectrolyte's complex membrane around the particle in presence of cationic polymer such as chitosan [14]. However substantial amount of drug may release in the upper part of GIT by erosion of coating and diffusion[15].

Response surface methodology (RSM) is widely practiced approach in the development and optimization of drug delivery devices [15-20]. Based on principle of design of experiment (DoE), the methodology encompasses the use of various type of experimental designs, generation of polynomial equations, and mapping of response over the experimental domain to determine the optimum formulation [17, 21]. The technique requires minimum experimentation and time, thus proving to be far more effective and cost effective than conventional methods of formulating dosage form.

The current study aims at developing and optimizing colon specific chitosan-calcium-pectinate beads of CLX using RSM. Chitosan and pectin were used as biodegradable polymers for formation of Microbeads. Previous study revealed that with chitosan and pectin, substantial amount of CLX get released at upper GI tract. Therefore, Eudragit RS100(ED) was embedded to prevent this dose loss in upper GI tract. Calcium ion was used as cross linking agent for gelation of microbeads. Computer aided optimization technique, using a full central composite design with 6 center points, was employed to investigate the effect of 3 independent variables (factors) *i.e.* amount of chitosan, pectin and calcium chloride on % drug entrapment efficiency and CLX release pattern in upper GI (acidic dissolution medium pH 1.2) tract and Lower GI tract (basic dissolution medium with fungus culture *Aspergillus niger*).

MATERIALS AND METHODS:

Materials: Celecoxib (CLX) (Batch No: 225/KL/CLX/2014/062), Polyvinylpyrrolidone K30 (average MW 40 kDa), were obtained from Lupin Research Park, Pune, India. Identity and purity of CLX was checked by means of spectroscopic methods (IR, UV) combined with powder X-ray diffractometry and DSC analysis. Chitosan (deacetylation degree 86%), pectin (degree of esterification 63-66%) and calcium chloride (solubility 74% w/v) were provided by Analab fine chemicals, Mumbai. Hydroxypropyl-β-cyclodextrin (HPB) (Batch no: E0240) was a kind gift from Roquette freres, France. *Aspergillus niger* (NCIM545) fungus culture was purchased from National chemical laboratories, Pune. All other solvents and chemical used were of analytical grade.

Methods:

Preparation and characterization of CLX-HPB-PVP ternary System: Solubility study of raw CLX, spray dried CLX, physical mixture (PM) and spray dried (SD) 1:3 weight ratio of CLX-HPB, CLX-PVP and 1:3:3 weight ratio of CLX-HPB-PVP in water was studied. The spray drying operation was performed using a spray dryer (Labultima LU-222). Appropriate amounts of CLX, PVP K30 and HPB were added to 99.8% methanol. The spray drying was performed with the following conditions: inlet temperature of 90 ^{0}C and outlet temperature 70 ^{0}C, solution flow rate 5 ml/min at speed 5 ml/min and aspirator 65%; feed rate 12%; atomization air pressure 1.75 Kg/cm^2. The spray dried particles were stored in a desiccator until used for further studies.

The product was characterized by X-ray powder diffractometry (Bruker, D8 advance, Germany diffractometer) using Ni filtered Cu K (α) radiations, a voltage of 35 kv, current of 30 mA and receiving slit of 0.2 In. Samples were analyzed over 2θ range of 5-700 and 5-500 for stability interpretation. DSC analysis done using Mettler-Toledo (Mettler-Toledo, Switzerland) equipped with a refrigerated cooling system and calibrated using indium standard.

Sample (4-5mg) were placed in aluminum pans hermetically sealed with aluminum lids. The program temperature was set from 30-300 °C and increased at a rate of 10 °C/min. The nitrogen gas flow rate was adjusted to 50 ml/min. Onset temperature and melting points of the samples were automatically calculated using the software provided (STARe Ver. 12.1 Mettler Toledo, Switzerland). Raw CLX, SD CLX, all PM and SD samples were evaluated for percent cumulative CLX release (USP apparatus 1 (basket method); 50 mg of CLX (or CLX equivalent) added to 900 mL pH 7.4 phosphate buffer basket rotation 100 rpm, $37 \pm 0.5\ ^0$C).

Software for experimental design: The software Design Expert V.10 was used for the generation and evaluation of the statistical experimental design. Full central composite design was employed to investigate the influence of four formulation components, namely chitosan, pectin, calcium chloride ($Cacl_2$) and eudragit RS100 (ED). The response variable were percent entrapment efficiency (% EE), CLX released in upper gastro intestinal tract (CDR in UGIT) and lower gastrointestinal tract (CDR in LGIT), Total 30 runs including 6 replicates of central points were performed. All other formulation and processing variables were kept invariant throughout the study. **Table 1** summarizes the experimental design. Multivariate linear regression was used to generate the models. Analysis of variance (ANOVA) was applied for testing the significance and validity of the models. Response surface and desirability function were used to define the design space and find the optimum conditions.

Design of experiments (DoE):

TABLE 1: FORMULATION DESIGN USING FULL CENTRAL COMPOSITE DESIGN

	Factor-1	Factor-2	Factor-3	Factor-4
Run	A:chitosan %	B:pectin %	C:Cacl2%	D:ED %
1	4	5	5	0
2	4	5	12	1
3	4	5	12	0
4	2	3.5	8.5	1.5
5	2	6.5	8.5	0.5
6	2	3.5	15.5	0.5
7	0	5	12	1
8	0	5	5	0
9	2	3.5	8.5	0.5
10	4	2	12	0
11	4	2	12	1
12	-2	3.5	8.5	0.5
13	0	2	12	1
14	6	3.5	8.5	0.5
15	2	3.5	8.5	0.5
16	4	2	5	0
17	2	3.5	8.5	0.5
18	0	5	12	0
19	0	5	5	1
20	2	3.5	8.5	-0.5
21	2	3.5	8.5	0.5
22	2	3.5	8.5	0.5
23	2	3.5	1.5	0.5
24	4	5	5	1
25	4	2	5	1
26	0	2	12	0
27	2	3.5	8.5	0.5
28	0	2	5	0
29	0	2	5	1
30	2	0.5	8.5	0.5

Factor -1 A = Chitosan (chitosan) 0 to 5% w/v; Factor -2 B = Pectin (pectin) 3 to 5% w/v; Factor -3 C = Calcium chloride ($CaCl_2$) 7 to 12% w/v; Factor -4 D = Eudragit RS100 (ED) 0 to 1% w/v.

Preparation and characterization of microbeads: The particles were prepared by complex coacervation method. Weighed amounts (3–5% w/v) of pectin were dispersed in distilled water (20 mL), 0 to 1% w/v ED were added and CLX (100 mg) was added as spray dried ternary system (CLX: HPB: PVP, 1:3:3). Chitosan was dissolved at different concentrations (0–5% w/v) in 0.1N glacial acetic acid under magnetic stirring, and Then 5% to 12% calcium chloride was added as cross linking agent. Dispersion of CLX with pectin and ED were dropped through a 2 mm needle into chitosan dispersion containing calcium chloride for the complex coacervation. Beads are made by continuous stirring at 300 rpm for 30 min by using mechanical stirrer and they were further kept for 1 hr in the same solution and removed by filtration.

The beads so obtained were washed with deionized water to remove excess of calcium chloride. The beads were then air-dried at room temperature. The microbeads obtained at the end of preparation of each batch were weighed, and the production yield was determined as percentage with respect to the initial amounts of excipients and CLX used for their preparation. Shape and surface morphology of microbeads were examined using Scanning electron microscopy (SEM) (JEOL JSM-6360A scanning microscope Japan). The microbeads were coated with Gold ion sputtering using auto fine coater JFC-1600 (JEOL, Japan) and coating was done for 5-6 minutes. The microbeads were kept on the sample holder and the scanning electron micrographs were taken.

Entrapment efficiency: Weighed amount of Microbeads of each batch were finely powdered and mixed with methanol. Mixture was maintained under stirring for 24 hrs at room temperature, to ensure complete dissolution of CLX. Samples were then filtered using Whatman filter paper (0.45nm pore size) and CLX content is determined by UV spectrophotometer at λmax 250 nm. The CLX entrapment efficiency (E.E %) was then calculated according to Eq.:

$$\% \text{ Entrapment efficiency} = Qr / Qt * 100$$

Where Qt is the total CLX amount initially added during the batch preparation and Qr is the CLX amount recovered in the microbeads.

***In-vitro* celecoxib release:** Release studies were performed using the basket apparatus (USP Apparatus 1). Microbeads equivalent to 50 mg of CLX were added to 900 ml of dissolution medium thermostatic at 37 ± 0.5^0C and stirred at 100 rpm, dissolution medium was varied according to the following sequence, 2hrs in 0.1N HCl, 3 hrs 6.8pH phosphate buffer solution (PBS) and 19 hrs in 7.4pH PBS with *Aspergillus niger*. At suitable time intervals, 5 ml aliquots were withdrawn from the dissolution medium and filtered through 0.25nm filter paper and absorbance of each aliquot were taken on 250nm using UV spectrophometer (Schimadzu 1700).

Data analysis and optimization: PCP Disso software and Design Expert software v10 were used for data analysis and optimization. Noted absorbance of CLX release were inserted in to PCP disso software and percent cumulative CLX released (% CDR) in 0.1N HCL, 6.8 pH PBS and 7.4 pH PBS, were determined. The data of %CDR in 0.1N HCL, 6.8 pH PBS (UGIT) and 7.4 pH PBS (LGIT), % entrapment efficiency were fed to design expert software for optimization.

RESULTS AND DISCUSSION:

Characterization of CLX-HPB-PVP ternary system: The solid CLX-HPB-PVP ternary system obtained by spray drying was characterized by DSC and X-ray powder diffractometer, to confirm the formation of the solid complex (**Fig. 1** and **Fig. 2**). The thermal curve of CLX was typical as that of an anhydrous crystalline substance, exhibiting a sharp fusion peaks (**Fig. 1E**), while HPB and PVP showed broad endothermic bands, consistent with their hydrated amorphous nature (**Fig. 1A**. **1B**). The CLX melting endotherm was still well detectable in the ternary physical mixture 162.35 ^{0}C (**Fig. 1C**), while it was completely absent in the spray dried product (**Fig. 1D**). This may be due to the interaction between the polymers and the drug; and the formation of an amorphous ternary complex.

The X-ray diffraction pattern of the CLX was characterized by the presence of several sharp and intense peaks, showing its crystalline nature while both HPB and PVP (**Fig. 2 A** and **B**) exhibited a flat pattern, like amorphous substances. Some of the most intense diffraction peaks of CLX, emerging from the amorphous profile of HPB and

PVP, can be seen in the ternary physical mixture indicating partial amorphous nature while they totally absent in the corresponding spray dried product (**Fig. 2D**), indicating its complete amorphous ternary system, in agreement with DSC results.

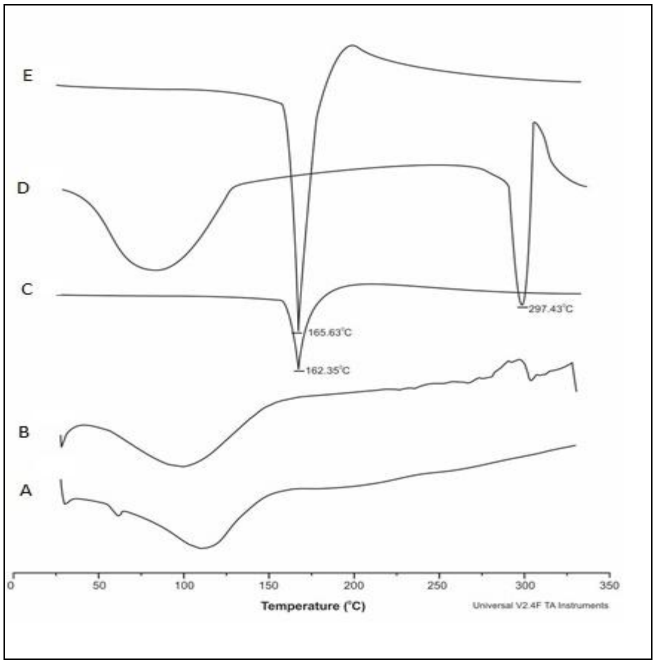


FIG. 1: DSC; A. PVP, B. HPB, C. PHYSICAL MIXTURE, D. SPRAY DRIED PRODUCT AND E. CLX.

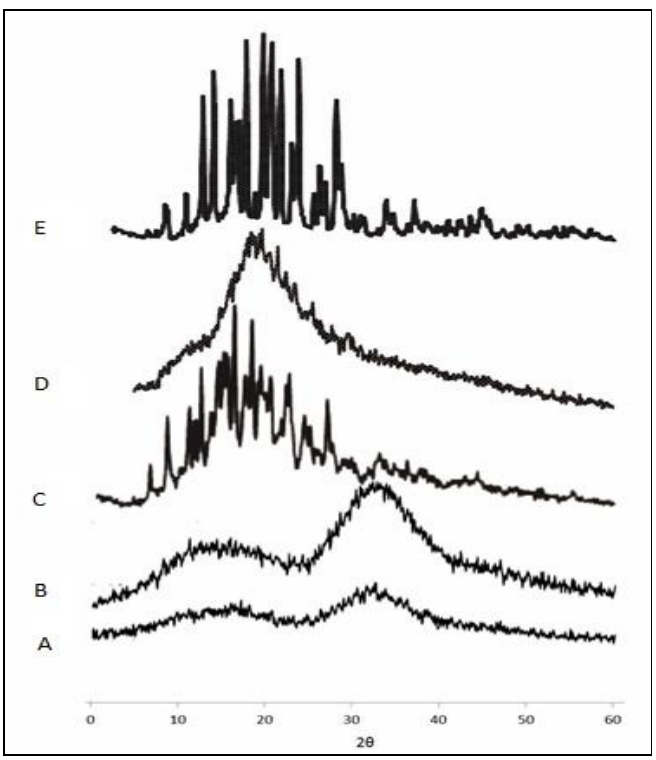


FIG. 2: X-RD; A. HPB, B. PVP, C. PHYSICAL MIXTURE, D. SPRAY DRIED PRODUCT AND E. CLX

Dissolution rate studies demonstrated that there was increase in dissolution of the ternary spray dried product.

The percent dissolution of this product by 2hrs was 15% higher than that of the CLX-HPB spray dried product, 17% higher than the CLX-PVP spray dried product and 44% higher than that of the pure CLX (**Fig. 3**). In fact, the higher dissolution rate of CLX-HPB-PVP spray dried system allowed its rapid dissolution in the pectin aqueous dispersion and, consequently, its homogeneous distribution within the microbeads during their formation, thus assuring high content uniformity.

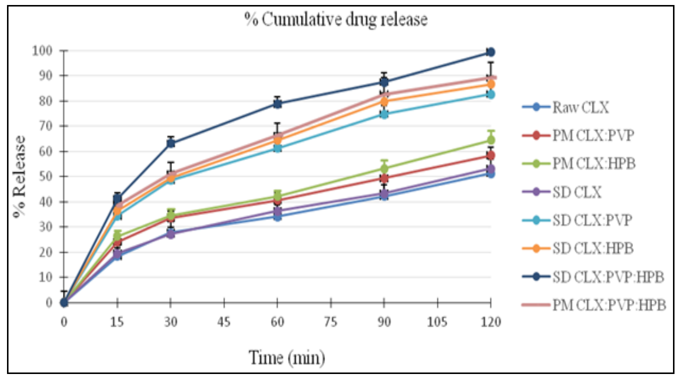


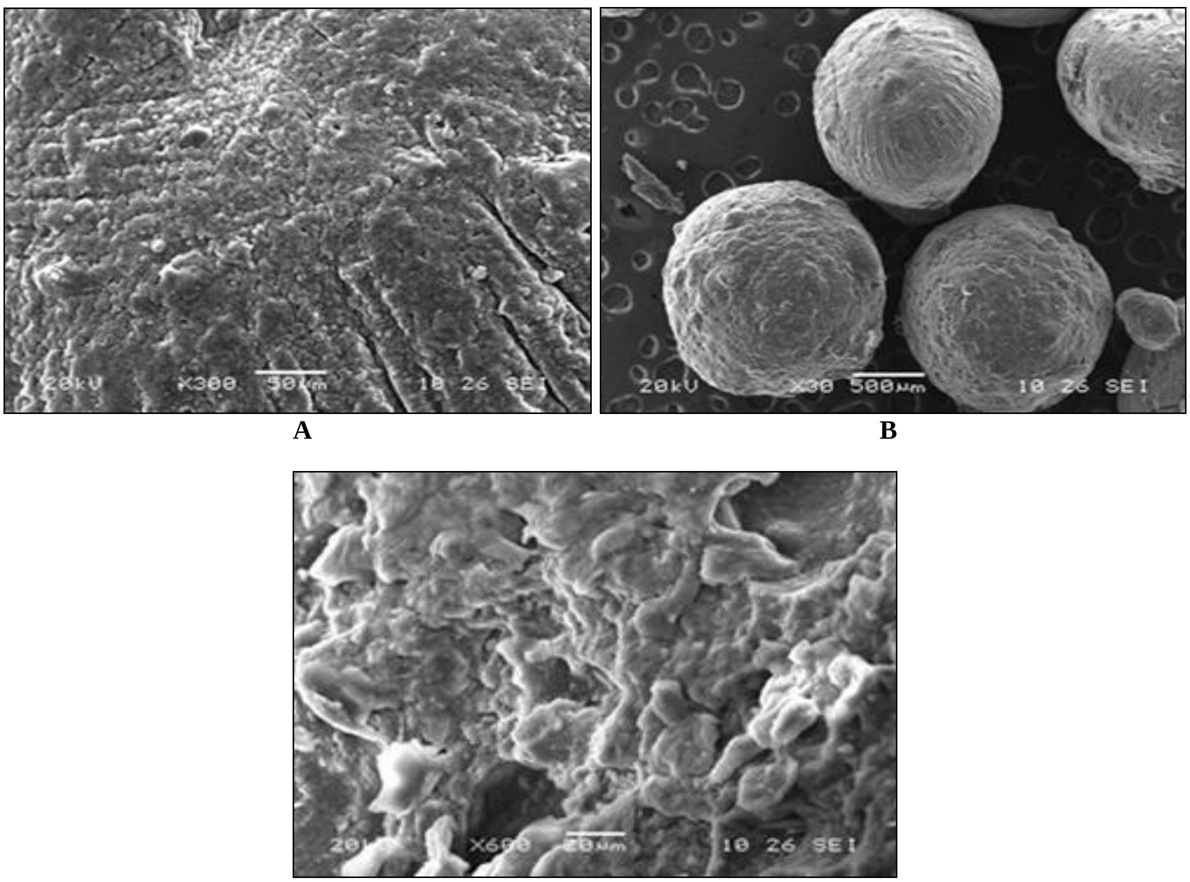
FIG. 3: DRUG RELEASE STUDY OF CLX AND PM AND SD OF CLX AND POLYMERS

Optimization of microbeads formulation: DoE was used for microbeads formulation optimization, to ensure the quality of the product. In order to define the ‘‘design space,’’ independent variables and their responses in terms of the product quality; were defined. The components used for the microbeads production like pectin, chitosan, $CaCl_2$ and ED were chosen as the independent variables since they were considered critical in determining the performance of the final product. The CLX entrapment efficiency (EE%), CLX release by 2 h in gastric pH, CLX release by 3 hrs. in small intestinal pH (%DR in UGIT) and later in colonic medium (% CDR in LGIT) were selected as the most important responses to improve the product quality.

The experimental domain of each of the selected independent variable was set on the basis of a preliminary screening, where few technological factors were considered such as size homogeneity, spherical shape regularity, yield, and consistency of the microbeads.

The pectin concentration range was set between 3 and 5% w/v. Pectin amount in solution less than 3% gave rise to irregular-shape sticky microspheres, while more than 5% amount crates viscous solution which was difficult to pass through syringe. In case of chitosan, 0% w/v was chosen as the lowest value, to observe the effect of its absence in the formulation. The 5% w/v was selected as higher level because it was making the solution to viscus to stir uniformly. As for the $CaCl_2$ concentration range, 12% w/v was considered higher value, in order to avoid negative effects of an excess of free Ca^{2+} ions. The 5% was chosen as the lower value, since values lower than 5% w/v produce poor quality microbeads. Other parameter like, 15 minutes of cross linking time, 25^0C temperature, 300 rpm stirring speed, and 50ml dispersion medium were fixed on the basis of literature survey and trial and error method.

Microbeads evaluation: All the formulations prepared within the layout of design of experiments. All formulation microbeads yielded with a uniform spherical shape, suitable consistency, and homogeneous size. There was no statistically significant variation ($p > 0.05$) in microbeads dimensions, whose mean diameter varied from 1.48 ± 0.07 mm to 1.61 ± 0.03 mm ($n = 20$). The product yields were around 88–96%.



A B

C

FIG. 4: SEM; A. MICROSPHERE SURFACE AT 300X, B. MICROSPHERE AT 30X, C. CROSS SECTION OF MICROSPHERE

In SEM analysis (**Fig. 4**) microbeads looked as almost spherical and regularly shaped, the surface texture of microbeads was rough. The typical formation of rectangular shapes, homogeneous distribution of them on the microbeads surface was an indicator for the presence of chitosan. A section of microbeads evidenced the absence of these rectangular structures in their internal core, confirming chitosan was dominantly present on the particle surface. All the batches were then evaluated in a randomized order for both encapsulation efficiency and CLX release profile under pH gradient. Analysis of variance (ANOVA) was applied for testing the significance and validity of the postulated model, using a 1% significance level.

ANOVA showed that the assumed regression model was significant and valid for all three the examined responses. This means that the found relationship was able to describe the response variation in function of factor variations and thus to carefully describe the design space. **Table 2** showed final equations in terms of coded factors, explain relationship of factor variables on response variables.

TABLE 2: FINAL EQUATIONS IN CODED FACTOR

CDR in UGIT=+1.17	CDR in LGIT=+92.00	EE=+94.00
-0.12* A	+7.87* A	+7.79*A
-0.042* B	+2.79* B	+3.46*B
-0.042* C	+1.37* C	+0.37*C
-4.87* D	+4.54* D	+0.29*D
+0.062* AB	+0.19*AB	+0.56*AB
-0.19* AC	+0.44*AC	+0.44*AC
+0.062* AD	+0.56*AD	+0.31*AD
+0.062* BC	-0.31*BC	+1.06*BC
+0.062* BD	+1.31*BD	+0.19*BD
+0.062CD	-1.94*CD	-0.69*CD
+0.66 *A^2	-4.26*A^2	-3.74*A^2
+0.53*B^2	-5.39*B^2	-5.11*B^2
+0.53*C^2	-1.01*C^2	-0.86*C^2
+1.78*D^2	-2.14*D^2	-0.61*D^2

All the batches showed high %EE, never less than 89%, except for microbeads without chitosan, where the EE% decreased until 70% and 60% in case of pectin was very low 0.5%. (**Fig. 5**) The high EE% values are attributable to the very limited aqueous solubility of CLX, even as ternary spray dried system with HPB and PVP, which prevents CLX diffusion from the gel network to the aqueous medium during the ionotropic gelation process.

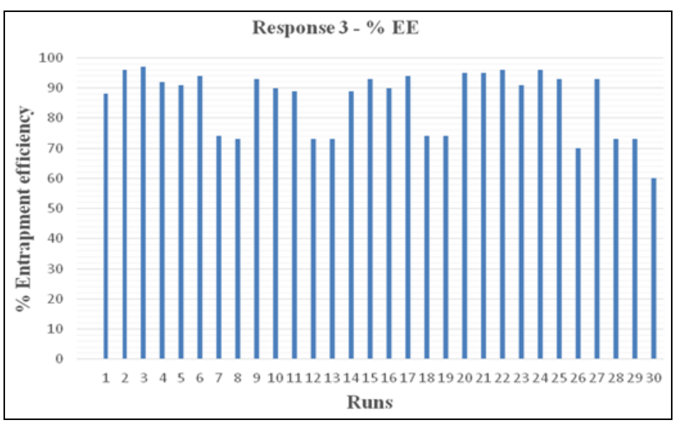


FIG. 5: RESPONSE 3 % DRUG ENTRAPMENT EFFICIENCY FOR ALL 30 RUNS

All microbeads formulations showed close to zero %CDR in UGIT, except formulations without ED, where the %CDR in UGIT was up to 12%. Moreover, %CDR in LGIT were more than 90% except for the formulations without ED and without chitosan where around 12% drug was release in UGIT and drug loading was very less respectively (**Fig. 6, 7** and **8**) These results indicating that responses were very strongly dependent upon the factor variables in the experimental domain under study. On the other hand, it is important to emphasize that all chitosan-Ca-pectinate microbeads batches proved to be able to carry the entrapped CLX up to the colonic fluid, preventing premature release of CLX in UGIT.

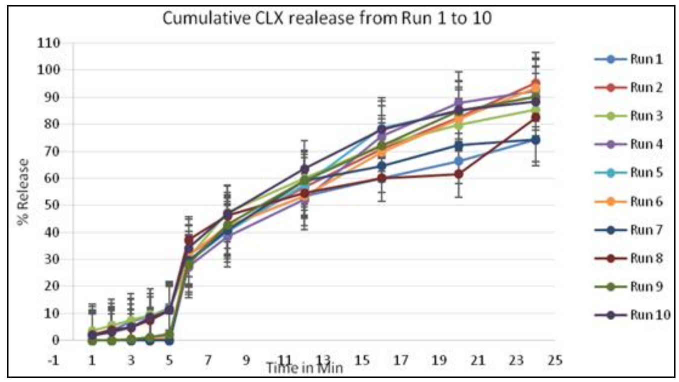


FIG. 6: RESPONSE 3 % DRUG ENTRAPMENT EFFICIENCY FOR ALL 30 RUNS

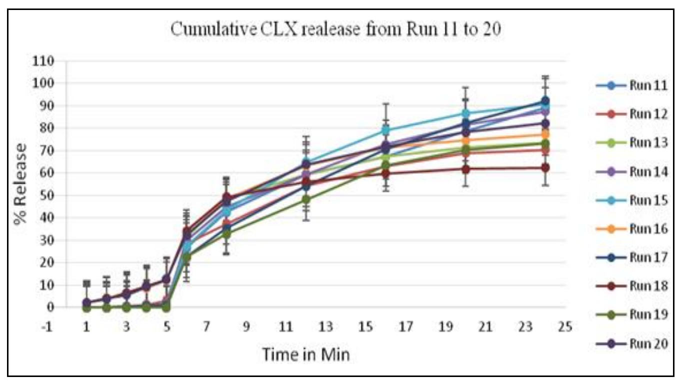


FIG. 7: RESPONSE 3 % DRUG ENTRAPMENT EFFICIENCY FOR ALL 30 RUNS

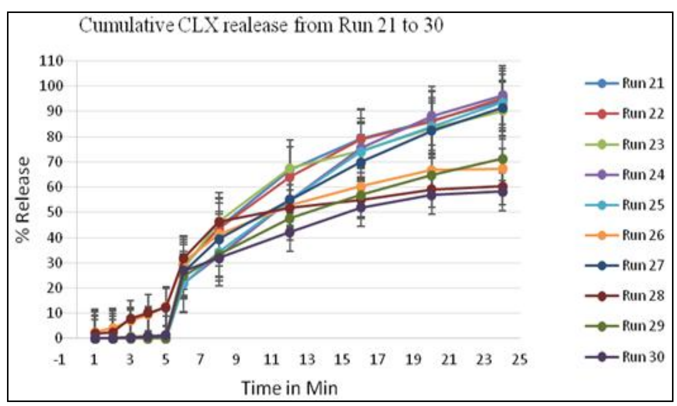


FIG. 8: RESPONSE 3 % DRUG ENTRAPMENT EFFICIENCY FOR ALL 30 RUNS

Response surface study: Three-dimensional response surface plots were then generated, using the quadratic model obtained from multiple regression analysis, in order to investigate the effects of independent variables on responses. The %EE was increased with increase in concentration of chitosan in formulation irrespective of concentration of pectin. However, when the chitosan concentration becomes too high, its positive effect on EE% was reduced, since the increased viscosity of the solution slows down the diffusion of chitosan chains, hindering their interaction with the pectin chains. The cross linking CaCl2 agent showed positive effect on % EE and presence of ED does not hampered drug entrapment efficiency. Concentration of pectin also showed positive effect on drug entrapment in microbeads. The highest DR% value was observed for the lowest levels of both polymers, and a dramatic reduction in CLX release was observed for high levels of pectin (**Fig. 9, 10** and **11**). Both these results showed that, there was formation of electrostatic interaction between the carboxyl groups of pectin and the positively charged amino groups of chitosan, leads to the formation of a poly-electrolytic complex between the polymers. As a consequence of this, a more tight structure of the microbeads was formed, which, results in to a more effective entrapment of the CLX, but, at the same time, it reduces the diffusion of drug through the matrix.

The presence of ED in microbeads reduced drug release in UGIT which explains that ED is successfully entrapped in microbeads. The concentration of cross linking agent *i.e.* $CaCl_2$ showed negative effect on %CDR as it increases cross linking and tightens the polyelectrolytes complex.

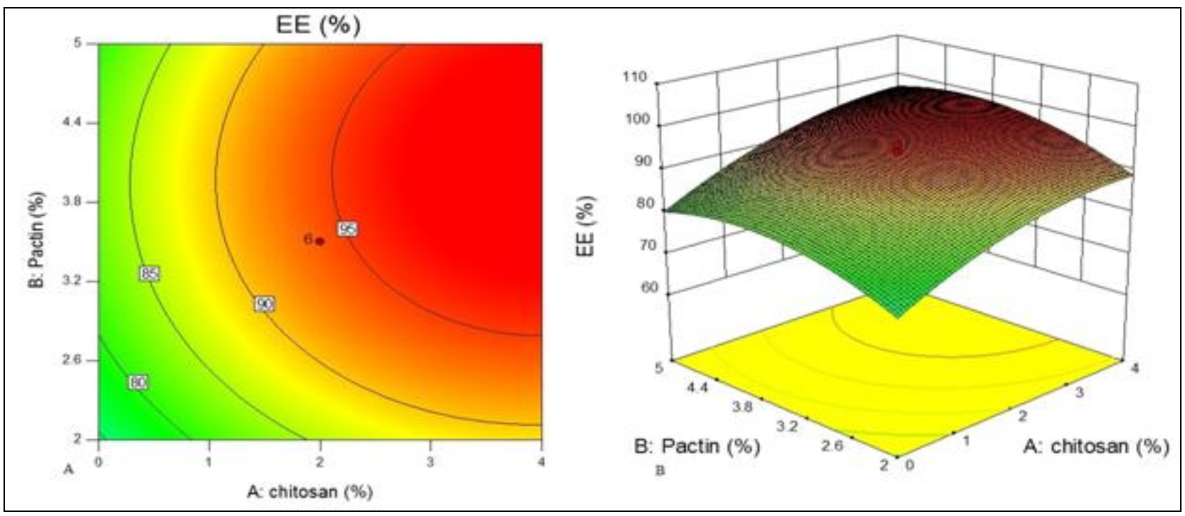


FIG. 9: RSM; 5A. COUNTOR PLOT FOR EE, 5B. 3D PLOT FOR EE

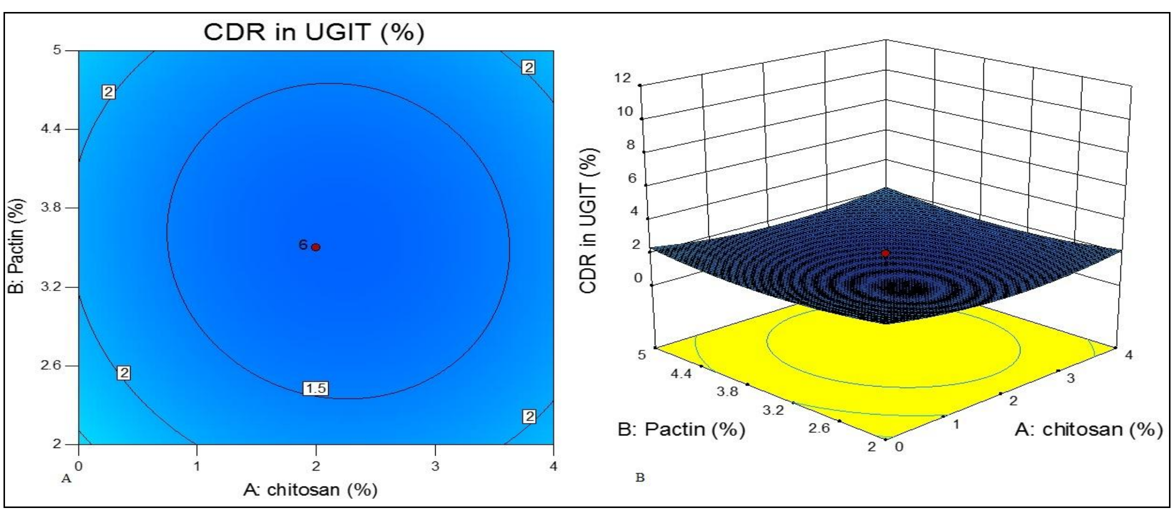


FIG. 10: RSM; 6A. COUNTOR PLOT FOR CDR IN UGIT, 6B. 3D PLOT FOR CDR IN UGIT

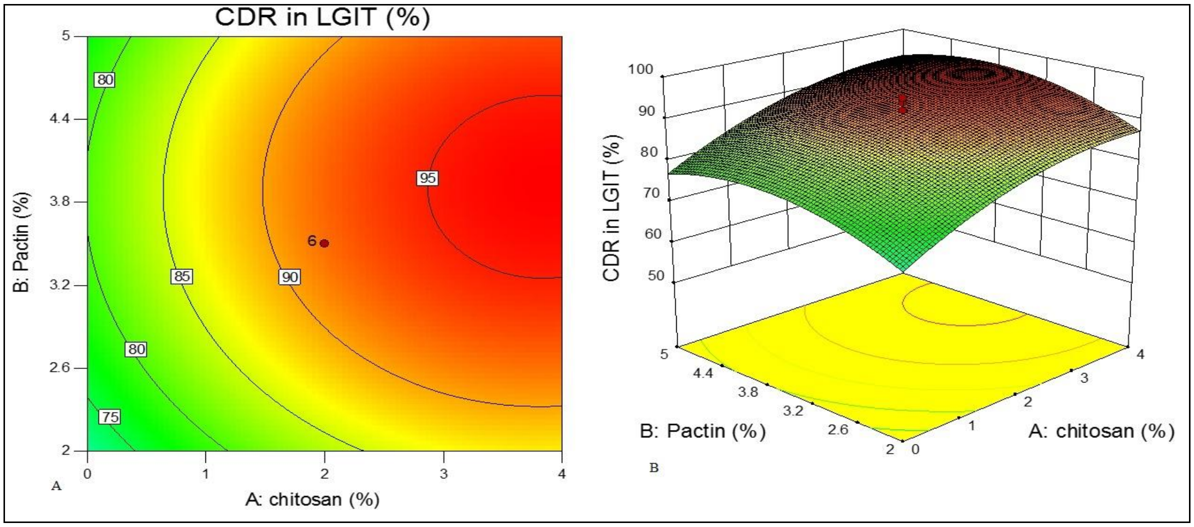


FIG. 11: RSM; 7A. COUNTOR PLOT FOR CDR IN LGIT, 6B. 3D PLOT FOR CDR IN LGIT

Desirability function: To find the best compromise between the values of the variables, in order to maximize at the same time both entrapment efficiency and CLX release in LGIT and minimize CLX release in UGIT. All responses were associated with their own partial desirability function, which varied from 0 to 1, according to the closeness of the response to its target value.

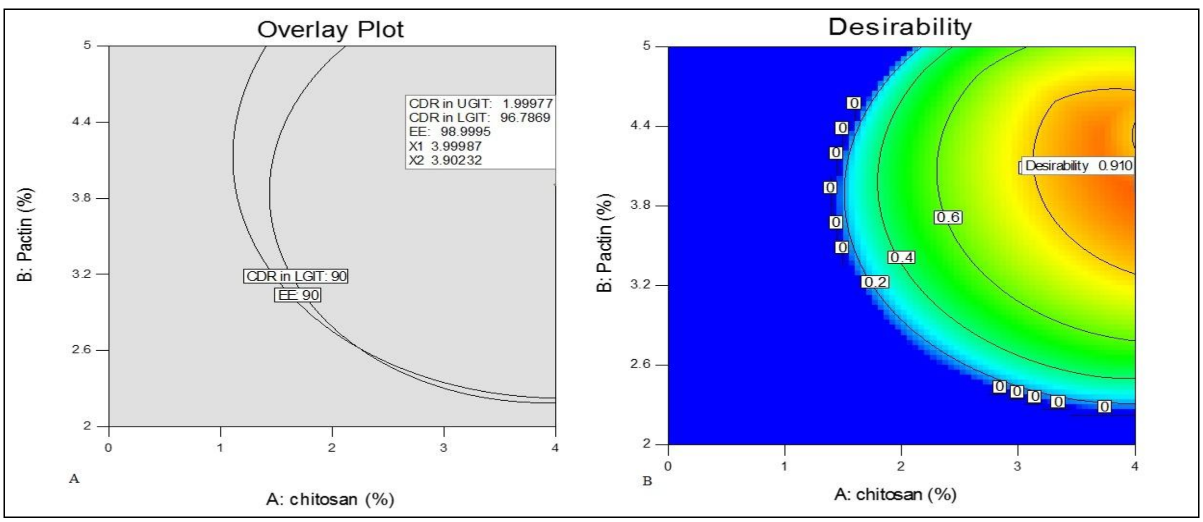


FIG. 12: RSM; 8A. OVERLAY PLOT, 8B. DESIRABILITY

The individual desirability functions were combined together, to obtain the overall desirability function (D) for the system whose maximum value could then be looked for within the experimental domain. In this case, the desirability was achieved to 0.910 X1 and X2 value were 3.99 and 3.90 respectively at same time expected responses CDR in UGIT 1.99 CDR in LGIT 96.78 and %EE was 98.99 (**Fig. 12**. overlay plot).

Evaluation of the optimized formulation: In order to validate the predictive ability of the hypothesized model for all responses around the optimized conditions, the agreement between predicted and measured responses was verified. Therefore, CLX microbeads were prepared according to the optimized conditions and characterized for % entrapment efficiency and % CLX released in UGIT and LGIT. The experimental values for responses *i.e.* % CDR in UGIT 1.18 ± 0.6%, % CDR in LGIT 97.36 ± 0.9% and % EE 99.19 ± 0.7%. The predicted values were inside the confidence interval for each response, thus indicating statistical equivalence between experimental data and the predicted data and therefore demonstrating the validity of the applied model.

CONCLUSION: It was shown that DoE approach can be successfully used in the development of colon-targeted microbeads formulation of CLX with predictable entrapment efficiency and CLX release properties in UGIT and LGIT. In particular, DoE allowed the simultaneous evaluation, by a response surface study, of the effects of the selected variables, *i.e.*, % of pectin, calcium chloride, and CTN, on the considered responses for optimization, *i.e.*, %drug EE, % of CLX released in UGIT and LGIT. Since the changes in the considered factors showed a general opposite influence on all responses, the use of desirability function was necessary, to find the best compromise which allowed simultaneous optimization of the considered responses. The experimental values of all responses obtained from the optimized formulations were very close to the predicted values, demonstrating the usefulness and reliability of the assumed model in the preparation of colon specific microbeads of CLX with optimized and predictable properties, to obtain the desired CLX release profile. It can be expected that the application of the DoE tools could be useful for further formulation studies, where microbeads with different CLX release profiles could be required.

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CONFLICT OF INTEREST: Authors declare that, there is no conflict of interest.

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