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Novel microbially triggered colon specific delivery system of 5-Fluorouracil: Statistical optimization, *in vitro*, *in vivo*, cytotoxic and stability assessment

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ABSTRACT

The present study aimed to statistically optimize a colon specific formulation of 5-Fluorouracil for the treatment of colon cancer. A 3^2 full factorial design was used for optimization. The independent variables employed were amount of pectin and amount of starch paste, each at three levels. The evaluated responses were hardness, percent cumulative drug release (% CDR) at 5th h and $t_{90\%}$ (time required for 90% of drug release). Drug release studies were carried out using change over media [pH 1.2, 7.4 and 6.5 in presence of 4% (w/v) rat caecal contents]. The optimized formulation was subjected to *in vivo* roentgenographic studies in New Zealand white rabbits to analyze the *in vivo* behaviour of the developed tablets. This formulation was also evaluated for cytotoxic potential using HT-29 human colon cancer cell lines. Pharmacokinetic studies in New Zealand white rabbits were conducted to determine the extent of systemic exposure provided by the developed formulation in comparison to an immediate release tablet. The optimized formulation consisting of pectin (66.67%, w/w) and starch paste (15%, w/w) released negligible amount of drug at pH 1.2 and pH 7.4 whereas significant ($p < 0.05$) drug release was observed at pH 6.5 in presence of 4% (w/v) rat caecal contents. Roentgenographic studies corroborated the *in vitro* observations, thus providing the "proof of concept". Pharmacokinetic studies revealed significant reduction in systemic exposure and cytotoxicity studies demonstrated enhanced cellular uptake of drug by the developed formulation. Shelf life of the formulation was found to be 2.83 years. The results of the study established pectin-based coated matrix tablet to be a promising system for the colon specific delivery of 5-FU so as to treat colon carcinoma.

1. Introduction

Colon provides a myriad of therapeutic opportunities for the treatment of various diseases like inflammatory bowel disease, colon carcinoma, and amoebiasis (Philip et al., 2008). Colorectal cancer manifests as cancerous growths in the colon, rectum and appendix. It is reported to be the third most common type of cancer accounting for 6,55,000 deaths globally per year and the second leading cause of cancer-associated deaths in the western world (Zhao and Li, 2010). 5-Fluorouracil (5-FU) is the drug of choice for the treatment of colon cancer (Calabresi and Chabner, 1996). Currently, only intravenous preparations of 5-FU are available in market for clinical use (5-FU-CBC, Dabur, India; Oncourcil, Sunpharma, India; Flurac, Cadila, India). Intravenous administration is associated with pain and formulations have to be sterile. Moreover, owing to skillfulness required for administration, it is time consuming for doctors and patients and self-administration is not possible. Psychological distress, hypertrophy or atrophy of the subcutaneous fat at the site of injection, occasional allergies are some of the additional factors responsible for non-conformity by the patient to the therapy using this route. Furthermore, intravenous administration of the drug has been reported to cause severe gastrointestinal, dermatological, hematological, cardiac and neural side effects (Diasio and Harris, 1989). Most of these side effects are due to exposure of the drug to the unwanted sites. Severe systemic toxic effects along with a short plasma half life of 10–20 min predominantly make this drug to be delivered by a local delivery system capable of providing a continuous sustained release (Wei et al., 2008). Delivery of drugs to the receptors at a particular site has the potential to reduce side effects and to increase pharmacological response (Sinha et al., 2004). Recently, it has also been demonstrated in mice that targeting improves mesenchymal stem cells treatment of inflammatory bowel disease (Ko et al., 2010). Amongst the different routes of targeting a drug, the oral route remains the choice of administration (Krishnaiah et al., 2002). Conventional oral dosage forms are ineffective in delivering drugs to the colon due to absorption or degradation of the active ingredient in the upper gastrointestinal tract (Sinha et al., 2004).

It was, thus, hypothesized that targeted delivery of 5-FU through oral route would not only reduce systemic exposure of drug but

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would also help to circumvent limitations associated with i.v. route. Moreover, reduced systemic exposure would help to decrease above mentioned side effects. It was also anticipated that minimal systemic exposure of drug achieved due to site-specific delivery of 5-FU to colon would lead to reduction in dose as well as the duration of therapy in comparison to conventional oral administration. Longer exposure to lower concentration of 5-FU has been reported to favor DNA-directed effects which is thought to contribute to its anti-tumour effect (Wei et al., 2008). All these factors are expected to significantly enhance safety and effectiveness of the therapy which in turn would contribute to increased patient compliance. Literature review reveals that mainly tablets and multiparticulate systems have been investigated for colonic delivery of 5-FU (Sinha et al., 2004; Rahman et al., 2008; Patel et al., 2008). Tablets owing to the merits of being cost effective and requiring minimal processing steps during manufacture have been more preferred.

Pectin is a non-starch, linear polysaccharide extracted from the plant cell walls. It largely remains intact in the physiological environment of the stomach as well as small intestine, but is degraded by the bacterial inhabitants of the human colon which contain pectinolytic enzymes (Sinha and Kumaria, 2001). Various systems using pectin as a carrier have been investigated in order to develop oral formulations intended to release their active substance in the colon (Wakerly et al., 1996; Adkin et al., 1997; Monin and Pundarikakshudu, 2007; Ahrabi et al., 2000).

However, most of the studied approaches either employ compression coating having demerits of multiple granulation, improper centration, capping and multitude of steps required for processing or employ polymers having undesirable effects. Polymer like guar gum causes suppression of appetite and obstruction of esophagus due to premature swelling (Rowe et al., 2006) while chitosan causes loss of weight (Egras et al., in press). Such untoward effects are likely to further aggravate the debilitated condition of a patient suffering from colon cancer thereby decreasing the patient compliance to the therapy. Pectin, being one of the components of dietary fiber, was selected as the carrier for matrix formation since an inverse relationship has been reported between risk of colorectal cancer and intake of dietary fiber (Dahm et al., 2010). Hence, the present research work was aimed to formulate a colon specific drug delivery system of 5-FU using pectin-based matrix, dually coated by spray coating technique, as the carrier to overcome limitations of the reported investigations. An attempt was also made to estimate shelf life of developed formulation.

2. Materials and methods

2.1. Materials

5-FU (98–99% pure) was obtained as a gift sample from Shalaks Pharmaceuticals (P) Ltd., New Delhi, India. Eudragit S 100 was gifted by Degussa India (P) Ltd., Mumbai, India. Other materials namely lactose, starch, magnesium stearate, talc and barium sulphate were purchased from s.d. fine-chem. Ltd., Mumbai, India. New Zealand white rabbits used for the animal studies were procured from the animal house of Rajiv Academy for pharmacy, Mathura, India.

2.2. Experimental design

A 3^2 full factorial design was used for the optimization procedure. The studied factors (independent variables) were: amount of pectin (X_1) and amount of starch paste (X_2), each at three different levels as mentioned in Table 1. The dependent variables were: hardness (Y_1) percent of drug released at 5th h (Y_2) and $t_{90\%}$ (time required for 90% of drug release) at pH 6.5 (Y_3). A total of nine experimental runs (F1–F9) were performed and the composition of matrix tablet prepared on the basis of experimental design is shown in Table 2. Tablets weighing 300 mg were prepared in each case as per the method detailed in the Section 2.4.

2.3. Statistical analysis of data

The effect of independent variables on the responses was modeled with the help of design expert software version 8.0.2 (Stat-Ease, Inc., USA) using following polynomial equation:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_2^2 + b_{22}X_2^2 + b_{12}X_1X_2 \quad (1)$$

where Y is the measured response, X_i is the level of the ith factor; b_0 represents the intercept, and b_i, b_{ij}, ... represent coefficients computed from the responses of the formulations in the design.

2.4. Preparation of pectin-based colon-targeted 5-FU tablets

Pectin-based colon-targeted matrix tablets of 5-FU were prepared by wet granulation method using 10% (w/v) starch paste as binder. Lactose was used as diluent and a mixture of talc along with magnesium stearate at 1:1 (w/w) ratio was used as lubricant. Pectin was sieved through sieve no. 60 and mixed with 5-FU (passed through sieve no. 16) and lactose (passed through sieve no. 60. The powders were blended and granulated with 10% (w/v) starch paste. The wet mass thus obtained was passed through sieve no. 16 to obtain granules which were dried at 50 °C for 2 h. The dried granules were then passed through sieve no. 16 and were lubricated using a mixture of talc and magnesium stearate (1:1, w/w). The lubricated granules were then compressed at a compression force of 4000–5000 kg using 11 round punches.

2.5. Coating of matrix tablets of 5-FU

For optimization of the coating level, various concentrations of Eudragit S 100 [4% (w/v), 5% (w/v), and 6% (w/v)] in 1:3 (v/v) mixture of ethanol 95% (v/v) and isopropyl alcohol were investigated. 50 ml of coating solution at each level was used to coat a batch of hundred tablets. Coating level was optimized on the basis of weight of coated tablet and efficiency of the coat in sealing the drug release in gastric environment. The target build up weight was set at not more than 8% by weight of the core tablet. The prepared matrix tablets were finally enteric coated using a 6% (w/v) solution of Eudragit S 100 in a 1:3 (v/v) mixture of ethanol 95% (v/v) and isopropyl alcohol for further studies. Dibutyl phthalate (10%, v/v) was added as the plasticizer and magnesium stearate was added to reduce the tackiness of the tablets. Tablets were coated using a side-vented pan coater (Glatt coater, New Delhi, India). The pan was rotated at the speed of 15 rpm and the coating solution was sprayed at a rate of approximately 2 g/min. Spraying pressure was adjusted to 3 bars. Inlet and outlet temperatures were maintained at 40 and 30 °C respectively. The process was continued until the whole solution was sprayed onto the tablets whereupon the tablets were rotated for further 5 min. Thereafter, the tablets were dried in an oven at 40 °C for 2 h.

2.6. Determination of drug content

Pectin-based matrix tablets of 5-FU were evaluated for their drug content in triplicate. Ten tablets were finely powdered and quantity of the powder equivalent to 50 mg of 5-FU was accurately weighed and transferred to a 100 ml volumetric flask containing 50 ml of distilled water. The flask was shaken to solubilize the drug and the volume was made up to 100 ml with distilled water. The solution was filtered through a 0.22 μm membrane filter and analyzed for drug content using HPLC at 266 nm as mentioned in Section 2.13.

Table 1
Experimental design: factors and responses.

Factors employed (independent variables)	Levels used			Responses observed (dependent variables)
	−1 (low)	0 (medium)	+1 (high)	
X_1 = amount of pectin	100 mg	150 mg	200 mg	Y_1 = hardness
X_2 = amount of starch paste	15 mg	30 mg	45 mg	Y_2 = percent of drug release at 5th h
				$Y_3 = t_{90\%}$

2.7. Swelling or water uptake

The core tablets were subjected to swelling studies. One tablet from each formulation was randomly selected, weighed (W_1) and placed in petridish containing 10 ml of phosphate buffer (pH 7.4). At regular time intervals (20, 40, 60, 80, 100, 120, 140, 160 and 180 min) the tablet was carefully removed from petridish and excess of water was removed using filter paper. The swollen tablets were reweighed (W_2) and swelling index of each tablet was calculated using the Eq. (2). The analysis was performed in triplicate.

$$\text{Swelling index} = \frac{W_2 - W_1}{W_1} \times 100 \quad (2)$$

2.8. In vitro drug release

These studies were carried out in using a USP XXVI dissolution rate test apparatus I (basket) at 100 rpm. The dissolution media was maintained at 37 ± 2 °C. The tablets were tested for drug release for 2 h in 0.1 N HCl (900 ml). Then the dissolution medium was changed to phosphate buffer (pH 7.4) (900 ml) and drug release was studied for 3 h. Finally, the dissolution medium was changed to 200 ml of phosphate buffer (pH 6.5) containing 4% (w/v) of rat caecal contents and the drug release was studied for 19 h. The amount of drug released at each time interval was determined by HPLC as mentioned in Section 2.13. For preparation of rat caecal contents, Wistar rats weighing between 200 and 250 g were selected and maintained on a normal diet. One hour prior to the drug release studies, sufficient numbers of rats were killed by spinal traction and their abdomens were opened. The caecum were traced and ligated at both the ends. Thereafter, the caecum were dissected and immediately transferred to phosphate buffer (pH 6.5) previously bubbled with nitrogen. The caecal bags were opened and their contents were individually weighed. The contents were pooled, and suspended in the phosphate buffer (pH 6.5) which was continuously bubbled with nitrogen. These were eventually added to the dissolution media to obtain a final caecal dilution 4% (w/v). All of the above procedures were performed under nitrogen so as to maintain anaerobic condition (Sinha et al., 2004).

2.9. Validation of experimental design

Polynomial equations were generated using Design expert software version 8.0.2 (Stat-Ease, Inc, USA) for selected responses like hardness, % CDR at 5th h and $t_{90\%}$. The generated polynomial equations were further reduced on the basis of significant terms obtained by applying ANOVA. The 3^2 full factorial design was validated by preparing an extra check point formulation (F10). The predicted values for hardness, % CDR at 5th h and $t_{90\%}$ for F10 were determined on the basis of respective polynomial equations whereas the experimental values were determined by evaluating F10 for the selected dependent variables. The predicted and experimental values of the responses were compared for statistical significance using paired *t*-test.

2.10. Selection of optimized formulation

Optimized formulation was selected on the basis of maximum hardness, minimum % CDR at 5th h, minimum $t_{90\%}$ at pH 6.5 and with good desirability.

2.11. In vivo roentgenographic analysis

The protocol for *in vivo* roentgenographic study was approved by the Institutional Animal Ethics Committee of Rajiv Academy for Pharmacy, Mathura, India. As per the protocol no. IAEC/RAP/3040, three New Zealand white rabbits weighing between 3 and 3.5 kg were used for the study. Core tablets (6 mm) were prepared as per the optimized formula by replacing the drug with the radiopaque compound barium sulphate. Thereafter, the tablets were coated similarly to the optimized batch. Before administration of tablets to the rabbits for *in vivo* roentgenographic study the tablets were subjected to *in vitro* drug release studies in order to determine the intactness of the coat. Tablets were examined visually to check rupturing the coat at regular time intervals. The tablets in which the coat was found to remain intact for 5 h were selected for final *in vivo* roentgenographic analysis. All the rabbits used for the study were fasted overnight with free access to water *ad libitum*. After an overnight fasting, the selected tablets were administered to the rabbits with 15 ml of water. Roentgenographic images of the

Table 2
Composition of pectin-based matrix tablets of 5-FU.

Code	Drug (%, w/w)	Pectin (%, w/w)	Percentage of starch paste (10%, w/v)	Magnesium stearate (%, w/w)	Talc (%, w/w)	Lactose (%, w/w)
F1	16.67	33.33	5	0.16	0.16	44.67
F2	16.67	33.33	10	0.16	0.16	39.67
F3	16.67	33.33	15	0.16	0.16	34.67
F4	16.67	50.00	5	0.16	0.16	28.00
F5	16.67	50.00	10	0.16	0.16	23.00
F6	16.67	50.00	15	0.16	0.16	18.00
F7	16.67	66.67	5	0.16	0.16	11.33
F8	16.67	66.67	10	0.16	0.16	6.33
F9	16.67	66.67	15	0.16	0.16	1.33
F10[a]	16.67	58.33	12.5	0.16	0.16	12.17
F11[b]	16.67	–	15	0.16	0.16	68.01

[a] Extra check point formulation.
[b] Immediate release tablets formulation.

abdomen of the rabbits were taken at the end of 2, 3.5, 4, 5 and 7 h to trace the movement and behaviour of the tablet in the GIT of rabbits. To precisely locate the position of the tablet in the GIT of the rabbits, a barium meal study was performed before the actual study where by 10 ml of standard barium meal was administered to the rabbits. X-ray images of the rabbits in prone position were captured using Siemens X-ray machine, with 64 mAs and 63 kV techniques (Patel and Amin, 2011).

2.12. In vivo pharmacokinetic evaluation

The protocol for *in vivo* pharmacokinetic evaluation was approved by the Institutional Animal Ethics Committee (IAEC) of Rajiv Academy for Pharmacy, Mathura, India, as per the protocol no. IAEC/RAP/3040, Six New Zealand white rabbits weighing between 3 and 3.5 kg were used for the study. All rabbits were fasted overnight with free access to water. The rabbits were divided into two groups, namely standard and test, with three animals in each group. The standard group received in-house developed immediate release (IR) tablets (Table 2) where as the test group received optimized formulation (F9), each containing 50 mg of 5-FU. After oral administration of formulations, 2 ml of blood samples were collected from the marginal ear vein at 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 18 and 24 h. The plasma fraction used for 5-FU assay was obtained by centrifugation of the blood samples at 5000 rpm for 30 min (REMI International, Mumbai, India). The plasma samples were immediately stored at −20 °C until further analysis.

2.13. HPLC determination of 5-FU

Concentration of 5-FU in the samples was determined using HPLC method reported by Krishnaiah et al. (2003) with slight modification as per system requirement. The system used was Shimadzu LC-10AT VP having a UV detector (Shimadzu, Kyoto, Japan) and the software used was Class VP, version 5.032. A RP C-18 column (250 ± 4.0 mm I.D., particle size 5 μm; Merck Lichrospher, USA) was used as the stationary phase and HPLC grade water as the mobile phase. The flow rate was maintained at 1 ml/min and detection was carried out with UV–vis detector at 266 nm. For analysis of plasma samples, 0.5 ml samples were measured in glass vials and 7.5 ml of acetonitrile was added. The samples were then centrifuged at 4000 rpm for 15 min and supernatant was filtered through a 0.2 μm membrane filter and injected into the column. The retention time of 5-FU was found to be 7.02 ± 0.1 min. Run time employed for the method was 10 min.

2.14. Pharmacokinetic parameters

The pharmacokinetic parameters were calculated by using Quick calc. software (developed by Dr. Shivprakash, Plexus, Ahmedabad, India). The maximum concentration of drug in plasma (C_{max}), the time taken to reach C_{max} (t_{max}) and the time taken for the first appearance of 5-FU in the plasma (T_{lag} time) were obtained as directly measured values from the plasma – concentration versus time profile. The $AUC_{0\rightarrow\infty}$, K_a, K_e, total clearance (Cl_T) and elimination half life were calculated with the help of software. The pharmacokinetic parameters were analyzed for statistical significance using two-tailed unpaired *t*-test. A $p < 0.001$ was considered to be significant.

2.15. In vitro cytotoxicity studies

HT-29 (human colon cancer cell line) cell cultures were obtained from National centre for cell sciences, Pune, India. HT-29 cells were grown in Earl's minimal essential medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, penicillin (100 μg/ml), streptomycin (100 μg/ml) and amphoterecin B (5 μg/ml). The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 and were subcultured twice a week to examine the effects of 5-FU solution, 5-FU granules without pectin and 5-FU granules with pectin. Ten milligrams of drug was accurately weighed and dissolved in 1 ml of dimethyl sulfoxide. Volume was made up to 10 ml by adding double distilled water so as to achieve a final concentration of 1000 μg/ml. Similarly, granules corresponding to 10 mg of drug were accurately weighed and dissolved in 1 ml of dimethyl sulfoxide. Volume was made up to 10 ml by adding double distilled water in each case so as to achieve a final concentration of 1000 μg/ml.

2.16. Cytotoxicity assay involving determination of total cell protein content by sulphorhodamine B (SRB) assay

SRB is a dark pink amino xanthene dye with sulfonic groups. Under mild conditions, SRB binds to protein basic amino acid residues of protein in trichloro acetic acid (TCA) fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of at least two orders of magnitude. Color development in SRB assay is rapid, stable and visible. The developed color can be measured over a broad range of visible wavelength in either a spectrophotometer or a 96-well plate reader. When TCA-fixed, SRB stained samples are air-dried, they can be stored indefinitely without deterioration. The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0×10^5 cells/ml using minimal essential medium (MEM) containing 10% new born calf serum. To each well of the 96-well microtitre plate, 0.1 ml of diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off and the monolayer was washed once. 100 μl of the medium and different drug concentrations were added to the culture in microtitre plates. The plates were then incubated at 37 °C for 3 days in 5% CO_2 atmosphere. Microscopic examination was carried out and observations were recorded every 24 h. After 72 h, 25 μl of 50% TCA was added to the wells gently such that it formed a thin layer over the drug dilution to form an overall concentration of 10%. The plates were incubated at 4 °C for 1 h. The culture plates were flicked and washed five times with water to remove traces of medium, drug and serum, and were then air-dried. The air-dried plates were stained with SRB for 30 min. The unbound dye was then removed by rapidly washing four times with 1% acetic acid. The plates were then air-dried. 100 μl of 10 mM tris base was then added to the wells to solubilize the dye. The plates were shaken vigorously for 5 min. The absorbance was measured using micro plate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following equation:

$$\%\ \text{Growth inhibition} = 100 - \left(\frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}}\right) \times 100 \quad (3)$$

The results were reported in terms of CTC_{50} (cytotoxic concentration for 50% cells).

2.17. Stability studies

Stability studies for the optimized batch of 5-FU colon-targeted tablets were performed as per ICH Q1A R2 guidelines by storing the tablets at 40 ± 2 °C and 75% ± 5% RH for 3 months. The tablets were observed for change in physical appearance and drug content at the end of 0, 30, 60 and 90 days. Zero day samples were used as control. The results obtained were presented in the form of plot between percentage label claim (% drug remaining) versus time in days to determine shelf life. Shelf life was determined as the time at

which the 95% one-sided confidence limit for the mean curve intersected the acceptance criterion of 90% percentage label claim. The data was evaluated using Sigmaplot™ software (Cranes Software International, Bangalore, India). The tablets were also subjected to *in vitro* drug release studies. The drug release profiles of the optimized batch at the end of 30, 60 and 90 days were investigated for evaluation of the similarity factor (f_2) with the drug release profile at the end of zero day.

2.18. Statistical analysis

The cumulative percent of drug released from the pectin-based coated matrix tablets of 5-FU in the dissolution medium during the period of 24 h in presence and absence of rat caecal content were compared for statistical significance by using two-tailed unpaired *t*-test. A value of $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Selection of dose for colon specific delivery

As per the calculation by Chabner and Alabresi, the dose of 5-FU for colon specific drug delivery on the basis of colon surface area was found to be 75 mg. Considering the reports stating 5-FU to be highly toxic against the intestinal mucosa, only 50 mg of 5-FU was used by the authors (Sinha et al., 2004). So, 50 mg has been used as the dose of the drug for colon specific delivery in the present study as well.

3.2. Preparation of pectin-based colon-targeted 5-FU tablets

On the basis of 3^2 experimental design, nine formulations were prepared which were evaluated for weight variation, thickness, hardness, drug content, friability and swelling index. The results of these investigations are shown in Table 3. The tablets were found to be within the I.P. limits of the performed tests.

3.3. In vitro drug release

To assess the significance of coat in preventing the drug release in stomach as well as the role of pectin in modulating the drug release in small intestine and colon, a preliminary investigation involving comparative evaluation of the release profile of 5-FU from uncoated F1 and coated F1 (containing various levels of Eudragit S 100) at pH 1.2, 7.4 and 6.5 in presence and absence of rat caecal contents was performed. Average weight of coated tablets at 4% (w/v), 5% (w/v) and 6% (w/v) coat level was found to be 318.33 ± 1.53 mg, 321.67 ± 0.58 mg and 323.67 ± 0.58 mg respectively. No significant increase ($p > 0.01$) in the weight of the tablets was observed when the coating level was increased from 4% (w/v) to 6% (w/v). When evaluated for their effectiveness in sealing the drug release in the gastric environment, coating level of 4% (w/v) and 5% (w/v) released 39.67 ± 1.11% and 25.22 ± 0.89% of their drug content respectively in the gastric environment and were found to be ineffective in sealing the drug release in the gastric milieu in comparison to coat level of 6% (w/v). Since a coating level of 6% (w/v) exhibited satisfactory sealing of release profile in the gastric environment, higher levels of coating were not investigated. The release behaviour of uncoated F1 and coated F1 [containing 6% (w/v) Eudragit S 100] are shown in Fig. 1. F1 formulation was selected for the investigation since it contained minimum amount of pectin. In case of uncoated F1, 90.73 ± 1.23% of drug release was observed within 2 h of the study. This might be due to the hydrophilic nature of the prepared matrix owing to the presence hydrophilic polysaccharide pectin. The matrix tablets coated with Eudragit S 100 exhibited negligible amount of drug release in the gastric environment. This could be because of the fact that Eudragit S 100 solubilizes at pH above 7. The same tablets recorded 83 ± 1.23% of drug release in presence of rat caecal contents whereas only 62.78 ± 0.98% of drug release in its absence. Higher drug release in presence of rat caecal contents may be ascribed to the presence of pectinolytic enzymes in rat caecal contents which degrade pectin and thus facilitate drug release. Since F1 formulation contained minimum amount of amount of pectin and exhibited 83 ± 1.23% of drug release in colon, other formulations containing higher amount of pectin are likely to behave better.

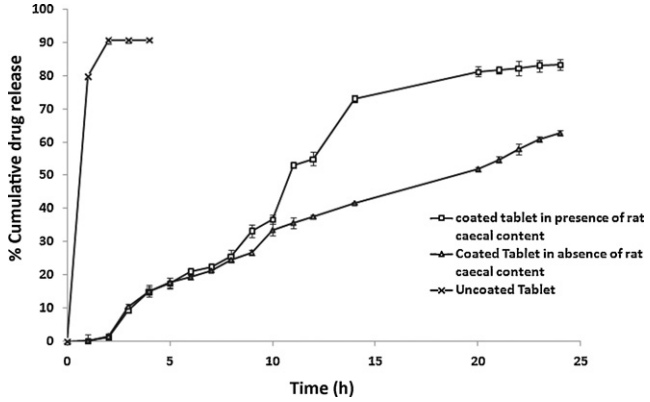

Fig. 1. *In vitro* drug release study showing the mean (±S.D.) percent of 5-Fluorouracil release from pectin-based uncoated tablets, coated tablet in presence of rat caecal content and coated tablet in absence of rat caecal content ($n = 3$).

Comparative release profile of 5-FU from the nine experimental formulations and the in-house developed IR (reference) formulation in media mimicking mouth to colonic transit is shown in Fig. 2. The in-house developed IR tablets released majority of their drug content 90.73 ± 1.24% in 0.1 N HCl where as the nine experimental formulations did not show any drug release in this medium. As discussed already, this can be attributed to the presence of coat of Eudragit S 100 in the nine experimental formulations. Eudragit S 100 solubilizes only at pH ≥7 and hence the experimental formulations remained intact at the gastric pH. Thus, a coat level of 6% (w/v) proved to be effective in sealing the release of 5-FU from the pectin-based matrix tablets in gastric environment.

On analyzing the % CDR (mean ± S.D.) of formulation F1–F9 in intestinal fluid (phosphate buffer, pH 7.4, 3 h), it was found that formulations F1, F2 and F3 containing 33.33% (w/w) of pectin demonstrated a % CDR of 40 ± 1.66%, 36.66 ± 1.09% and 32.55 ± 1.90% respectively. This showed that 33.33% (w/w) of pectin was not sufficient to minimize drug release in small intestine. As the level of starch paste increased from 5% (w/w) in F1 to 15% (w/w) in F3, the % CDR at 5th h decreased. This could be due to the reason that increased levels of starch paste in the tablets increased their ability to resist disintegration in the intestinal fluid. As the amount of pectin was increased from 33.33% (w/w) to 50% (w/w) in formulations F4–F6, the % CDR at 5th h decreased to 26.65 ± 0.9%, 22.65 ± 1.78% and 18.58 ± 2.01% for F4, F5, F6 respectively. This demonstrates that even the 50% (w/w) of pectin is not sufficient to minimize release of 5-FU in the small intestinal milieu. The reduction in the amount of % CDR at 5th h with an increase in the amount of pectin can be explained on the basis of the fact that pectin tends to swell and form a gel at higher pH which might be contributing towards the decrease in the release profile owing to formation of a diffusion control layer (Sriamornsak et al., 2007). On further increasing the amount of pectin to 66.67% (w/w) in formulations coded F7, F8, F9, reduction in the amount of % CDR at intestinal pH was observed. Formulations F7, F8 and F9 were found to release 10.87 ± 1.89%, 8.98 ± 1.87% and 8.01 ± 2.34% of drug respectively. This could be due to the reason that further increase in the amount

Table 3
Evaluation of coated matrix tablets ($n = 3$).

Code	Weight ± S.D. (mg)	Thickness ± S.D. (mm)	Hardness ± S.D. (kg/cm^2)	Friability (%)	Drug content ± S.D. (%)	Swelling index ± S.D. (%)
F1	297 ± 1.50	3.42 ± 0.00	4.20 ± 0.23	0.54	96.04 ± 1.32	21.34 ± 2.32
F2	300 ± 2.50	3.42 ± 0.11	4.60 ± 0.14	0.54	96.26 ± 1.54	19.65 ± 1.87
F3	295 ± 2.70	3.40 ± 0.00	4.75 ± 0.45	0.56	97.56 ± 0.65	16.0 ± 3.40
F4	298 ± 3.00	3.44 ± 0.00	4.10 ± 0.30	0.52	96.55 ± 2.43	25.96 ± 2.34
F5	300 ± 1.23	3.46 ± 0.23	4.33 ± 0.63	0.51	96.76 ± 1.32	23.43 ± 1.43
F6	300 ± 0.89	3.42 ± 0.34	4.70 ± 0.67	0.54	99.40 ± 1.45	20.55 ± 1.58
F7	299 ± 2.54	3.40 ± 0.31	4.23 ± 0.87	0.54	95.90 ± 2.32	34.32 ± 1.22
F8	299 ± 3.34	3.45 ± 0.43	4.42 ± 0.59	0.53	95.82 ± 1.78	31.34 ± 2.43
F9	298 ± 2.56	3.43 ± 0.54	4.80 ± 0.67	0.56	95.90 ± 2.32	28.45 ± 1.45
F10	299 ± 1.45	3.68 ± 0.42	4.28 ± 0.15	0.52	96.60 ± 1.78	30.58 ± 1.33
F11	298 ± 2.42	3.38 ± 0.28	4.80 ± 0.88	0.55	97.44 ± 2.11	–

of pectin in these formulations led to the formation of a stringent barrier owing to absorption of water leading to the development of a highly viscous diffusion control layer. Amongst the formulations F4, F5, F6 and F7, F8, F9, reduction in the % CDR at 5th h was observed with an increase in the proportion of starch paste as explained earlier for formulations F1, F2 and F3. Formulation F9 was found to release only 8.01 ± 2.34% of drug in the small intestinal environment at the end of 5 h of drug release study since it contained higher amount (15%, w/w) of starch paste. This could be responsible for maintaining integrity of tablets of batch F9 thereby delaying contact of matrix with water which is a pre requisite for the drug release to take place.

Eventually the release of the drug from the nine experimental formulations was analyzed in colonic environment [phosphate buffer pH 6.5, containing 4% (w/v) of rat caecal contents] to precisely delineate the release behaviour of these formulations in the colonic environment. Four percent (w/v) of rat caecal contents were included in the study to mimick the colonic environment. Amongst the formulations F1–F9, F9 was found to exhibit the maximum amount of % CDR of 96.34 ± 0.88 in the colonic environment. This could be attributed to the presence of highest amount of pectin in F9. Since pectinolytic enzymes in case of F9 had the maximum amount of substrate to act upon, hence maximum drug release was observed in F9 within 13.50 h of study in the colonic environment. It is the beauty of the present system that pectin is behaving as a bifunctional release controlling excipient since it is responsible for retarding the drug release in gastric and small intestinal environment but has the potential to maximize the drug release in colonic milieu.

Table 4
Experimental runs and observed results.

Run no.	Responses		
	Y_1 (kg/cm^2)	Y_2 (%)	Y_3 (h)
1	4.20 ± 0.23	40.54 ± 0.67	20.23 ± 0.34
2	4.60 ± 0.14	36.66 ± 0.83	22.10 ± 0.45
3	4.75 ± 0.45	32.55 ± 0.98	23.05 ± 0.65
4	4.10 ± 0.30	26.65 ± 0.45	20.10 ± 0.23
5	4.33 ± 0.63	22.65 ± 0.22	20.12 ± 0.21
6	4.70 ± 0.67	18.58 ± 1.43	21.04 ± 0.38
7	4.23 ± 0.87	10.87 ± 0.98	12.20 ± 0.11
8	4.42 ± 0.59	8.98 ± 1.78	14.00 ± 0.08
9	4.80 ± 0.67	8.01 ± 1.34	13.50 ± 0.014

After evaluating the experimental formulations for their efficiency in preventing drug release in the gastric and small intestinal milieu, their release behaviour in colonic environment was further analyzed using response surface methodology since it was desirable that the optimized formulation should exhibit minimum drug release in the stomach and small intestine where as maximum drug release was desirable in the colon. Moreover, the time taken to release majority of the drug in colon should be minimum.

Thus, the following parameters were selected as response variables for selection of final formulation. Y_1: hardness; Y_2: percent cumulative drug release (% CDR) at 5th h; Y_3: $t_{90\%}$ (time required for drug release up to 90%) at pH 6.5 in presence of 4% (w/v) rat caecal contents (Table 4).

In order to determine the levels of factors (X_1 and X_2) which yielded optimum drug release responses, mathematical relation-

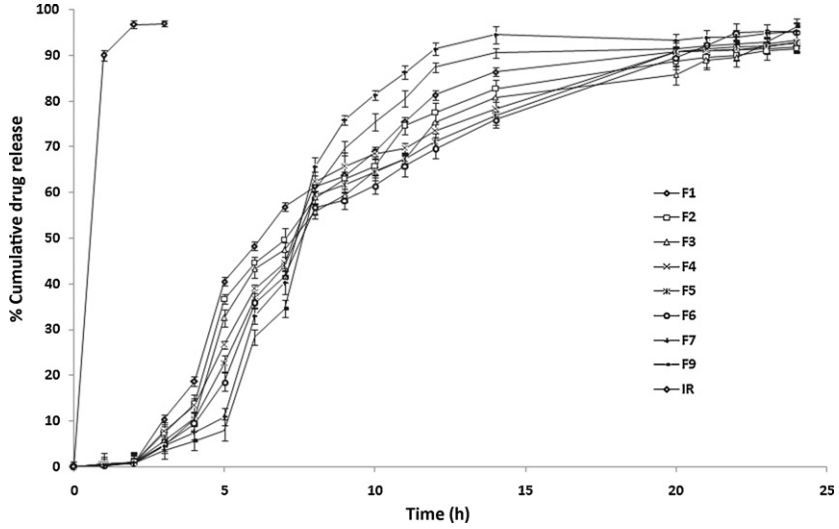

Fig. 2. Percent cumulative release (mean ± S.D.) of 5-FU from coated tablets and IR (reference) tablet in intestinal and colonic environment.

Table 5
Analysis of variance (ANOVA) of dependent variables.

Source	Sum of squares	df	Mean square	*F* value	*p*-value Prob > *F*
Y_1					
Regression	0.68	5	0.14	8.49	0.015
Residuals	2.42	3	0.016		
Total	0.73	8			
$R^2 = 0.99675$					
Y_2					
Regression	1184.11	5	236.82	288.65	0.0003
Residuals	2.46	3	0.82		
Total	1186.58	8			
$R^2 = 0.9945$					
Y_3					
Regression	111.58	5	22.32	27.70	0.0103
Residuals	2.42	3	0.81		
Total	114.00	8			
$R^2 = 0.99675$					

ships were generated between the dependent and independent variables (responses) using Design expert software version 8.0.2. Following reduced equations were generated for the observed responses (Y_1, Y_2 and Y_3) after application of ANOVA.

$$Y_1 = 4.41 - 0.025X_1 + 0.33X_2 - 0.007X_1X_2 + 0.055X_1^2 + 0.060X_2^2 \quad (4)$$

$$Y_2 = 22.56 - 13.65X_1 + 3.15X_2 + 1.28X_1X_2 + 0.31X_1^2 - 0.10X_2^2 \quad (5)$$

$$Y_3 = 20.33 - 3.83X_1 + 1.33X_2 - 0.25X_1X_2 - 2.5X_1^2 + 0.012\,X_2^2 \quad (6)$$

In the above equations, coefficients with more than one factor represent the interaction between factors while coefficients with second order terms indicate the quantitative effect of independent variables (X_1 and X_2) upon the responses (Y_1, Y_2 and Y_3). Analysis of variance indicated that the assumed regression models were significant and valid for each considered response shown in Table 5.

The three-dimensional response surface plots were drawn to estimate the effect of the independent variables on each of the response as shown in Fig. 3. Response surface plots depict concurrent effect of any two variables on response parameter keeping one variable at constant level. Fig. 3(a) shows the effect of pectin and starch paste on hardness. The amount of starch paste was found to have a significant effect on the hardness of the formulation in comparison to the amount of pectin. Increase in the amount of starch paste led to an increase the hardness of the formulation because starch paste in the concentration of 5–25% (w/w) in tablet granulation acts as a binder. No effect was seen on changing the level of pectin on the hardness of the formulation.

Fig. 3(b) shows the effect of independent variables on % CDR at 5th h. The effect of amount of pectin on % CDR at 5th h was found to be more prominent than the amount of starch paste. This could be attributed to the fact that pectin on hydration undergoes swelling to form a viscous gel layer which acts as a diffusion control layer for the release of drug. The same phenomenon might also be responsible for the reduction in release profile observed on increasing the amount of pectin in the matrix since with an increase in the amount of pectin in the matrix, viscosity of the gel layer formed on hydration of pectin is expected to increase thereby providing a stern barrier to the release of 5-FU which eventually retards the drug release in small intestine upto 5th h. Fig. 3(b) shows that there is no effect of changing the amount of starch paste on % CDR at 5th h. This may be explained on the basis of fact that starch paste acts as a binder in the present study. It is responsible for maintaining the integrity of the tablets and has no role in modulating the release of the drug from the tablets. Thus, no effect of varying the level of 10% (w/v) starch paste was seen on % CDR at 5th h.

Fig. 3(c) shows the effect of two formulation factors on $t_{90\%}$ at pH 6.5 in presence of rat caecal contents. This figure indicates that an increase in the amount of pectin decreases the time requirement to release upto 90% of the drug in phosphate buffer pH 6.5 containing 4% (w/v) rat caecal contents. This could be due to the reason that on increasing the amount of pectin in the matrix, pectinolytic enzymes present in the colon have more of substrate to act upon leading to rapid release of drug and thereby causing reduction in $t_{90\%}$. Fig. 3(c) also shows no significant effect of changing the amount of starch paste on $t_{90\%}$ as already explained in case of % CDR at 5th h.

3.4. Validation of experimental design

In order to check the validity of the generated equations in the optimization procedure, a new batch of tablets (extra check point formulation) was prepared. Comparative analysis of the predicted value and experimental values using paired *t*-test indicated

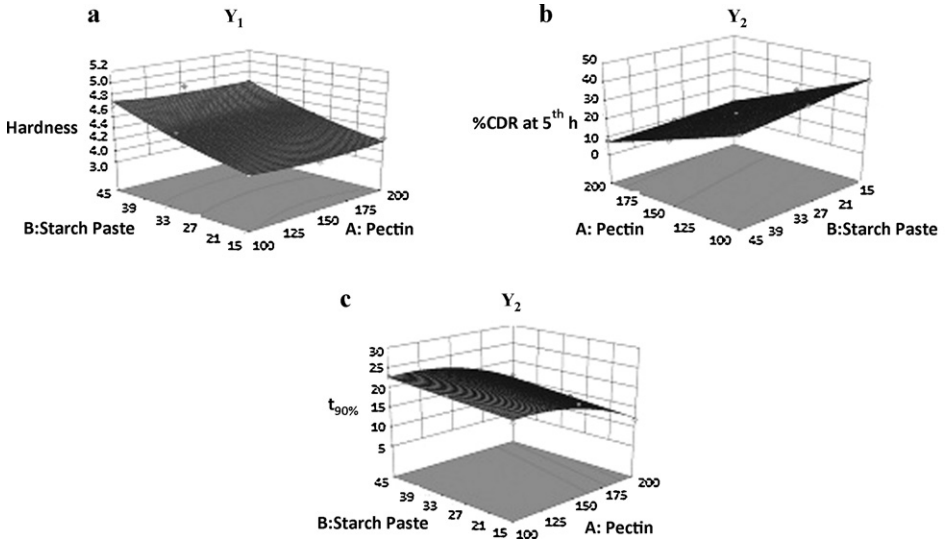

Fig. 3. . Response surface plots showing effect of pectin and starch paste on: (a) hardness, (b) % CDR at 5th h and (c) $t_{90\%}$.

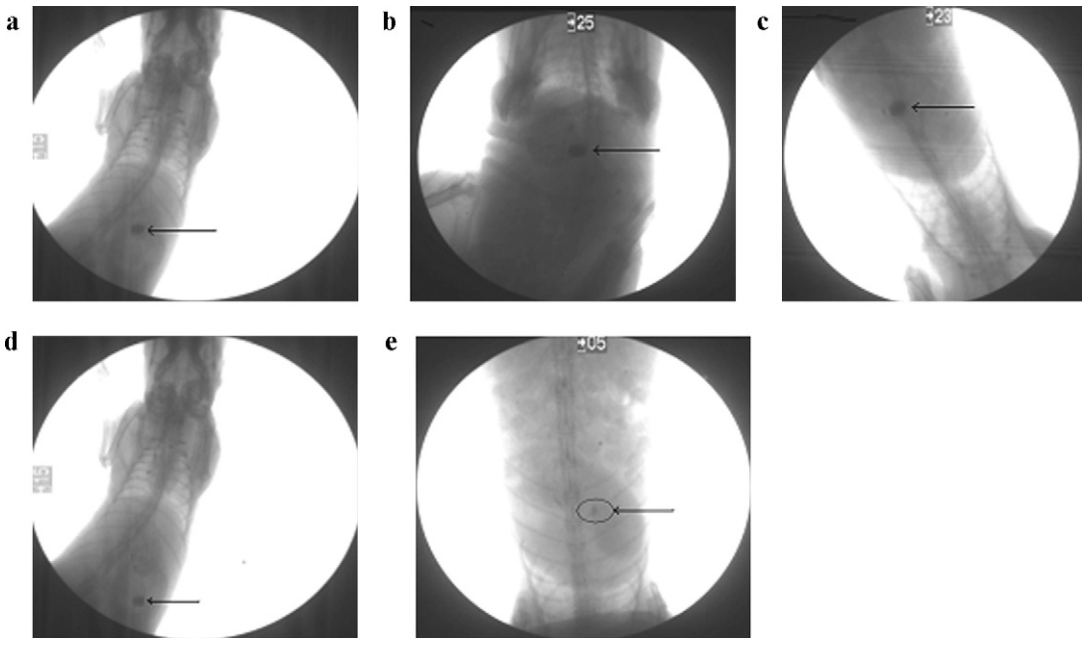

Fig. 4. Roentgenographic images showing *in vivo* behaviour of optimized formulation F9 after oral administration to rabbits: (a) after 2 h of administration in stomach (b) after 3.5 h of administration at pyrolic junction (c) after 4 h of administration in small intestine (d) after 5 h of administration in the colon (e) after 7 h of administration in the colon.

no significant ($p < 0.01$) difference between the two values (Table 6) thereby establishing validity of the generated model.

3.5. Selection of optimized formulation

Eventually, formulation F9 yielding desirability factor of 0.840 on analysis by design expert software was selected as the optimized formulation. The optimized formulation exhibited hardness of 4.80 ± 0.67 kg/cm^2, 8.01 ± 1.34% of CDR after 5 h and $t_{90\%}$ (90% of drug release) at 13.50 ± 0.014 h.

3.6. In vivo roentgenographic evaluation

Rabbits were selected as the animal model since variation in the pH of GIT of rabbits is analogous to that of humans (pH of stomach, small intestine and colon is reported to be 1.5–2.0, 7.2 and 6.5 respectively). Also, the mean colonic arrival time in rabbits (total time: 4–6 h, stomach transit time: 2–4 h, small intestine transit time: about 2 h) is almost similar to that of humans (Patel and Amin, 2011). No significant difference ($p > 0.05$) was observed between the results of *in vitro* drug release from the optimized colon-targeted system containing barium sulphate and the optimized 5-FU coated tablets (data not shown here) indicating suitability of the batch containing barium sulphate for *in vivo* roentgenographic analysis. The results of roentgenographic study are shown in Fig. 4. Fig. 4(a) shows that the tablet remains intact in the stomach establishing *in vivo* efficiency of the 6% (w/v) coating of Eudragit S 100 in preventing drug release in the gastric milieu. Fig. 4(b) and (c) exhibits no significant difference in the integrity of tablet in comparison to Fig. 4(a) thereby indicating intactness of the tablet in small intestine. Fig. 4(d) and (e) shows the behaviour of tablet after 5th and 7th h of administration i.e. in colon. Reduction in size of tablet was seen in Fig. 4(e) indicating release of drug in colon. These images clearly demonstrate that the optimized formulation F9 could be targeted specifically to the colon, without any premature drug release in the small intestine thus providing the 'proof of concept'.

Table 6
Predicted and experimental value of responses for extra check point formulation.

Response	Predicted value	Experimental value
Y_1	4.18	4.28 ± 0.15
Y_2	15.27	15.08 ± 0.98
Y_3	14.51	15.0 ± 1.04

3.7. In vivo pharmacokinetic evaluation

The mean (±S.D.) plasma levels of 5-FU following oral administration of optimized colon-targeted 5-FU tablets and immediate release tablets, both at a dose of 7.47 mg (as per the body weight of rabbit), are shown in Fig. 5. 5-FU appeared almost instantaneously after oral administration of immediate release tablets exhibiting a C_{max} of 208.67 ± 14 ng/ml within 0.5 h of sampling (t_{max}). In case of colon-targeted tablets, 5-FU appeared in plasma after 4 h (t_{lag}) of drug administration and demonstrated significantly reduced ($p < 0.001$) C_{max} of 169.56 ± 12 ng/ml at 6th h (t_{max}). A lag time of 4 h in the appearance of drug in the plasma in case of colon-targeted tablets indicates that the colon-targeted formulation was able to prevent the release of 5-FU in the stomach and small intestine (Table 7). The reduction in C_{max} in case of colon-targeted tablets may be due to the controlled release of drug in colon leading to a decreased systemic exposure of the drug. $AUC_{0-\infty}$ for immediate release tablets and colon-targeted tablets was found to be 244.83 ± 0.24 ng/ml/h and 777.16 ± 0.18 ng/ml/h respectively. $AUC_{0-\infty}$ for immediate release tablets was found to be significantly increased ($p < 0.001$) from that of $AUC_{0-\infty}$ of colon-targeted tablets. Increased $AUC_{0-\infty}$ in case of colon-targeted tablets despite reduction in C_{max} may be attributed to the increase in duration of absorption in colon-targeted tablets in comparison to immediate release tablets since the formation of swollen matrix in colon by colon-targeted tablets leads to release and subsequent absorption of drug in a controlled manner thereby increasing the duration of absorption of drug. K_a in case of immediate release tablets was found to be 2.99 ± 0.04 h^{-1} whereas K_a in case of colon-targeted tablets was found to be 1.54 ± 0.02 h^{-1}. K_a for colon-targeted tablets was significantly reduced in comparison to immediate release

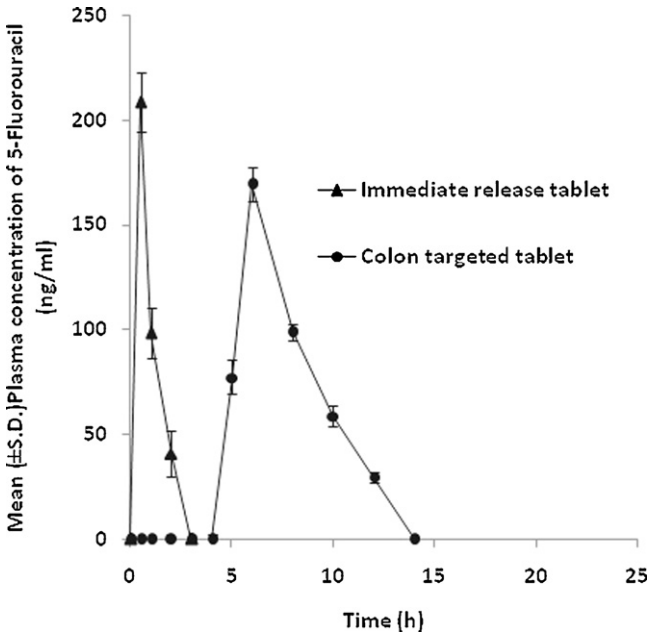

Fig. 5. Mean ± S.D. ($n = 3$) plasma concentration of 5-FU in New Zealand white rabbits following oral administration of immediate release (IR) tablets (dose 7.47 mg) and pectin-based colon-targeted tablets (dose 7.47 mg).

tablets ($p < 0.01$). As already explained this may be due to slower absorption of drug in case of colon-targeted tablets comparison to immediate release tablets. Elimination rate constant (K_{el}) in case of immediate release tablets was found to be $0.88 \pm 0.07\ h^{-1}$ whereas its value for colon-targeted tablets was found to be $0.29 \pm 0.05\ h^{-1}$. K_{el} of colon-targeted tablets was significantly ($p < 0.001$) reduced in comparison to immediate release tablets. This may be attributed to the reduced and slower appearance of drug in blood circulation in case of colon-targeted tablets leading to a slower contact of the drug with organs of elimination and thus resulting in slower rate of elimination. Elimination half life of 5-FU after oral administration of immediate release and colon-targeted tablets was observed to be 0.79 h and 2.32 h respectively. Since elimination half life of a drug is inversely related to its elimination rate, elimination half life of 5-FU after oral administration of colon-targeted tablet was found to be significantly increased ($p < 0.001$) in comparison to that of immediate release tablets. Total clearance (Cl_T) of 5-FU from immediately released and colon-targeted tablet was found to be 0.03 ± 0.05 mg/h and 0.0096 ± 0.08 mg/h respectively. Cl_T of 5-FU from colon-targeted tablets was found to be significantly reduced in comparison to that from immediate release tablets. Drug clearance refers to the volume of plasma fluid that is cleared of drug per unit time. Lesser and slower systemic exposure of drug per unit time in case of colon-targeted tablet in comparison to immediate release tablets may be responsible for the reduced clearance of drug from colon-targeted tablets over immediate release tablets. Thus, *in vivo* pharmacokinetic studies of pectin-based colon-targeted 5-FU coated tablets in New Zealand white rabbits exhibited delayed T_{max}, decreased C_{max} and absorption rate constant indicating that the drug was not released considerably in stomach and small intestine, but was released significantly in colon to show local action with lesser systemic exposure.

Table 7
Mean (±S.D.) pharmacokinetic parameters of 5-fluorouracil following oral administration (dose 7.47 mg) of immediate release tablets and colon-targeted tablets in New Zealand white rabbits ($n = 3$).

Pharmacokinetic parameters	Type of formulation	
	Immediate release tablet	Colon-targeted tablet
[a]T_{lag} time (h)	[b]0.5	4.0 ± 0.56
[a]C_{max} (ng/ml)	208.67 ± 14.0	169.56 ± 12.0
[a]T_{max} (h)	[b]0.5	6.0 ± 0.57
K_a	2.99 ± 0.02	1.54 ± 0.04
K_{el}	0.88 ± 0.07	0.29 ± 0.05
$AUC_{0-\infty}$ (ng/ml/h)	244.8 ± 0.14	777.2 ± 0.28
Elimination half life	0.79 ± 0.67	2.32 ± 0.88
Total clearance (Cl_T) (mg/h)	0.03 ± 0.05	0.0096 ± 0.08

[a] Significant difference with respect to immediate release tablet ($p < 0.001$).
[b] First time point of blood sampling at which drug was detected in plasma.

Table 8
CTC_{50} value by SRB assay.

S. no.	Sample	CTC_{50} (μg/ml)
1.	C-1 (5-FU solution)	175
2.	C-2 (5-FU loaded pectin-based granules)	280
3.	C-3 (granules without pectin)	350

3.8. Cytotoxicity studies

In vitro cytotoxicity of 5-FU loaded pectin-based granules, granules without pectin and 5-FU solution was investigated using HT-29 human colon cancer cell lines by SRB assay. CTC_{50} value for different samples was determined and the results are shown in Table 8. CTC_{50} value for the 5-FU solution (C-1) was found to be the lowest amongst C-1, C-2 and C-3 indicating highest potency of the drug in this dosage form. This may be ascribed to the fact that 5-FU in the solubilized form in C-1 ensures maximum uptake of the drug by the cancerous cells. CTC_{50} value for the 5-FU granules along with pectin (C-2) was found to be lower as compared to the CTC_{50} value without pectin (C-3). This could be explained on the basis that pectin shows mucoadhesive property and its mucoadhesion is more in large intestine than small intestine (Thirawong et al., 2007). Moreover, the degree of esterification of pectin used in the present study was low as indicated by presence of low methoxy groups (6%). Thus, increased number of carboxyl groups in the pectin used in present study might also be contributing towards increased mucoadhesion from granules containing pectin, leading to increased contact time with the cells thereby facilitating better uptake of drug by the cancerous cells.

3.9. Stability studies

Considering the potential utility of optimized formulation for targeting 5-FU to the colon, stability studies were carried out at

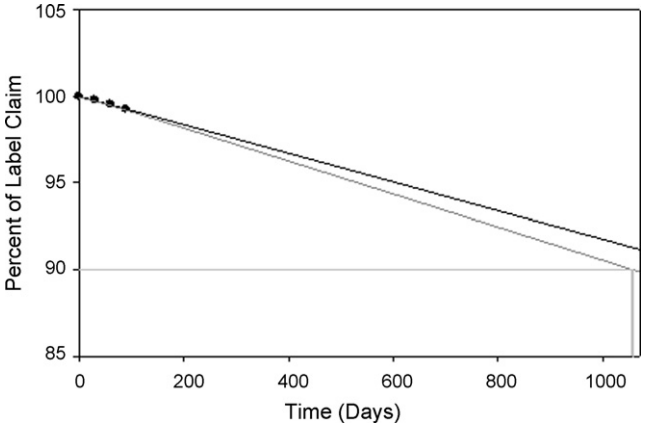

Fig. 6. Shelf life of pectin-based coated matrix tablet.

$40 \pm 2\,^{\circ}C/75 \pm 5\%$ RH for 3 months. No change was observed in the physical appearance and drug content during the storage period. Mean (±S.D) drug content at the end of 1, 2 and 3 months was found to be $99.85 \pm 0.53\%$, $98.25 \pm 0.33\%$ and $96.66 \pm 0.56\%$ respectively, indicating no significant change ($p > 0.05$) in the drug content. Similarity factor (f_2) for the % CDR during the drug release study conducted in the simulated physiological environment of stomach, small intestine and colon after storage of 1 month, 2 months and 3 months was found to be 87.84, 78.44 and 76.89 respectively. These observations indicate chemical stability of the drug and physical stability of the formulation during the storage period. On the basis of the accelerated stability testing, shelf life of the formulation was calculated to be 2.83 years with the help of sigma plot 10 software (Fig. 6).

4. Conclusion

Colon targeted, pectin-based matrix tablets of 5-FU dually coated with Eudragit S 100 were successfully optimized using 3^2 full factorial design. The optimized tablets contained 66.67% (w/w) of pectin as the matrix former and 15% (w/w) of 10% (w/v) starch paste as the binder. Six percent (w/v) solution of Eudragit S 100 was found to be capable of preventing the drug release in the gastric environment. The optimized tablets released only $8.01 \pm 2.34\%$ of their drug content in the small intestine environment. *In vivo* roentgenographic studies in New Zealand white rabbits revealed intactness of the core in stomach and small intestine thereby providing the "proof of concept". Pharmacokinetic studies in rabbits exhibit a lag time of 4 h as well as significant reduction in the systemic exposure of the drug through formulation F9. *In vitro* cytotoxicity studies in the HT 29 human colon cancer cell lines revealed significant reduction ($p < 0.01$) in CTC_{50} value through granules with pectin signifying increased potency of the formulation C-2 over granules without pectin. Accelerated stability studies established physical integrity of the formulation and chemical stability of the drug. Shelf life of the optimized tablet was calculated to be 2.83 years. The present study corroborates microbially triggered pectin-based matrix tablet of 5-FU dually coated with Eudragit S 100 to be a potential system so as to restrict the release of drug like 5-FU to colon with the merits of reduced systemic exposure and enhanced potency.

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