Original Research Article

Factorial designed 5-fluorouracil-loaded microsponges and calcium pectinate beads plugged in hydroxypropyl methylcellulose capsules for colorectal cancer

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Abstract

Introduction: The work was aimed to develop an enteric-coated hydroxypropyl methylcellulose (HPMC) capsules (ECHC) plugged with 5-fluorouracil (5-FU)-loaded microsponges in combination with calcium pectinate beads. **Materials and Methods:** The modified quasi-emulsion solvent diffusion method was used to prepare microsponges. A 3^2 factorial design was employed to study the formulation and the effects of independent variables (volume of organic solvent and Eudragit-RS100 content) on dependent variables (particle size, %entrapment efficiency, and %cumulative drug release). The optimized microsponge (F4) was characterized by scanning electron microscopy, powder X-ray diffraction, and thermogravimetric analysis. F4 was plugged along with the calcium pectinate beads in HPMC capsules coated with enteric polymer Eudragit-L100 (Ed-L100) and/or Eudragit-S100 (Ed-S100) in different proportions. An *in vitro* release study of ECHC was performed in simulated gastric fluid for 2 h, followed by simulated intestinal fluid for next 6 h and then in simulated colonic fluid (in the presence and absence of pectinase enzyme for further 16 h). The optimized formulation was subjected to *in vivo* roentgenographic and pharmacokinetic studies in New Zealand white rabbits to analyze the *in vivo* behavior of the developed colon-targeted capsules. **Results:** Drug release was retarded on coating with Ed-S100 in comparison to a blend of Ed-S100:Ed-L100 coating. The percentage of 5-FU released at the end of 24 h from ECHC3 was 97.83 ± 0.12% in the presence of pectinase whereas in the control study, it was 40.08 ± 0.02%. **Conclusion:** Thus, enteric-coated HPMC capsules plugged with 5-FU-loaded microsponges and calcium pectinate beads proved to be a promising dosage form for colon targeting.

Key words: *In vivo* roentgenograph, microsponges, numerical optimization, powder X-ray diffraction studies, thermogravimetric analysis studies

INTRODUCTION

Cancer, one of the most challenging diseases to treat is the second leading cause of death in developed countries. Over the past few decades, it continued to be a worldwide health problem although several techniques were developed.[1] Colorectal cancer (cancer in colon and rectum) form life-threatening tumors. More than 80% of colorectal tumors develop from adenomatous polyps.

Demographic studies show that 25% and 50% people have polyps in the age range of below 50 years and below 75 years, respectively. Less than 1% of smaller polyps (slightly less than half an inch) become cancerous while 10% of larger polyps become cancerous within 10 years and about 25% of these larger polyps become cancerous after 20 years.[2]

5-fluorouracil (5-FU), an antineoplastic anti-metabolite, inhibits RNA function and/or processing and synthesis of

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thymidylate. The precise mechanism of action is the binding of the deoxyribonucleotide of the drug (5-FdUMP) and the folate cofactor, N5-10-methylenetetrahydrofolate, to thymidylate synthase to form a covalently bound ternary complex. This results in the inhibition of the formation of thymidylate from uracil, which leads to the inhibition of DNA and RNA synthesis and cell death. 5-FU can also be incorporated into RNA in place of uridine triphosphate, producing a fraudulent RNA and interfering with RNA processing and protein synthesis.[3]

The major challenge associated with the efficient delivery of 5-FU is its erratic oral bioavailability, due to which the intravenous administration of this drug is currently in clinical use. However, intravenous administration leads to the quick distribution of 5-FU and elimination with an apparent terminal half-life of 8-20 min. It also produces severe systemic toxic effects of gastrointestinal (GIT), hematological, neural, cardiac, and dermatological origin. Most of these systemic side effects are due to the cytotoxic effect of 5-FU after it reaches the unwanted sites.

Therefore, the targeted delivery of 5-FU through oral route would not only reduce the systemic exposure of drug but also provide an effective and safe therapy for colon cancer with reduced dose and reduced duration of therapy.

Drug delivery systems based on calcium pectinate beads have been recently investigated for specific targeting drugs to the colon. Such systems, obtained by pectin gelatinization in the presence of calcium salts, are less water-soluble than natural pectins, since calcium ions induce noncovalent associations of carbohydrate chains through the formation of the so-called "egg box complexes" but they maintain the selective biodegradation by pectinolytic enzymes of colonic bacteria microflora.[4]

Microsponges are the porous microparticulate polymeric drug delivery systems composed of the tiny sponge-like spherical particles that consist of a myriad of interconnecting voids within a noncollapsible structure with the large porous surface. Microsponges with <200 μm may efficiently be taken up by the macrophages present in the colon, thus exhibiting effective localized drug action at the desired site. Apart from being site specific, the retention of drug or its carrier system on the colonic surface is yet another important consideration to guide the selection of microsponges as the drug carrier system in the research work.[5] They can also increase the lag time for absorption of the drug as these get entrapped on the surface of the colon and thus have the potential for being developed as colon-targeted drug delivery system. Longer exposure to a lower concentration of 5-FU has been reported by researchers to favor DNA-directed effects which is thought to contribute to its anti-tumor effect. Microsponges give a sudden drug release initially, that helps in achieving the required drug concentration. But a sustained release helps in maintaining the concentration. Microsponges also help in drug retention in the local tissue, thus the selection of microsponges as the drug delivery system is justified.

A hydroxypropyl methylcellulose (HPMC) capsule was preferred over gelatine capsules because a HPMC capsule had slower drug-release profile in acidic media and the fast release profile at a pH of 5 and above. This can result in lower quantities of polymer coat compared to that required for tablets to achieve the desired release in the small intestine or colon. Surprisingly it was also found that enteric-coated HPMC capsules offer much higher resistance against acid solutions as compared to enteric-coated gelatine capsules.[6]

The objective of our research work was to design novel enteric-coated hydroxypropyl methylcellulose capsules (ECHC) plugged with 5-FU-loaded microsponges in combination with calcium pectinate beads. 3^2 full factorial designs were used to optimize microsponges prepared by the modified quasi-emulsion solvent diffusion method using sodium chloride (NaCl) as porogen.

MATERIALS AND METHODS

Materials
5-FU (Shalaks Pharmaceutical Pvt. Ltd, New Delhi); HPMC capsules (ACG Associated Capsules Pvt. Ltd, SciTech Centre, Mumbai); Eudragit-RS100, Eudragit-S100 (Ed-S100) and Eudragit-L100 (Ed-L100) (Evonik Labs, Mumbai) were obtained as gifts from the suppliers. All other chemicals used were of analytical grade and purchased from authentic suppliers.

Method development for the fabrication of microsponges
Previously described method of microsponge preparation by quasi-emulsion solvent diffusion technique using porogen was modified to employ an aqueous solution of NaCl as porogen. One percentage (w/v) aqueous solution of the porogen was prepared and sufficient amount of Span 80 was added to it with agitation to obtain 1% (v/v) dispersion.[7,8] A solution of the Ed-RS100 and 5-FU was prepared in mixture of ethanol:dichloromethane (7:3) and the porogen solution (0.1 mL) was uniformly emulsified in it to form a W/O emulsion. Five percentage (w/v) aqueous hot polyvinyl alcohol solution (external phase) was prepared separately, and the previously prepared W/O emulsion was emulsified in it. This W/O/W emulsion was stirred for 24 h to get microsponges that were filtered, dried at 60°C, and stored in a desiccator.[9]

Optimization of microsponges via 3^2 factorial designs
A 3^2 randomized full factorial design was run to optimize the variables. In this design, two independent factors (volume of organic media [X_1] and amount of polymer content [X_2]) were evaluated, each at three levels to develop nine formulations (F1-F9) and experimental trials were performed using Design Expert Software 8.0.7.1 (Stat-Ease, Inc., Minneapolis, USA). Table 1 represents the design model. The dependent variables that are particle size, % entrapment efficiency (%EE) and %cumulative drug release (%CDR) were evaluated and polynomial equations were generated for the dependent variables

Table 1: 3² factorial design model for the fabrication of 5-FU-loaded microsponges			
Formulation code	Independent variables		Dependent variables
	Volume of organic media (mL) [X₁]	Polymer content (mg) [X₂]	
F1	(+1) 8	(+1) 1200	Particle size (Y₁)
F2	(−1) 6	(−1) 400	%EE (Y₂)
F3	(0) 7	(0) 800	%CDR (Y₃)
F4	(+1) 8	(−1) 400	
F5	(−1) 6	(+1) 1200	
F6	(0) 7	(−1) 400	
F7	(−1) 6	(0) 800	
F8	(0) 7	(+1) 1200	
F9	(+1) 8	(0) 800	
F10 (extra design check point)	(+0.5) 7.5	(−0.5) 600	

EE: Entrapment efficiency, CDR: Cumulative drug release, 5-FU: 5-Florouracil

that were reduced by removing nonsignificant coefficients by applying one-way analysis of variance (ANOVA) ($P < 0.05$). To demonstrate graphically, the influence of each factor on responses, the response surface plots and three-dimensional (3D) bar graph were generated.

Validation of experimental design

The formulation developed was evaluated for the responses and the experimental values obtained were compared with those predicted by the mathematical models generated (F 10).

Selection of optimized formulation

A numerical optimization technique using the desirability approach was employed to select optimized formulation with desired responses. Constraints like maximizing EE and %drug release at the end of 8 h as well as minimizing particle size were set as goals to select the optimized formulation using Design Expert Software 8.0.7.1 (Stat-Ease, Inc., Minneapolis, MN, USA). Optimized formulation was plugged in enteric-coated colon-targeted capsules.

Characterization of microsponges
Morphological studies and rheological characterization

Morphological characteristics of microsponges were studied by scanning electron microscopy (SEM) (Hitachi, S 3000H Japan). The microsponges were dotted on an adhesive tape attached to an aluminum stub and excess microsponges were detached. To render the particles electrically conductive, the stub sputter was coated with gold using a vacuum evaporator. The coated microsponges were viewed at 10 kV.

Powder X-ray diffraction studies

The crystalline behavior of the drug before and after encapsulation was evaluated by an X-ray powder diffractometer (Bruker D8 Discover, Germany) using a CuK alpha radiation source with Ni-filter. A scanning rate of 5°/min, tube voltage of 35 kV and current of 35 mA over a range of 16-60° were used in the measurement.

Thermal analysis

Differential scanning calorimetry and thermogravimetric analysis of the pure drug and drug-loaded microsponges were carried out with simultaneous thermogravimetric analysis (TGA)/ differential scanning calorimetry (DSC) analyzer (Mettler Toledo TGA/ DSC, USA). An amount 13.714 mg of drug and 9.35 mg of drug-loaded microsponges was placed in aluminum pans and sealed prior to the test. All samples were run at a heating rate of 10°C/min over a temperature range 30-600°C in an atmosphere of nitrogen.

Particle size

All formulations of the microsponges were analyzed for particle size by an optical microscope. The instrument was calibrated and found that 1 unit of eyepiece micrometer was equal to 7 μm. Sizes of 100 microsponges were calculated in ×10.

Determination of drug content, drug loading and entrapment efficiency

A sample of drug-loaded microsponges (50 mg) was dissolved in 50 mL of phosphate buffer (pH 7.4) using an ultrasonicator and kept for overnight. Filtered samples were appropriately diluted and analyzed spectrophotometrically at 266 nm. %Drug content, %EE, and %drug loading were calculated.

Percent entrapment efficiency was indirectly measured by the spectrophotometric assay (Ultraviolet-visible spectrophotometer) at 266 nm of the residual drug content in the aqueous solution after separating microsponges according to the following equation:

$$\%EE = (Q_e/Q_t) \times 100$$

where Q_t is the drug content initially added during preparation and Q_e is the drug content recovered in the aqueous solutions after separating microsponges that are entrapped drug.

In vitro drug-release studies of microsponges

In vitro drug release of the prepared microsponges was performed using USP apparatus I. Microsponges equivalent to 50 mg of drug were filled in HPMC capsules and placed in the basket (mesh #230 = 63 μm) and the study was performed in the 900 mL of simulated colonic fluid (SCF) without pectinase for 8 h at 50 rpm, maintained at 37 ± 0.5°C. Aliquots were withdrawn periodically and sink conditions were maintained by adding an equal amount of release medium. The samples were analyzed spectrophotometrically and %CDR versus time plots was constructed. Data obtained from *in vitro* release study was evaluated to check the goodness of fit to various release kinetics equations for quantifying the phenomena controlling the release of drug from microsponges.[10]

Modification of pectin and its characterization

Pectin was crosslinked by the ionotropic gelation method. 10% (w/v) aqueous pectin dispersion was added drop wise into gently stirred 100 mL of 10% (w/v) aqueous solution of calcium

chloride preadjusted to pH 5.5. The gelled particles were left in the medium for 10 min, washed with deionized water and dried at 60°C. The particles were screened through sieve no. 44 and kept in the dessicator to remove the traces of moisture and then evaluated for morphological, rheological, and swelling characteristics.[11]

Preparation and evaluation of enteric-coated hydroxypropyl methylcellulose capsules

Each batch of HPMC capsules was coated by enteric polymer Ed-S100/Ed-L100 or bend of Ed-S100 and Ed-L100 in different ratios, by the dip coating method into the polymeric solution of Ed-S100 and Ed-L100 to ensure the formation of a uniform and thin covering over the capsule. In order to enhance the elasticity of Eudragit films, 1.25% of dibutyl phthalate as a plasticizer was added to the coating solution. HPMC bodies and caps were coated separately to ensure the coverage of the body with polymer, especially where the cap overlaps.[12] Coated bodies were filled by formulation matrix consisting of 50 mg drug-loaded microsponges plugged along with 5, 10, 15% w/w of calcium pectinate beads and remaining volume was adjusted by inert lactose and finally closed with the coated caps. The joint of the capsule was sealed with a small amount of 5% Ed-S100 solution. For each polymeric solution coating of capsules was made with different thickness ratios. The capsules were allowed to dry with the help of dryer with an inlet temperature of 35-40°C and observed for coat uniformity, pores, and cracks under a microscope.

Integrity test of enteric-coated hydroxypropyl methylcellulose capsules

Prior to the dissolution test, enteric-coated HPMC capsules were subjected to disintegration test in 0.1 M HCl for 2 h to determine the ability of the coat to withstand the acid environment. Afterward, the capsules were dried at 25°C for 45 min, thereafter; the capsules were subjected to microscopic examination to check for the integrity of the coat. The enteric-coated capsules which passed the disintegration test were further evaluated for the integrity of coating in pH 5.5 since the dissolution of coating in pH lower than 6.8 may alter the required lag time. On the basis of solubility in pH 5.5 buffer, coating compositions and levels that fell short were rejected to avoid alteration of lag time. Only coating levels and compositions which were not dissolved at all or dissolved very slowly in pH 5.5 buffer were selected for further *in vitro* drug-release studies.

In vitro drug-release study of enteric-coated hydroxypropyl methylcellulose capsules

The study was performed in USP drug-release apparatus in various release media sequentially simulated gastric fluid (SGF) (pH 1.2) for 2 h, followed by simulated intestinal fluid (SIF) (pH 7.4) for next 6 h and then in SCF (presence and absence of pectinase, for further 16 h). The test conditions were 900 mL of medium stirred at 50 rpm and maintained at 37 ± 0.5°C. Aliquots were withdrawn at regular intervals and replaced with equal amount of the fresh medium to maintain sink condition. The samples were

analyzed for 5-FU content released at 265.4 nm. Concomitantly, the swelling characteristics and physical behavior of capsules were observed at 0, 2, 5, 8, 10, 14, 18, and 24 h and recorded visually. The systematic evaluation of ECHC-3, ECHC-14, and ECHC-15 led to identification of optimized formulation.

Animal studies

All the animal experiments have been conducted in full compliance with the institutional ethical and regulatory principles and as per the spirit of Association for Assessment and Accreditation of Laboratory Animal Care and International's expectations for animal care and use/Ethics Committees. The investigations were performed after obtaining approval by the Institutional Animal Ethical Committee of PSIT, Kanpur, India (IAEC no: IAEC/PSIT/1273/ac/13).

In vivo roentgenographic study

The colon-targeted capsule plugged with microsponges was partially replaced with barium sulfate. The reformulated enteric polymer-coated capsules was administered orally with 15 mL of water to New Zealand white rabbits ($n = 3$; 3-3.5 kg) that were fasted overnight with free access to water. Fluoroscopic images of the abdomen of the rabbits were taken before administration (0 h) and at 1.5 h, 3.5 h, 5 h, 7.5 h, and 10 h to trace the *in vivo* movement and behavior of capsules in the GIT tract. X-ray images of the rabbits in the prone position were captured using L and T vision 100 (C-arm) X-ray machine (Larsen and Toubro Limited, Mumbai, India), at 64 mAs and 63 kV.

In vivo pharmacokinetics study

New Zealand white rabbits weighing 3-3.5 kg were classified into two groups, namely standard and test with three animals in each group. The animals were fasted overnight with free access to water. The standard group received 5-FU aqueous solutions (10 mg/kg) while the test group received colon-targeted capsules (ECHC-3). At appropriate intervals, blood samples (~1 mL) were withdrawn from the marginal ear vein. 5-FU was extracted from plasma by mixing rabbit plasma with ethyl acetate:isopropyl alcohol (85:15, v/v). The samples were then dried with N_2 at 37°C, and the dehydrated samples were dissolved in 400 µL of mobile phase for subsequent HPLC. The concentration of released 5-FU was measured using reversed-phase HPLC (HP1100 liquid chromatography, agilent). A hypersil C18 (5 µm, ID 4.6 mm × 300 mm) analytical column was used with a mobile phase of 0.01 M/L phosphate buffer (pH 3.0) and an elution rate of 1.0 mL/min at room temperature. Absorbance at 269 nm was monitored, and pharmacokinetic parameters were determined from the absorbance-time curves. This method provided a complete separation with a corresponding retention time of 7.0 min for 5-FU. The area under the plasma drug concentration-time curve was calculated using the trapezoidal rule. The maximal plasma concentration of drug (C_{max}) and the time to reach maximum plasma concentration (T_{max}) were directly obtained from plasma data. The data from different formulations were compared for statistical significance by one-way ANOVA. All results were expressed as a mean ± standard deviation.

RESULT AND DISCUSSION

Formulation of drug-loaded microsponges

In the present research work, NaCl was used as porogen, based on the consideration that use of hydrogen peroxide and sodium bicarbonate as porogen resulted in the evolution of gases such as nascent oxygen or carbon dioxide that may be incompatible with active pharmaceutical ingredients.[13,14] NaCl as porogen is inert in terms of the evolution of gas, and its high aqueous solubility would facilitate its easy extraction in the outer aqueous phase during the microsponge formation. Therefore, using NaCl as porogen, nine formulations (F1-F9) were fabricated and characterized.

Characterizations of microsponges
Morphological studies and rheological characterization

Micrographs of optimized drug-loaded microsponges F4 confirmed the highly porous nature of microsponges. SEM of microsponges before *in vitro* release test in Figure 1a and b depicted spherical, smooth surfaced, and uniformly porous particles. Figure 1c and d magnified the surface view of microsponges F4 showing its highly cross-linked polymeric surface containing numerous interconnected voids loaded with drug thus supporting the spongy nature of microsponges. Figure 1e and f are the micrographs of microsponges F4 after the *in vitro* drug release for 8 h, and it depicted irregular microsponges with numerous regular striations due to the erosion of surface-associated drug molecules.

Powder X-ray diffraction studies

The X-ray diffraction patterns of 5-FU and 5-FU-loaded microsponges plotted using origin software are shown in Figure 2. The powder X-ray diffraction (PXRD) pattern of 5-FU showed peaks at 29°, 33°, 16°, 19°, and 21° which were intense and sharp indicating its crystalline nature also supported by literatures.[15] Some of these 5-FU peaks are also present in diffractogram of 5-FU-loaded microsponges, due to the surface-adsorbed drug. Since Eudragit-RS100 has ammonio methacrylate units that confer a positive charge on the polymer surface while 5-FU has a negative charge on its surface. Therefore, the drug molecule can undergo ionic adsorption on the surface of the polymer. However, 5-FU-loaded microsponges presented most of the peaks of diminished intensity indicating that there might be partial or complete transitions of drug entrapped in microsponges from crystalline state to amorphous state.

Thermal analysis

TGA-DSC thermograms of pure 5-FU and drug-loaded microsponges are depicted in Figure 3. The extrapolated onset temperature in thermograms denotes the temperature at which the weight loss begins. The extrapolated onset temperature of 5-FU is 286.9°C. The DSC thermogram of the pure 5-FU also showed a sharp melting endotherm peak at ∼286.9°C followed by decomposition, which was in agreement with those reported previously.[16] The red curve is the %weight loss curve, and it indicated that %weight loss occurs in one step with 90% decomposition of drug between 280°C and 350°C. Drug-loaded optimized microsponges showed TGA extrapolated onset temperature at 280°C and 365°C indicating the presence of small drug in crystalline form which might be due to small amount of drug adsorbed onto the surface of the microsponges, whereas a shifted small broad endothermic peak at 351°C suggested that drug was either totally or partially converted into amorphous form and further no characteristic peak of 5-FU was observed. The reduction of height and sharpness of the endotherm peak may be due to the presence of polymers in the microsponges. TGA thermograms of microsponges also showed downward shift which indicated
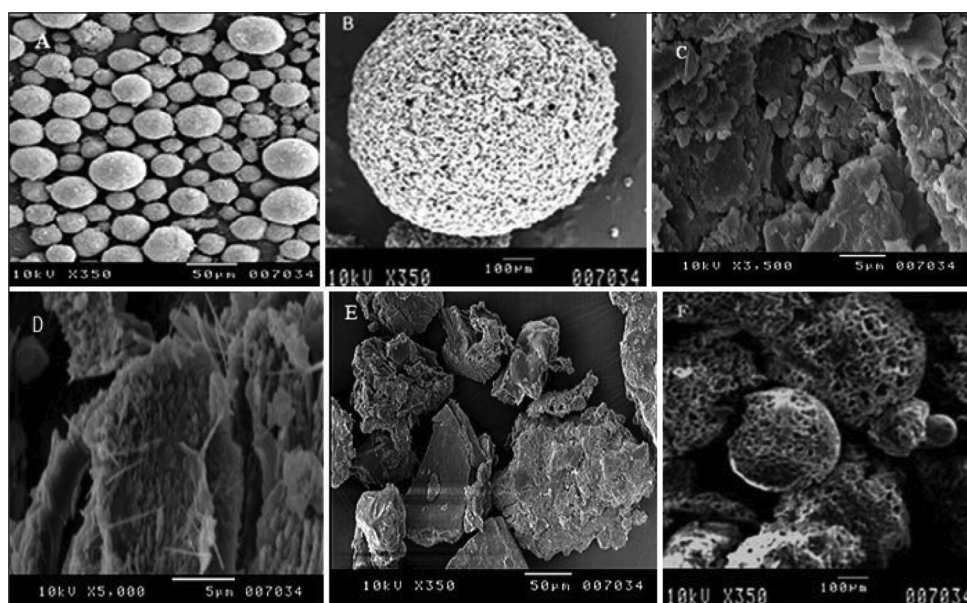

Figure 1: Scanning electron microscopy of microsponges F4 before drug release (a and b), surface view of F4 before drug release (c and d), F4 after drug release (e and f)
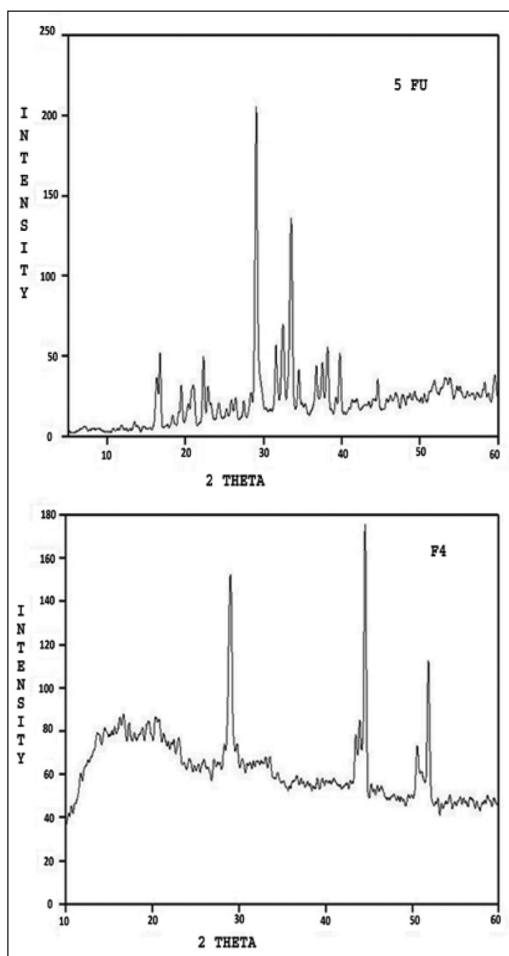
Figure 2: X-ray diffraction patterns of 5-fluorouracil and F4 (optimized 5-fluorouracil-loaded microsponges)

the loss of mass (due to solvent evaporation, loss of moisture and degradation) upon heating. The red curve, which is the %weight loss curve, showed a %weight loss in two steps. In the first step, 20% weight loss between 260°C and 290°C indicated decomposition of surface adsorbed drug present in crystalline form and in the second step 70% weight loss in temperature range of 340-410°C suggested the decomposition of amorphous form of drug entrapped within the microsponges. Therefore, the physical status of the drug present in microsponges was in well correlation with PXRD data.

Particle size

The particle size of the microsponges ranged between 41.48 μm (F2) and 75.02 μm (F9) [Table 2]. On applying one-way ANOVA, it was observed that the experimental design had a significant influence on the particle size. As the amount of the drug to be incorporated was kept constant, any change in particle size was influenced by the variation in the levels of ERS100 (polymer) and the volume of organic solvent. At a lower level of organic solvent, the microsponges F2, F5, and F7 with a mean diameter 61.22 μm, 71.48 μm, and 75.02 μm, respectively, were obtained. However, on increasing the volume of organic solvent, microsponges with smaller diameter were formulated. Thus, F4 and F9 yielded smaller microsponges of 42.15 and 58.31 μm size, respectively. Also, at a low level of polymer content, microsponges F2, F4, and F6 with a mean diameter of 61.22 μm, 42.15 μm, and 55.32 μm, were obtained. However, on increasing the polymer content, microsponges with larger diameter were formulated that is F1, F5, and F8 with a mean diameter of 69.40 μm, 71.48 μm, and 65.31 μm, respectively, were obtained. Therefore, it was interpreted that a higher concentration of polymer or a lower level of organic solvents produced a more viscous dispersion, which
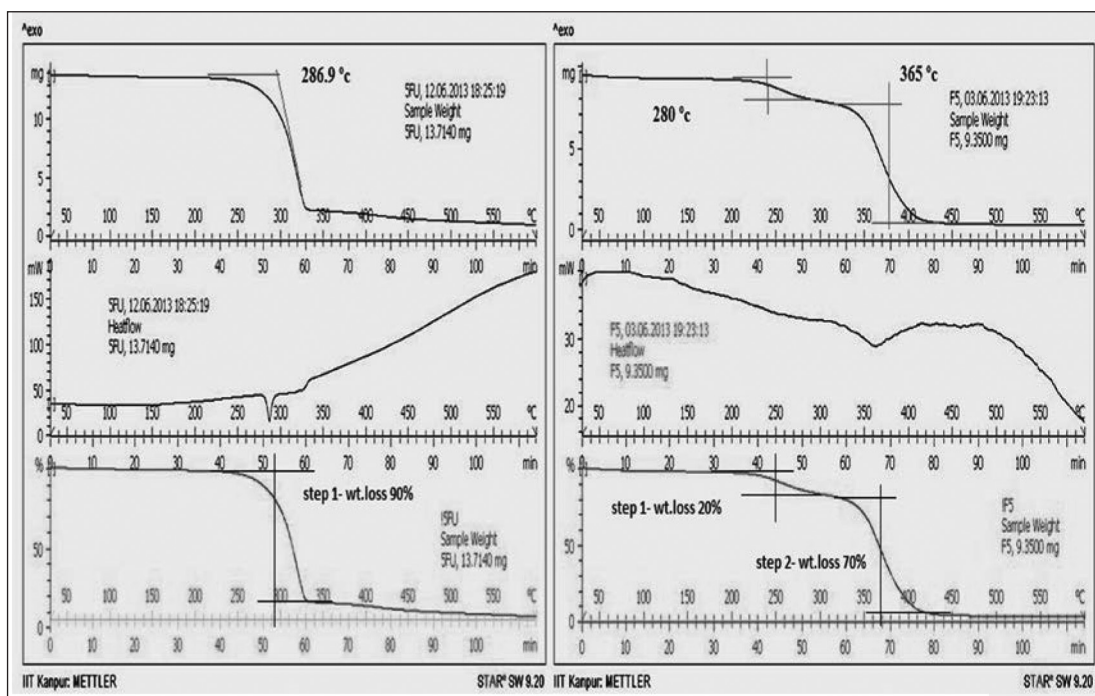

Figure 3: Thermogravimetric analysis-differential scanning calorimetry thermograms of pure 5-fluorouracil and 5-fluorouracil-loaded microsponges

Table 2: Compilation of evaluation parameters of 5-fluorouracil-loaded microsponges

Formulation code	Particle size (µm)	Drug content (%)	Drug loading (%)	EE (%)	% CDR
F1	69.40±1.3	79.01±0.5	21.97±0.2	58.02±0.2	95.92±0.12
F2	61.22±0.6	52.20±1.6	25.48±0.4	35.56±0.3	93.69±0.07
F3	61.34±0.3	84.54±0.2	23.76±0.5	50.27±0.2	92.54±0.11
F4	42.15±0.2	69.29±0.4	39.75±0.1	59.05±0.3	97.07±0.13
F5	71.48±0.2	72.43±1.1	15.98±0.2	50.02±0.1	94.54±0.03
F6	55.32±0.2	57.65±0.2	31.32±0.0	47.40±0.1	90.52±0.02
F7	75.02±0.4	58.39±0.7	26.98±0.1	46.14±0.3	93.48±0.06
F8	65.31±0.2	76.58±0.1	19.71±0.2	54.87±0.3	91.78±0.15
F9	58.31±0.2	85.01±0.29	27.93±0.14	60.82±0.15	97.58±0.02
F10+	50.5838	—	—	59.3045	97.2196

+ Predicted Value, %EE: Percent Entrapment Efficiency, %CDR: Percent Cumulative Drug Release. *All the values are expressed in Mean ± SD (Standard Deviation)

formed larger droplets and consequently larger microsponges obtained.

Entrapment efficiency
The level of organic solvent and level of the polymer also had a significant influence on the EE. The EE was observed to increase with an increase in the level of organic solvent that can be attributed to better solubilization of the drug in the organic medium. The higher volume of the organic solvent resulted in the most uniform mixing of the drug and polymer resulting in a more uniform matrix with high drug EE. Microsponges F1, F4, and F 9 with a high level of organic solvent had high %EE 58.02%, 59.05%, and 60.82%, respectively. Whereas microsponges F2 and F7 had low %EE, that is, 35.56% and 46.14% due to the low level of organic solvent. Similarly on increasing polymer content %EE increased that is (F1) 58.02% and (F8) 54.87% had high %EE, whereas microsponges with low level of polymer content had less %EE, that is, (F2) 35.56% and (F6) 47.40% [Table 2].

Along with the above-mentioned two factors, a third factor that is particle size has also been found to have an impact on the EE. The observations suggested that EE of the microsponges increased with the decrease in the particle size. The smaller particle size of the microsponges leads to the increased contact surface area that causes the increased drug entrapment. Consequently, F4 and F9 with a particle size 42.15 µm and 58.31 µm had high %EE, that is, 59.05% and 60.82%, whereas F2 and F7 with a particle size 61.22 µm and 75.02 µm had low %EE, that is, 35.56% and 46.14%, respectively. Therefore, particle size also had an impact on the EE but it was not statistically significant. Drug content and drug loading of microsponges ranged from 52.2% (F2) to 85.2% (F9) and 15.98% (F6) to 39.75% (F4), respectively [Table 2].

In vitro drug-release study
The %CDR in the SCF was observed between 90.52% (F6) and 97.58% (F9) suggesting the ability of the microsponges to release the drug completely [Table 2]. All the formulations followed zero-order release kinetics [Figure 4] except F2 and F8 that followed Higuchi and Peppas release kinetics. Zero-order release of drug from microsponges has been reported by researchers.[17] The mechanism of drug release from microsponges, as explained by various researchers, is co-relatable to its porous surface. The porous surface of the carrier particle enables easy penetration
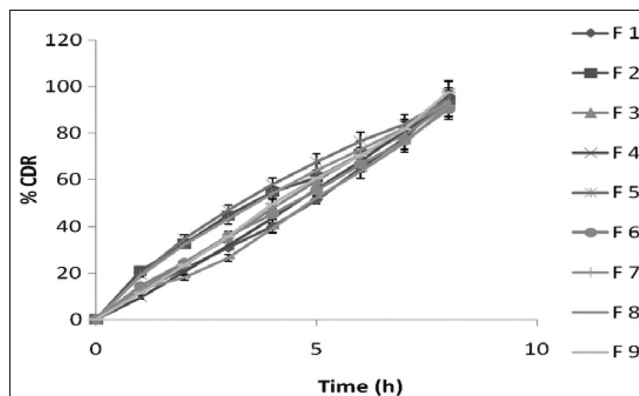

Figure 4: *In vitro* drug-release profile of 5-fluorouracil-loaded microsponge formulations (F1-F9)

of the release media and its accessibility to the entrapped drug molecule. In the case of F2 and F4, a high initial rate of the drug release in 2-3 h was observed due to the surface-adsorbed drug molecule that underwent quick release to attain the equilibrium with the dissolved drug molecule, additionally, as suggested by various research reports, control on the drug release can also be achieved by uniform mixing of the drug and polymer in organic media to form a homogenous phase. F2 and F7 formulated with low levels of polymer and organic phase might have resulted in the improper encapsulation of the 5-FU in the Ed-RS100 matrix. Due to this, the majority of the drug molecules resided on the particulate surface as adsorbed molecules and had the probability of undergoing quick solubilization and hence quick drug release. But after achieving equilibrium, the rate of drug release decreased. This effect was not predominant in F9 as it is constituted of the intermediate level of Ed-RS 100 and a high level of the organic phase. While in F5, the release fitted first-order model that may be due to the fact that the drug release from the polymeric microsponges occurs on transition of the polymer, and as the amount of polymer in microsponges increases the time required for phase conversion also increases. Thus in F8, initial drug release was due to the adsorbed molecules but with the lapse of time, Ed-RS100 layer underwent transition resulting in the abrupt or burst release of 5-FU.

Statistical analysis
Statistical analysis was done using Design expert software version 8.0.7.1 (Stat-Ease, Inc., Minneapolis, MN, USA) and the second
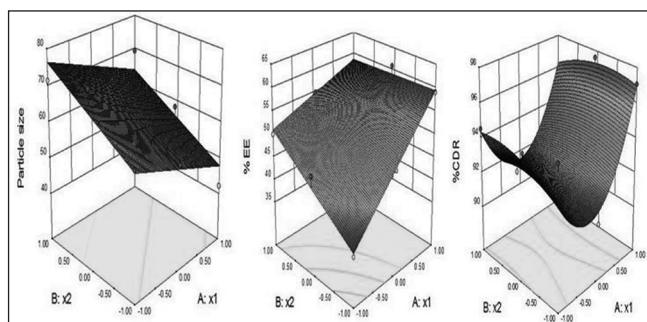
Figure 5: Response surface plot depicting the influence of independent variables over dependent variables

Figure 6: Three-dimensional bar graph depicting the influence of independent variables over dependent variables

order polynomial equations were derived. The statistical model incorporating interactive and polynomial terms was utilized to evaluate the responses. Response surface plot [Figure 5] and 3D bar graph [Figure 6] clearly depicts the effects of independent variables that is varying the levels of volume of organic solvent and polymer content over dependent variables (i.e., particle size, %EE, and %CDR).

Factorial equation for particle size

The response surface linear model generated for particle size was found to be significant with an F-value of 11.30 ($P < 0.0500$) and a correlation coefficient of 0.9824.

Particle size $(Y_1) = 62.17 - 6.31\,X_1 + 7.92\,X_2 - 1.52\,X_1^2 + 2.72\,X_2^2 + 3.94\,X_1^2X_2 + 3.06\,X_1X_2^2 - 2.03\,X_1^2X_2^2$

The co-efficient of X_1 is negative indicating that when the volume of organic solvent increased, the particle size decreased whereas the positive coefficient of X_2 indicates that particle size increased on increasing polymer content. The P values for variables X_1 and X_2 were 0.0252 and 0.0099, respectively, ($P < 0.0500$) indicating that both the independent variables show a significant effect on the dependent variable that is particle size.

Factorial equation for %entrapment efficiency

The response surface linear model generated for particle size was found to be significant with an F-value of 63.59 ($P < 0.0500$) and a correlation coefficient of 0.9745.

%EE $(Y_2) = 51.350 + 7.695\,X_1 + 3.483\,X_2 - 3.872\,X_1X_2$

The positive co-efficient of X_1 and X_2 indicates that %EE increases on increasing volume of organic content as well as polymer content, whereas the combination of independent variables X_1X_2 had a negative influence on %EE. The P value for variable X_1, X_2, and X_1X_2 were 0.0001, 0.0031, and 0.0047, respectively, ($P < 0.0500$) showing that both the independent variables and combination of independent variable had a significant effect on the dependent variable that is %EE.

Factorial equation for %cumulative drug release

The response surface linear model generated for %CDR was found to be significant with an F-value of 11.55 ($P < 0.0500$)
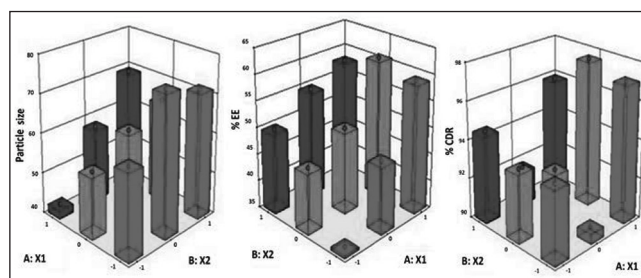
and a correlation coefficient of 0.9506.

%CDR $(Y_3) = 92.022 + 1.476\,X_1 + 3.766\,X_1^2 + 0.160\,X_2 - 0.613\,X_2^2 - 0.500\,X_1X_2$

According to above-factorial equation, independent variables X_1, X_1^2 and X_2 had a positive influence on %CDR, whereas the combination of independent variables X_1X_2 and X_2^2 had a negative influence on %CDR. X_1 and X_1^2 with a P value 0.0250 and 0.0087, respectively, were significant model terms ($P < 0.0500$), whereas X_2, X_1X_2, and X_2^2 were insignificant model terms.

Validation of the experimental design

The results of experimentally observed responses and those predicted by the mathematical models along with the percentage prediction errors were compared. The prediction error for the response parameters ranged between 0.51% and 1.15% with the value of the absolute error of 1.28 ± 0.70%. The low values of error indicate the high prognostic ability of factorial equation and counter plot methodology. "Adeq Precision" measures the signal to noise ratio. A ratio >4 is desirable. The ratio of 8.505 indicates an adequate signal. Thus, this model can be used to navigate the design space.

Selection of optimized formulation

Microsponge formulation F4 with the highest desirability factor of 0.944 having a smallest particle size of 42.15 ± 0.28 μm, maximum %EE and %CDR of 59.05 ± 0.31 and 97.07 ± 0.13, respectively, was selected as optimized formulation. Ramps reports clearly depict criteria's for the selection of optimized formulation [Figure 7]. F4 was further used for the preparation of colon-targeted capsules.

Modification of pectin to calcium pectinate

Use of calcium pectinate beads as a plug in colon-target capsules was a novel step and the rationale behind this was that calcium pectinate (the insoluble salt of pectin) is not degraded by gastric or intestinal enzymes, but will be degraded by colonic pectinolytic enzymes and hence the formulation is microbial triggered. Calcium pectinate is a natural polymer which can be used as a major component or filler in a pharmaceutical composition that could be degraded by only colonic enzymes. Pectin is one of the most widely investigated polysaccharides in the colon-specific drug delivery. It can be broken down by pectinase enzymes produced by anaerobic bacteria of the colon and control the
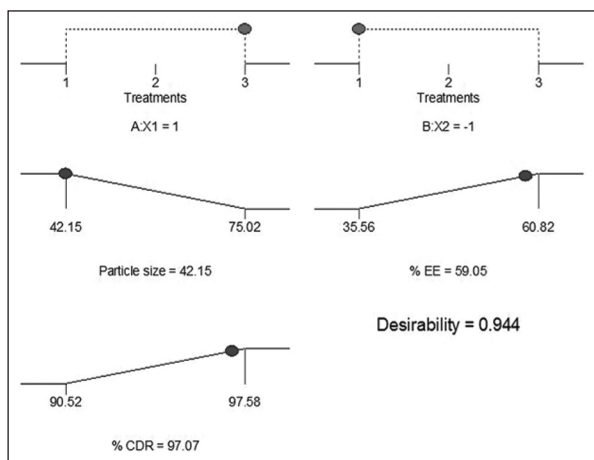
Figure 7: Numerical optimization ramps report

Figure 8: Scanning electron microscopy of pectin (g) and calcium pectinate beads (h)

drug release by this principle. It can also act via the pH and time-controlled mechanisms. However, due to high water solubility and swelling, pectin is not capable of shielding the drug effectively during the passage through the stomach and small intestine. Therefore, pectin with low degrees of methoxylation (i.e., low methoxypectins or low methoxyl [LM] pectin) was cross-linked with calcium ions (Ca^{2+}, divalent cations) to produce Ca-pectinate networks that were less water soluble.[18] Indeed, Ca^{2+} ions form "bridges" between the free carboxylated groups of galacturonic acid moieties. The network that is formed has been described by Grant *et al.* under the name of egg-box model. Pectin with DM <50% (i.e., LM pectin) was used because it contains more free carboxylic group than high methoxy pectin (high methoxyl [HM] pectin). Therefore, more cross-linking between divalent cations (e.g., Ca^{2+}) and free carboxylic groups of the pectin is evident in LM pectin than HM pectin.[17] The spherical beads were easily prepared without any sophisticated instrument due to ionic interaction between the negatively charged carboxylic groups on pectin molecules and the positively charged divalent calcium ions, which led to intermolecular cross-linking and instantaneously produced gelled sphere.[19]

Characterization of calcium pectinate beads
Size, surface morphology and rheological characterization
The particle size of calcium pectinate was of $\sim$105.22 ± 3.36 μm. SEM of pectin demonstrated smooth doughnut-shaped particles more or less of uniform size [Figure 8g] and calcium pectinate beads were spherical with smooth surfaced [Figure 8h]. Analysis of the rheological characteristics of calcium pectinate confirmed good rheological properties with bulk and tapped density of 0.504 g/cm³ and 0.558 g/cm³, Carr's compressibility index of 9.67% and angle of repose of 20.56º indicated good flow properties of calcium pectinate beads.

Swelling characteristics
Pectin and calcium pectinate were compared for their swelling characteristics. No significant swelling was observed for calcium pectinate (swelling ratio of 0.11 ± 0.04) as compared to pectin
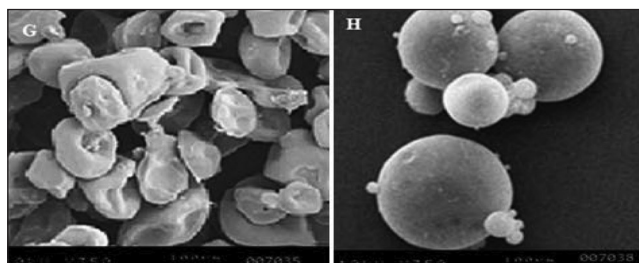
with a swelling ratio of 0.98 ± 0.02. The reduction in swelling of the calcium pectinate in SIF (pH 7.4) presents its feasibility to be used as a carrier for the colon-targeted drug delivery.

Preparation of enteric-coated hydroxypropyl methylcellulose capsules
Eudragit® L and S grades are suitable for enteric coatings and are specifically used for controlled release in the colon.[20] Coatings on gelatin capsules often suffer from insufficient adhesion between the shell and the coating. Thus, previous workers in the area of enteric coating have found it necessary to precoat gelatin capsules with a cellulose derivative, either to promote adhesion of polymers to the capsule shell or to improve gastro-resistance.[21] When the capsule itself is made of a cellulose derivative than a precoating step will be eliminated. Gelatin capsules have a very glossy surface due to the fact that the amount of regular reflection from the surface is high, and the amount of diffuse reflection is low. In contrast, HPMC capsules have a visually matt surface with a greater amount of diffuse reflection, suggesting a more irregular surface. SEM's of the surface of HPMC and gelatin capsules in Figure 9i and Figure 9j clearly shows this difference. During the coating process, the temperature of the capsule bed reaches 25-27ºC. At this temperature, HPMC is soluble and will start to dissolve in the eudragit film providing a strongly adhesive surface. Gelatin, on the other hand, is only slightly soluble at this temperature and its surface characteristics will remain virtually unchanged. Figure 9k shows SEM of the cross-section of a cleaved surface through an HPMC capsule coated with Ed-S100 solution. The contours of the coating material are seen to follow the irregular surface of the HPMC capsule. It is suggested that the high strength of the bond between HPMC and the film is a combination of the irregular surface and the tackiness of the partially dissolved surface. No pores or cracks can be observed, due to the well-controlled coating process. In addition, the critical area of overlap between the cap and body of the capsule is also covered with Ed-S100 polymers to ensure gastric integrity.

Evaluation of colon-target enteric-coated hydroxypropyl methylcellulose capsules
Integrity test of enteric-coated hydroxypropyl methylcellulose capsules
Table 3 displays results of the integrity test. ECHC-3, ECHC14 and ECHC15 that did not dissolve in pH 5.5 buffers were selected for a further *in vitro* drug-release drug study in SCF.

In vitro drug-release study

An *in vitro* drug-release study was conducted by pH change method as per USP protocol (2 h in SGF, 2-5 h in SIF and 5-24 h in SCF) and results are shown in Table 4. The immediate release marketed formulation showed almost total release in 4 h and pure drug in 3 h. Nearly complete drug release of microsponges in HPMC capsules (97.07%) in 8 h indicated the need for enteric coating of capsules for colon delivery [Figure 10]. Enteric-coated HPMC capsules ECHC-3, ECHC-14, and ECHC-15 showed

no drug release in simulated gastric fluid (pH 1.2) up to 2 h indicating the intactness of the applied coat. On exposures to simulated intestinal media, capsules coated with a blend of Ed-S100:Ed-L100 showed earlier drug release as compared to capsules coated with only Ed-S100. As the pH of solubilization of Ed-L100 is 6 and that of Ed-S100 is 7.0, Ed-L100 gets dissolved first and form pores. Calcium pectinate beads also get hydrated and forms a viscous gel layer that slows down further seeping in of dissolutin fluids and therefore, the release of 5-FU takes place by diffusion along with the erosion of eudragit layers. The formualtions ECHC-14 and ECHC-15 released 15.01% and 14.56% of 5-FU, respectively, at the end of 5-h dissolution study
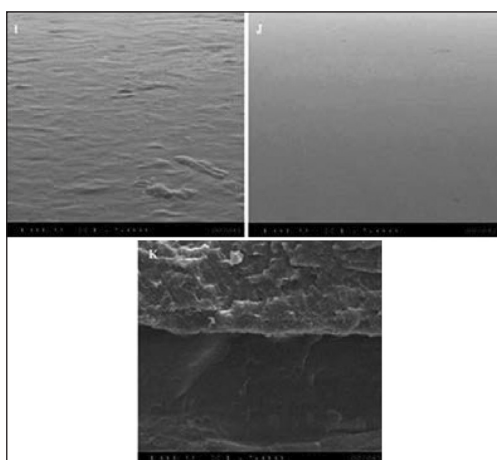

Figure 9: Scanning electron microscopy of cross section of hydroxypropyl methylcellulose capsule surface (i), gelatine capsule surface (j), and hydroxypropyl methylcellulose capsule surface coated with Ed-S 100 (k)
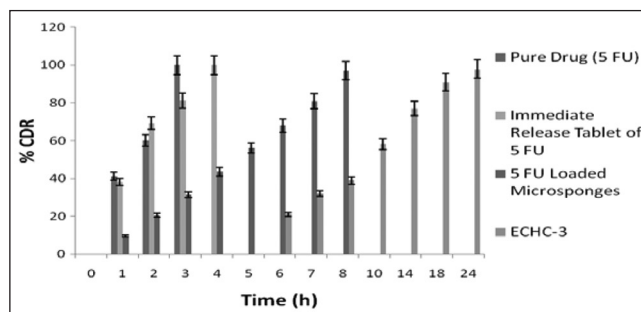

Figure 10: Comparison of *in vitro* drug-release profile of immediate release marketed tablet of 5-fluorouracil, pure 5-fluorouracil drug, 5-fluorouracil-loaded microsponges and enteric-coated hydroxypropyl methylcellulose capsules plugged with 5-fluorouracil-loaded microsponges (enteric-coated hydroxypropyl methylcellulose capsules-3) and calcium pectinate beads

Table 3: Integrity test of ECHC

Parameters	Formulation code														
	ECHC1	ECHC2	ECHC3	ECHC4	ECHC5	ECHC6	ECHC7	ECHC8	ECHC9	ECHC10	ECHC11	ECHC12	ECHC13	ECHC14	ECHC15
Coating ratio	S100			L 100			S100: L 100 (1:1)			S100: L 100 (2:1)			S100: L 100 (4:1)		
Calcium pectinate Plug level	5	10	15	5	10	15	5	10	15	5	10	15	5	10	15
Disintegration test in 0.1 N HCL	F	P	P	F	P	P	F	P	P	F	P	P	P	P	P
Solubility in pH 5.5	—	S	NS	—	S	S	—	S	S	—	S	S	S	NS	NS

*F: Fail, P: Pass, S:Soluble, NS: Not Soluble, ECHC: Enteric-coated hydroxypropyl methylcellulose capsules

Table 4: *In vitro* drug-release profile of ECHC

Time	Cumulative % drug release					
	ECHC 3		ECHC 14		ECHC 15	
0-2 h in gastric pH(1.2)	No release		No release		No release	
2-5 h in intestinal pH(6.8)	No release		12.01±0.02		10.56±0.07	
Simulated colonic pH(7.4)	Pectinase	Control study	Pectinase	Control study	Pectinase	Control study
6	21.03±0.01	8.17±0.02	38.02±0.04	18.03±0.02	35.12±0.05	14.06±0.01
8	39.04±0.12	15.02±0.14	50.04±0.06	26.04±0.11	49.07±0.13	22.15±0.08
10	58.22±0.03	22.08±0.13	63.21±0.08	31.21±0.07	66.15±0.04	28.07±0.13
14	77.01±0.06	29.01±0.05	76.14±0.01	35.08±0.06	79.22±0.11	34.12±0.06
18	91.02±0.04	33.09±0.06	88.11±0.03	39.01±0.11	90.23±0.02	40.01±0.02
24	97.83±0.12	40.08±0.01	96.05±0.12	44.84±0.03	97.01±0.08	46.43±0.09

*All the values are expressed in Mean± SD

in intestinal media. This indicates that inspite of the high water solubility of 5-FU there was tight control of drug release in the physiological environment of the stomach and small intestine. However, the integrity of ECHC-3 was maintained with no drug release up to 5 h indicating that drug released was highly retarded on coating with Ed-S100 (ECHC-3) in comparison to a blend of Ed-S100: Ed-L100 coating. Visual observation revealed that small flakes of coatings occurred about 3.5 h from the beginning of the release experiment. The release of drug from the enteric-coated capsules can be explained by the pore formations and bursting/flake formation of the coat due to the presence of high alkaline pH of dissolution media.

The ECHC-3 containing 15% calcium pectinate plug in ES100-coated HPMC capsules appears to be promising as it did not release the cytotoxic 5-FU in the physiological environment of the stomach and small intestine. The relative advantage of ECHC-3 over the other formulations ECHC-14 and ECHC-15 also depends on its ability to release the drug in the physiological environment of the colon. Hence, after completing the dissolution study in 1.2 pH and 6.8 pH, the dissolution study was carried out in SCFs (with pectinase enzyme and without pectinase enzyme, i.e., control study) for another 19 h. The percentage of 5-FU released from ECHC-3 at the end of 24 h with pectinase enzyme was found to be 97.83 ± 0.12%, whereas in the control study (without pectinase enzyme in the dissolution medium) only 40.08 ± 0.02% of 5-FU was released. This difference was found to be statistically significant ($P < 0.001$). This study shows that the release of 5-FU in the physiological environment of the colon is due to the microbial degradation of calcium pectinate plug in the presence of pectinase enzyme. The dissolution study was also conducted without pectinase enzyme (control study) to ensure that the drug was released not due to the mechanical erosion which is likely to occur because of bowel movements in humans. Similarly, formulations ECHC-14 and ECHC-15 released 96.05 ±0.05% and 97.01 ± 0.08% of 5-FU, respectively, in the presence of pectinase enzyme, whereas in the control study they released only 44.84 ± 0.03% and 46.34 ± 0.09%, respectively. A significant difference was observed ($P < 0.001$) in the amount of 5-FU released from formulations ECHC-14 and ECHC-15 at the end of 24 h with pectinase enzyme as compared to *in vitro* drug release without pectinase enzyme.
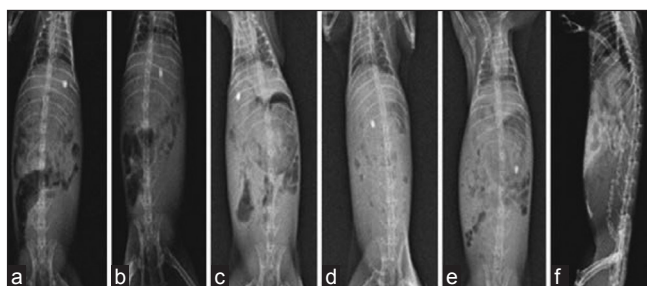
All the three formulations ECHC-3, ECHC-14, and ECHC-15 released almost complete drug by the end of the 24-h *in vitro* release study in the presence of pectinase enzyme. The release profiles of all the three formulations were similar at the end of 24 h with no significant difference ($P ≤ 0.05$). HPMC capsule provided a system of low permeability and a good barrier to drug diffusion at the pH, where the protection is required. Variation in coating ratios had little influence on the dissolution profiles of microsponges confirming the robustness of the formulation and the good compatibility between HPMC and the polymethacrylate films. Thus, enteric-coated HPMC capsules plugged with a matrix of 5-FU-loaded microsponges and calcium pectinate proved to be a potential candidate for targeted colonic delivery to treat colorectal cancer.

Animal studies
In vivo roentgenography study
In vivo X-ray imaging allows the visualization of *in vivo* functioning of a colon-specific drug delivery system, thereby ascertaining the location of drug release. The results of X-ray imaging studies are shown in Figure 11. Figure 11a shows the position of capsule just after the administration, followed by its passage to stomach after 1.5 h in Figure 11b indicating that the capsule remains intact in the stomach, establishing *in vivo* efficiency of the coating of Ed-L100 and Ed-S100 in preventing drug release in the gastric milieu. Figure 11c depicts no significant difference in the integrity of capsules in comparison to Figure 11b, thereby indicating the intactness of capsule in the small intestine at 3.5 h. The capsule was traced in the small intestine after 3.5 h [Figure 11c], in intestinal colon junction after 5 h [Figure 11d] and finally in the colon after 7.5 h [Figure 11e]. After 10 h, [Figure 11f] the intact capsule could not be traced demonstrating its erosion in the colon. These images clearly demonstrate the efficiency of selected formulation (ECHC-3) for its capacity to traverse the intestine intact and of 5-FU microsponges to the colon.

In vivo pharmacokinetics study
Figure 12 shows the plasma concentration versus time profiles of 5-FU after administration of ECHC and 5-FU aqueous

Figure 11: X-ray images of New Zealand rabbit administered with colon-targeted formulation (a) immediately after administration, (b) 1.5 h (stomach), (c) 3.5 h (small intestine), (d) 5 h (intestinal colon junction), (e) 7.5 h (colon) and (f) 10 h (rectum)
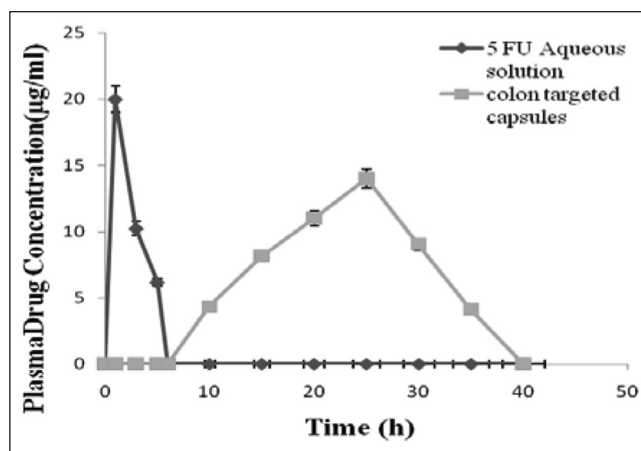

Figure 12: *In vivo* pharmacokinetic profile of 5-fluorouracil in New Zealand white rabbits following administration of 5-fluorouracil aqueous solution and developed colon-targeted capsules

solution to New Zealand white rabbits at a dose of 10 mg/kg, respectively. Colon-specific absorption of released 5-FU affected its pharmacokinetic parameters. After oral administration of the 5-FU solution, the drug was detected rapidly in plasma. The maximum concentration of 5-FU was 20 μg/mL after about 1 h. Thereafter, the plasma concentration decreased quickly, and the drug was not detectable as soon as 8 h. The elimination half-life was 0.12 ± 0.03 h. While in case of colon-targeted enteric-coated capsules (ECHC), 5-FU appeared in plasma after 6 h (lag time) of administration with a C_{max} of 14 μg/mL at 25 h. A lag time of 6 h for the developed colon-targeted enteric-coated capsules indicated the ability of the colon-targeted formulation to prevent the release of 5-FU in the stomach and small intestine. The C_{max}, T_{max}, $t_{1/2}$, and $AUC_{0-\infty}$ were significantly different from those of the aqueous solution. The C_{max} of the colon-targeted formulation was significantly ($P < 0.001$) less than that of the C_{max} of the 5-FU solution, suggesting reduced systemic absorption of the drug from ECHC-3. This means that larger fraction of the drug was available on the colonic surface for local action. $AUC_{0-\infty}$ for an aqueous solution and colon-targeted formulation was found to be 62.5543 μg h/mL and 49.3412 μg h/mL, respectively. Consequently, the Fr (relative bioavailability) of colon-targeted tablet was determined as 70.18%. Thus, *in vivo* pharmacokinetic studies of enteric-coated HPMC capsules plugged with calcium pectinate beads and 5-FU-loaded microsponges exhibited increased lag time, delayed T_{max}, decreased C_{max}, and reduced bioavailability. It can thus be concluded that the developed colon-targeted formulation has the ability to avoid the drug release in the upper GIT, but can release the active agent specifically in the colon exert local action with reduced systemic exposure.

CONCLUSION

A novel colon-targeted drug delivery system that combined two approaches that are pH-sensitive delivery and biodegradation by bacterial enzymes in the colon environment was successfully developed. This study clearly indicated that enteric-coated HPMC capsules plugged with 5-FU-loaded microsponges along with 15% calcium pectinate beads provided a tight control over drug release in the physiological environment of stomach and small intestine in spite its high water solubility. Matt surface of HPMC capsule provided a good substrate for adhesion of enteric-coated polymers and calcium pectinate beads also proved to be a suitable carrier for the colon-targeted system. Thus, the developed colon-targeted drug delivery system proved to be more patient compliant by providing a better mode of treatment over the present intermittent chemotherapy by injection or infusion.

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