Development and characterization of pellets for targeted delivery of 5-fluorouracil and phytic acid for treatment of colon cancer in Wistar rat

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ABSTRACT

The present study was designed to investigate the therapeutic efficacy of metal chelator and anticancer drug in the treatment of colorectal cancer (CRC). Pellets containing Phytic acid, 5-Fluorouracil (5-FU), Microcrystalline cellulose (MCC) PH 101, Hydroxypropyl Methylcellulose (HPMC) and Barium sulfate were prepared by using extrusion spheronization technique. Prepared pellets were coated with Eudragit S100 to achieve colon-specific drug delivery. Pellets were characterized for various pharmaceutical and micromeritic attributes. The in vivo therapeutic efficacy comprising of both pharmacokinetic and pharmacodynamic parameters was determined in Ehrlich ascites carcinoma (EAC) induced cancer animal model. Phytic acid and 5-FU combinations seem to exert higher cytotoxic activity via increased reactive oxygen species (ROS) level by chelating manganese. Further pharmacokinetic studies revealed approximately 50% lower Cmax in the finished formulation, indicates lower systemic exposure to the drug. X-ray radiography ensures the localized delivery of the encapsulated drug. Histopathological studies indicated no significant local toxicity compared to the uncoated formulation. Results inferred that the proposed combination has superior anticancer activity with minimum systemic and local toxicity and it opens a new avenue in the treatment of colorectal cancer.

1. Introduction

The incidences of colorectal cancer (CRC) have increased rapidly in young patients. Further, drinking alcohol increases the risk of developing CRC (Vuik et al., 2019). The second leading cause of death responsible all over the world is cancer, accounting for 8.9 million deaths globally per year (Fitzmaurice et al., 2018). 5-fluorouracil (5-FU) has been widely used as oral chemotherapy for early and advanced CRC. Although 5-FU presents to be a promising drug for CRC however, short elimination half-life requires multiple administration, limits the therapeutic potential of the drug (Alibolandi et al., 2017). Multiple administration of 5-FU increases the risk of chemotherapy toxicity and resistance. Colon-specific sustained drug delivery system improves the efficacy and safety of the drug. Further sustained-release minimizes the risk of drug plasma fluctuation and reduced systemic toxicity (Sreelatha and Bramha, 2013). Therefore, specific Eudragit® acrylic polymers have been developed for peroral dosage forms with the step-wise release of active ingredients in the digestive tract (Amidon et al., 2015). Eudragit® coatings get dissolved as a function of environmental pH and release of active ingredients occurs near the colon in a pH range 6.5–7.5. Eudragit® S 100 has been extensively used as a pH-sensitive polymer for colon drug targeting (Wairkar and Gaud, 2016). In the current study, we have formulated Eudragit® S100 coated pellets to achieve colon-specific sustained drug delivery system. Superoxide dismutase (SOD) is a critical enzyme responsible for the elimination of superoxide radicals and is considered to be a key anti-oxidant in aerobic cells (Hwang et al., 2007). Reactive oxygen species (ROS) production including the superoxide radicals (O2−) and hydrogen peroxide (H2O2) occurs when oxygen is taken up by cells for oxidative phosphorylation during ATP (adenosine triphosphate) generation in mitochondria. Accumulation of ROS results in cellular oxidative stress and if not corrected, can lead to damage of important biomolecules such as membrane lipids, proteins, and DNA (deoxyribonucleic acid). Prolonged accumulation of high levels of free radicals in cells may cause irreversible cellular injury and ultimately results in cell death. Since SOD is the key enzyme involved in the management of free radicals (Hileman et al., 2001), silencing of SOD causes increased accumulation of O2− subsequently lead to cell death. Thus, silencing of SOD might offer a promising alternative to kill cancer cells (Birben et al.,...
Phytic acid (myoinositol-hexaphosphate, IP6) is a constituent of dietary fiber in cereals. It has a high capacity to form insoluble complexes (phytates) with divalent metal ions present in food (Persson et al., 1998). The relative order of metal chelation with phytic acid at pH 7.4 was found to be Cu2+ > Zn2+ > Co2+ > Mn2+ > Fe2+ > Ca2+ (Reddy et al., 1982). Phytic acid supplementation is known to exhibit anti-cancer, anti-inflammatory and antioxidant activity, therefore, metal chelates are of considerable interest to improve the anticancer activity of chemotherapeutic drugs in combination or alone (Narayanawamy and Mohd, 2018). Accordingly, the present study focused on investigating the therapeutic potential of combination strategies (phytic acid with 5-FU) to treat cancer in xenograft animal model.

In addition to the above, the proposed formulation consists of a contrast agent, barium sulfate to track the drug carrier in the gastrointestinal tract. In the present study, we have formulated Eudragit coated pellets containing phytic acid, 5-FU and barium sulfate using extrusion spheronization technique. Critical formulation parameters including spheronizer speed and time were optimized for the spheronization technique. Critical formulation parameters like weight of pellets obtained

$\text{Weight of pellets obtained} = \frac{\text{Batch yield} (%) \times \text{Weight of drug and excipient}}{100}$

2.3. Preparation of coated pellets

Eudragit S100 was used for the coating of 5-FU loaded pellets. Accurately weighed enteric coating polymer (Eudragit S 100) was taken to make three different concentrations i.e. 5%, 10% and 15% to get optimized coating concentration as given in Table 2. Eudragit S 100 was dissolved in isopropyl alcohol. The solution was homogenized until it became clear. Then, the pipe of the peristaltic pump was rinsed with an organic solvent. Solution Pipe was filled with a coating solution and ensured that there were no air bubbles in the pipe and pipe was attached with spray gun of Accela Cota. The door was closed of coating pan and all the process parameters like; flow rate, temperature, drum speed and air pressure were then adjusted. 5.9 g of 5-FU loaded pellets of F10 formulation were firstly heated in the coating pan and then these preheated pellets were subjected to coating. A total of 1 ml coating solution was atomized by spray coating for 1 min, in time intervals of 1 min to allow sufficient time for solvent evaporation. Likewise, this was repeated 3 times and the resulted formulation was labelled as F11 (Pandey et al., 2018).

2.4. In-vitro characterization of 5-FU loaded pellets

The optimization of the amount of HPMC, barium sulfate and MCC and selection of the best formulation was done on the basis of the shape & size of pellets, percentage yield, friability and angle of repose.

2.4.1. Pellets morphology

Pellet size and morphological defects of the prepared pellets were examined under the Motic microscope. Pellet size was determined by using a 10X objective lens. A clean glass slide was taken and few drops of glycerin were spread over the slide. A pellet was placed over the slide and the size of 20 pellets was determined likewise (Pundlikrao and Rajput, 2017; Wairkar and Gaud, 2016).

| Table 1. Composition of different pellet formulations. |
|---------------|-----|-----|-----|-----|-----|
| Formula Code | Phytic acid (g) | Barium sulfate (g) | HPMC (g) | MCC (g) | Water (ml) | Drug (g) |
| F1 | 0.2 | 0.3 | 2 | 4 | 2 | - |
| F2 | 0.2 | 0.3 | 1 | 5 | 3 | - |
| F3 | 0.2 | 0.3 | 0.5 | 5 | 4 | - |
| F4 | 0.2 | 0.3 | 0.5 | 5.5 | 4.5 | - |
| F5 | 0.2 | 0.3 | 0.5 | 5.5 | 5 | - |
| F6 | 0.2 | 0.3 | 0.5 | 5 | 6 | - |
| F7 | 0.2 | 0.3 | 0.5 | 5.5 | 5 | - |
| F8 | 0.2 | 0.3 | 0.5 | 5.5 | 5 | - |
| F9 | 0.2 | 0.3 | 0.5 | 5.5 | 5 | - |
| F10 | 0.2 | 0.3 | 0.5 | 5.5 | 5 | 0.12 |
2.4.2. Sphericity studies

The shape and the area of pellets were investigated by optical microscopic image analysis. Pellet size and shape were measured using an optical DMW2-223 digital microscope (Motic Instruments) equipped with a 1/3" CCD camera imaging accessory and computer-controlled image analysis software (Motic Images, 2000, 1.3 version). 20 pellets from each batch were selected and analyzed by microscopic image analysis technique and a variety of parameters like aspect ratio, roundness and circularity have been used to assess the shape of the pellets. The pellet size data was further processed to get aspect ratio, circularity factor and roundness using following equations:

\[ \text{Aspect ratio} = \frac{D_{\text{max}}}{D_{\text{min}}} \]

Where, \( D_{\text{max}} \) --maximum diameter of the pellet, \( D_{\text{min}} \) --the minimum diameter of the pellet

\[ \text{Circularity factor} = \frac{4\pi A}{P^2} \]

\[ \text{Roundness} = \frac{P^2}{12.56 \times A} \]

Where, \( P \) – perimeter of the pellet, \( A \) – area of the pellet.

2.4.3. Micrometric properties

Pellets were characterized for flow properties such as the angle of repose, bulk density, tapped density, Hausner’s ratio, Carr’s index compressibility index and flow behavior was studied. The results of the above parameters were expressed as mean values of three observations.

2.4.3.1. Angle of repose. The flow property could be decided from the angle of repose as given in Table 4. Briefly, the sample was allowed to fall gently through a funnel on to a hard surface from the height of 2.5 cm. The height and diameter of the pile were noted. The angle of repose was determined by using the following formula.

\[ \tan \theta = \frac{r}{h} \]

Where \( h \) is the height of the pile and \( r \) is the radius of the pile.

2.4.3.2. Bulk density. Pellets were accurately weighed to 10 g and were gently poured through a glass funnel into a calibrated 100 ml measuring cylinder. The surface was made smooth carefully with the application of pressure. The volume occupied by the sample was recorded and bulk density (g/ml) was calculated and recorded in the table by using the following formula.

\[ \text{Bulk density} = \frac{\text{Weight of the sample taken}}{\text{initial volume of sample}} \]

2.4.3.3. Tapped density. Similar to the bulk density, tapped density was observed by tapping the cylinder 100 times from 3-inch height by using Electrolab tapped density apparatus after pouring the pellets into the measuring cylinder and the tapped volume was recorded. Finally, the tapped density was recorded by using the formula.

\[ \text{Tapped density} = \frac{\text{Weight of the sample taken}}{\text{minimum volume occupied after tapping}} \]

2.4.3.4. Hausner’s ratio. The Hausner’s ratio is a number that is correlated to the flowability of pellets. It was calculated by the following formula:

\[ \text{Hausner’s ratio} = \frac{\rho_t}{\rho_b} \]

Where \( \rho_t \) is tapped density and \( \rho_b \) is bulk density.

2.4.3.5. Carr’s index. The Carr’s index is an indication of the compressibility of pellets. It was calculated by the following formula:

\[ \text{Carr’s index} = \frac{100}{1 - \frac{\rho_t}{\rho_b}} \]

Where \( \rho_t \) is the tapped density of the powder and \( \rho_b \) is the freely settled bulk density of the powder.

2.4.4. Friability

Accurately weighed 5.9 g of pellets were placed on a sieve having 0.85 mm aperture with 20 pellets of 3 mm diameter and then placed in Roche’s friabilator (Veego Scientific, India). Then friabilator was subjected for 100 revolutions at 25 rpm speed. The pellets were collected from the friabilator and again placed on the sieve. The pellets having a smaller diameter than the aperture of sieve will pass through the sieve and then the pellets have reweighed. The friability was determined as the percentage loss of mass of pellets after the test was recorded.

\[ \text{Friability} = \frac{\text{Initial weight of sample} - \text{final weight of the sample}}{\text{Initial weight of the sample}} \]

Table 2. Optimization of formulation variables involved in spherization process.

| S. no. | Concentration (%) | Flow rate (ml/min) | Drum speed (rpm) | Temperature (°C) | Air pressure (kg/cm²) | Inference |
|--------|-------------------|--------------------|------------------|------------------|----------------------|-----------|
| 1.     | 5                 | 1                  | 20               | 50               | 5                    | No significant difference in in vitro drug release profile |
| 2.     | 10                | 1                  | 20               | 50               | 10                   | Required in vitro drug release                      |
| 3.     | 15                | 1                  | 20               | 50               | 10                   | Droplet formation on the tip of atomizer            |

Table 3. Optimization of formulation variables to obtain coated pellets.

| S. no. | Concentration (%) | Flow rate (ml/min) | Drum speed (rpm) | Temperature (°C) | Air pressure (kg/cm²) | Inference |
|--------|-------------------|--------------------|------------------|------------------|----------------------|-----------|
| 1.     | 5                 | 1                  | 20               | 50               | 5                    | No significant difference in in vitro drug release profile |
| 2.     | 10                | 1                  | 20               | 50               | 10                   | Required in vitro drug release                      |
| 3.     | 15                | 1                  | 20               | 50               | 10                   | Droplet formation on the tip of atomizer            |

Table 4. Empirical relation between the flow properties and angle of repose (Al-Hashemi and Al-Amoudi, 2018).

| Flow properties | Angle of repose (°) |
|-----------------|---------------------|
| Excellent       | <25                 |
| Good            | 25–30               |
| Passable        | 30–40               |
| Very poor       | >40                 |
2.4.5. Karl Fischer titration

The moisture content of prepared formulations was chemically quantified by Karl Fischer (KF) coulometric titration. The sample (0.1 g) was placed in a titration cell. The iodine is generated from oxidation of iodide contained in the KF reagent. Then reaction was started. Once the reaction consumes all the water present, the presence of excess iodine was detected by the indicator electrode. Then percentage of water present in the sample was calculated automatically and displayed on the screen of the KF titration apparatus.

2.4.6. Determination of entrapment efficiency

Sustained release pellets equivalent to 5 mg of the drug were dissolved in a suitable medium i.e. PBS 7.4. The resulting solution was briefly vortexed and treated in a bath sonicator for 10 min and then centrifuged for 10 min at 5000 rpm. The amount of drug in the supernatant was determined using the formula shown by the standard curve shown in Figure 1 and Table 5 at max. 266 nm by using UV-Spectrophotometer (Shanmugam et al., 2013). Using the following formula:

\[
\%\text{ Entrapment} = \frac{\text{Entapped drug}}{\text{Total drug}} \times 100
\]

2.4.7. Uniformity of drug content

The drug content for the 5-FU loaded pellets was determined by soaking pellets in water for 30 min, the pellets were broken with the spatula, vortexed for 5 min, centrifuged for 10 min at 2000 rpm, diluted to 50 ml with PBS 7.4 pH and then the drug was estimated by Shimadzu UV/visible spectrophotometer at 266 nm.

2.4.8. In vitro drug release study

In vitro release study of 5-FU loaded pellets was carried out using the dialysis bag method (Zhao et al., 2018). In this method firstly the dialysis bag was activated by washing it in running water for 3–4 h for removal of glycerin. Further, it was treated with 0.3 % (w/v) sodium sulfide solution at 70 °C for 1 min to remove sulfur compounds and then it was again washed with hot water (60 °C) for 2 min followed by acidification with 0.2% sulfuric acid and rinsed with hot water to remove the acid. Accurately weighed 0.24 g pellets were placed into a previously activated dialysis bag in a 5 ml solution of the donor compartment. The receptor compartment was filled with 100 ml of simulated gastric fluid (SGF) pH 1.2 in a conical flask. This flask was kept in an incubator shaker and speed of the shaker was maintained at 60 rpm at 37 ± 1 °C. At specific time intervals for 2 h, samples (5ml) were withdrawn and filtered. The same volume (5ml) was replaced after each sampling with SGF. Then, after 2 h the dialysis bag was placed into PBS pH 7.4. The same sampling procedure was followed for specific time intervals for 10 h and sink condition was maintained. The drug content in the sample was determined by the UV method being developed at 266 nm (Kamal et al., 2017, Shanmugam, S et al., 2013).

2.4.9. Compatibility studies and physical state of formulation (XRD and DSC)

The crystallinity of 5-FU loaded pellets was evaluated using an X-ray Diffractometer. The X-ray diffraction (XRD) patterns were determined for the 5-FU loaded pellets. Samples were exposed to monochromatic nickel-filtered copper radiation (45 kV, 40 mA) in a wide-angle X-ray Diffractometer (D8 Advance, BRUKER, Germany) with 20 angle. A thermo-analytical technique that is DSC, which is used to examine the degree of crystallinity and polymorphic transitions or thermal transitions involving energy changes throughout the method of formulation. The phase transition temperature of 5-FU in drug-loaded pellets was analyzed by Differential Scanning Calorimetry (SIIO 6300, SIIO, Japan) in perforated aluminum sealed pans at a heating rate of 5 °C/min from 30 to 400 °C using nitrogen as blanket gas (50 ml/s) (Tan et al., 2018).

2.4.10. In vitro cytotoxicity assay and cancer cell growth inhibition

In the present studies, EAC cells were used to determine the cytotoxicity of the experimental formulation. Briefly, EAC cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 50 μg/ml Gentamicin. The cells were cultured in a 5% CO2 atmosphere at 37 °C. Culture flasks were transferred to a sterile working area and the medium was discarded. Trypsin (0.1 ml/cm²) was added to the side of the flasks. Flasks were then turned over and laid down. Ensuring the monolayer was completely covered, the flask, were left stationary for 10–30s. Flasks were then rinsed to remove the trypsin from the monolayer. Then it was incubated in the flasks lying flat until the cell's rounded up when the flask was tilted. RPMI 1640 medium was added (0.1–0.2 ml/cm²) and cells were dispersed by repeated pipetting over the surface bearing the monolayer. Finally, the cell suspension up and down was pipetted out for a few times, with the help of the pipette resting on the bottom corner of the bottle, taking care not to create foam. Cells were then counted using hemocytometer.

2.4.11. MTT assay

% cell viability of prepared formulations was determined against EAC cell lines purchased from NCCS, Pune. EAC cell lines were seeded in 96-well microculture plates at 1 × 10⁷ EAC cells in RPMI 1640 media supplemented with 10% heat-inactivated FBS and incubated for 24 h at 37 °C and CO2 was maintained at 5%. Plain drug, drug-loaded coated and uncoated Pellets were dissolved in PBS pH 7.4 to prepare a solution. This solution was added in culture medium and EAC cells were incubated for 24 h. Then the percent cell growth inhibition was measured using MTT assay standard protocol.

2.5. In vivo studies

2.5.1. Induction of colon cancer and regimen of treatment

Colon cancer was induced in Wistar rats weighing 200–250 g and the dose of 1 × 10⁷ EAC cells was injected intrarectal, procured from NCCS Pune. Wistar rat was considered to have colon cancer after 14 days of

![Figure 1. Standard curve of 5-FU in PBS 7.4.](image-url)

**Table 5. Calibration curve of 5-FU in PBS 7.4.**

| Concentration (μg/ml) | Absorbance (nm) |
|-----------------------|-----------------|
| 0                     | 0.00 ± 0.00     |
| 10                    | 0.60 ± 0.05     |
| 12                    | 0.69 ± 0.05     |
| 14                    | 0.83 ± 0.06     |
| 16                    | 0.93 ± 0.05     |
| 18                    | 0.99 ± 0.04     |
| 20                    | 1.14 ± 0.06     |
2.5.2. Bioanalytical method development by HPLC for pharmacokinetic studies

High performance liquid chromatographic (HPLC) technique for the quantification of 5-FU in rat plasma has been developed. Chromatographic analysis was performed in the isocratic mode. The sample was analyzed by reverse-phase C18 Grace Vydac (250 × 4.6 mm, 5 μ) as a stationary phase. The mobile phase consisted of a mixture of methanol and water (92:8, v/v), which was pumped at a rate of 0.9 ml/min and 1.1 ml/min and using a similar C18 column (5 μm particle size, 4.6 mm internal diameter, and 250 mm length; Vertical Chromatography).

3. Results and discussions

3.1. Preparation and optimization of coated 5-FU loaded pellets

Process variable i.e. weight ratio of 5-FU, phytic acid, barium sulfate, HPMC and MCC were optimized on the basis of their effect on % yield, moisture and pellet shape as shown in Figure 2. In this study, the collective level of formulation variables, rotation speed & rotation time of spherization and extrusion conditions was required to produce 5-FU loaded pellets with desirable pharmaceutical properties (% highest yield, optimum moisture content and lowest pellet size). At the initial stage, the concentration of binder, water and bulking agent was optimized under a defined extrusion spherization parameters (rotation speed and time). Results show that as the concentration of HPMC decreases from 10.5% to 5% w/w against a fixed concentration of MCC, it affects the pellet morphology and percentage yield. However, percent yield was found to decrease with an increase in MCC concentration. Further, the higher water content increases inter particular attractive force which is enough to cause the spherization of pellets with decreasing particle size. From the results, 5% w/w concentration of binder was taken forward for further optimization. Keeping the binder concentration constant then MCC concentration was optimized. MCC concentration at 47.8% w/w has shown the highest product yield with...
optimum moisture content and lowest pellet size. As the MCC concentration was increased further, there was a decrease in % yield with an increase in particle size. 47.8% w/w percentage of MCC considered optimized and taken forward for further optimization. Further, the formulation was screened for the water level on the basis of pellets size and shape keeping the concentration of binder and bulking agent constant. Results suggested that the pellet size was significantly reduced above a total water level of 43.4% w/v. Further, the formulation was screened for rotation speed and time for spheronization. The changes in rotation speed and time caused the reduction in % yield. Therefore optimized speed and time rotation were considered to formulate drug loaded pellets as described in Table 2. Particle size, moisture content and percentage yield were the criteria considered for the selection of final formulation. It has been observed that the shape and size of the pellets were reduced with increasing concentration of water. The results of the optimized process suggested that the formulation F9 (containing 3% w/w phytic acid, 4.5% w/w barium sulfate, 7.5% w/w HPMC and 83.19% w/w MCC) demonstrated all the required characteristics and was therefore selected for drug-loaded formulation development.

Table 7. Shape and size analysis of different pellet formulations.

| Formula Code | Shape                      | Aspect ratio  | Roundness Factor | Circularity factor | Average diameter (μm) |
|--------------|----------------------------|---------------|------------------|--------------------|-----------------------|
| F1           | Rod shaped                 | 1.359 ± 0.15  | 1.059 ± 0.11     | 0.964 ± 0.39       |                       |
| F2           | Rod shaped                 | 1.325 ± 0.21  | 1.050 ± 0.25     | 0.969 ± 0.22       | 660 ± 20.6            |
| F3           | Rod shaped                 | 1.305 ± 0.11  | 1.047 ± 0.29     | 0.971 ± 0.27       | 680 ± 22.3            |
| F4           | Dumbbell                   | 1.286 ± 0.23  | 1.045 ± 0.06     | 0.975 ± 0.18       | 730 ± 23.7            |
| F5           | Dumbbell                   | 1.272 ± 0.21  | 1.039 ± 0.17     | 0.976 ± 0.21       | 680 ± 24.3            |
| F6           | Elongated spheroids        | 1.245 ± 0.16  | 1.024 ± 0.10     | 0.977 ± 0.15       | 740 ± 24.2            |
| F7           | Elongated spheroids        | 1.230 ± 0.24  | 1.021 ± 0.23     | 0.979 ± 0.26       | 610 ± 24.5            |
| F8           | Elongated spheroids        | 1.225 ± 0.21  | 1.019 ± 0.19     | 0.981 ± 0.10       | 560 ± 25.4            |
| F9           | Spheroids                  | 1.120 ± 0.19  | 1.0015 ± 0.13    | 0.988 ± 0.27       | 500 ± 24.1            |
| F10          | Spheroids                  | 1.109 ± 0.24  | 1.0009 ± 0.11    | 0.988 ± 0.16       | 480 ± 23.5            |
| F11          | Spheroids                  | 1.102 ± 0.09  | 1.0005 ± 0.09    | 0.990 ± 0.09       | 530 ± 24.2            |

Figure 2. Steps involved in pellets manufacturing and shape heterogeneity with time. First step in the process of palletization is to form extrude then with the help of minute and sharp blades in the disc of spheroniser, the extrude is cut into small pieces. As we continue spheronization process with the time rod shaped extrudes results into spherical shaped pellets.

Figure 3. Microscopical analysis of different pellet formulations. a) F2, b) F3, c) F4, d) F5, e) F6, f) F7, g) F8, h) F9, i) Drug loaded uncoated pellets and j) drug loaded coated pellets. This figure indicates the improvement in sphericity of pellets due to variations in the binder and vehicle (water).
Table 8. Micrometric properties for different pellet formulations.

| Formula code | Angle of repose (θ) | Bulk density (g/cm³) | Tapped density (g/cm³) | Hausner’s ratio | Carr’s index (%) | % Friability |
|--------------|---------------------|----------------------|------------------------|-----------------|-----------------|--------------|
| F1           | 36.300 ± 0.360      | 0.711 ± 0.003        | 0.823 ± 0.003          | 1.267 ± 0.005   | 19.780 ± 0.208  | 0.82 ± 0.15  |
| F2           | 33.600 ± 0.360      | 0.704 ± 0.003        | 0.818 ± 0.001          | 1.253 ± 0.004   | 19.003 ± 0.325  | 0.77 ± 0.19  |
| F3           | 32.033 ± 0.208      | 0.716 ± 0.003        | 0.845 ± 0.002          | 1.256 ± 0.004   | 17.263 ± 0.482  | 0.68 ± 0.11  |
| F4           | 31.566 ± 0.251      | 0.735 ± 0.002        | 0.844 ± 0.003          | 1.197 ± 0.003   | 15.976 ± 0.123  | 0.56 ± 0.18  |
| F5           | 31.333 ± 0.416      | 0.727 ± 0.003        | 0.837 ± 0.002          | 1.188 ± 0.003   | 14.170 ± 0.056  | 0.45 ± 0.14  |
| F6           | 30.666 ± 0.416      | 0.744 ± 0.002        | 0.850 ± 0.002          | 1.142 ± 0.006   | 12.453 ± 0.331  | 0.46 ± 0.10  |
| F7           | 29.433 ± 0.472      | 0.755 ± 0.003        | 0.861 ± 0.002          | 1.140 ± 0.004   | 12.296 ± 0.388  | 0.32 ± 0.12  |
| F8           | 26.633 ± 0.472      | 0.774 ± 0.003        | 0.823 ± 0.002          | 1.126 ± 0.004   | 11.903 ± 0.651  | 0.36 ± 0.10  |
| F9           | 25.066 ± 0.404      | 0.767 ± 0.002        | 0.843 ± 0.002          | 1.101 ± 0.008   | 8.573 ± 0.285   | 0.19 ± 0.07  |
| F10          | 25.005 ± 0.351      | 0.764 ± 0.002        | 0.832 ± 0.002          | 1.097 ± 0.005   | 8.023 ± 0.332   | 0.18 ± 0.06  |
| F11          | 24.332 ± 0.312      | 0.775 ± 0.003        | 0.837 ± 0.003          | 1.077 ± 0.002   | 7.320 ± 0.633   | 0.18 ± 0.05  |

Table 9. Percentage moisture content and percentage yield of all the formulation prepared while optimization of formula.

| Formulation code | % Yield | % Moisture content |
|------------------|---------|-------------------|
| F2               | 89.3 ± 0.15 | 3.33 ± 0.01 |
| F3               | 81.62 ± 0.21 | 3.44 ± 0.02 |
| F4               | 81.36 ± 0.21 | 3.52 ± 0.02 |
| F5               | 81.48 ± 0.17 | 4.21 ± 0.04 |
| F6               | 71 ± 0.28   | 4.23 ± 0.06 |
| F7               | 86 ± 0.08   | 4.27 ± 0.03 |
| F8               | 83.5 ± 0.09 | 4.31 ± 0.04 |
| F9               | 89.5 ± 0.06 | 4.32 ± 0.03 |
| F10              | 89.5 ± 0.05 | 4.32 ± 0.04 |

3.3. In-vitro characterization of 5-FU loaded pellets

In-vitro characterization of 5-FU loaded pellets included parameters like particle morphology, in-vitro drug release, entrapment efficiency, X-ray diffraction studies, differential scanning calorimetry and morphology.

3.3.1. Pellets morphology

The particle morphology is shown in Figure 3 and the mean particle size of pellets is shown below in Table 7. The size of optimized pellets of F9, F10 and F11 was found to be 500 μm, 480 μm and 530 μm respectively.

3.3.2. Sphericity studies

Pellets with the sphericity value of 1 were considered to be exactly spherical and pellets with sphericity values near to 1 were considered nearly spherical. The aspect ratio, roundness factor and circularity factor for the optimized formulation F9 were found to be 1.120 ± 0.19, 1.0015 ± 0.13 and 0.988 ± 0.27 respectively as shown in Table 7.

3.3.3. Micrometric properties

The results of flow properties like Angle of repose, Bulk density, Tapped density, Hausner ratio and Carr’s index of various batches of pellet formulations are shown in Table 8. The angle of repose (θ) for the pellets was observed in the range of 18.51° ± 1.1–25.13° ± 1.26 which indicated excellent flow properties of 5-FU loaded sustained pellet formulations. Similarly, values of Carr’s index and Hausner ratio were found in between 7.320 ± 0.63% to 20.31 ± 0.02% and 1.00 ± 0.04 to 1.38 ± 0.22 respectively. Carr’s index and Hausner ratio are commonly used

Figure 4. In vitro drug release profile of the plain drug, 5-FU uncoated pellets and 5-FU enteric coated pellets in SGF and SIF. Plain drug shows release upto 3 h and 60% of drug gets released into SGF. In case of uncoated pellets the release gets sustained upto 5 h due to incorporation of drug into carrier system while coated pellets exhibit sustained release of 5-FU upto 8 h due to enteric polymer coating.

3.2. Optimization of the pellet coating process

Eudragit S100 was used to coat 5-FU loaded targeted and sustained release pellets. Eudragit S100 was used at three different concentrations. From three different conc. 10%w/v Eudragit S100 coating was found to be the best coating for targeted and sustained release of 5-FU from pellets. The concentration of coating material above 10% leads to agglomeration and that could be related to slow drying under the specified experimental conditions. Further, a significant change in sphericity and morphology was observed with increased polymer concentration that could be attributed to improving atomization as a function of polymer viscosity. 10% w/v polymer conc. produced and pellets were coated (Tan et al., 2018).
parameters to predict flow characteristics and can be correlated with size, shape, surface area and cohesiveness of the substance. Pellets containing a low quantity of HPMC, an appropriate amount of water and MCC have shown lower values of Carr’s index as well as Hausner ratio and can be attributed to their regular and spherical shape. Therefore, it is evident that water and MCC can improve the shape and size of sustained release pellets which will further show better micrometric properties (Kamalaldin et al., 2017).

### 3.3.4. Friability

The friability test was carried out on all optimized formulation(s) to check their mechanical strength. The F10 and F11 formulations
possessed excellent mechanical strength and were observed in the range of 0.18 ± 0.05% w/w to 0.82 ± 0.15% w/w. Highest friability was seen in the F1 formulation in which hard, rod-shaped pellets were formed as shown in Table 8.

3.3.5. Karl Fischer titration

The percentage moisture content of each formulation was evaluated by the Karl Fischer (KF) coulometric titration method. The results of percentage moisture content and percentage yield are tabulated in Table 9.

3.3.6. Entrapment efficiency

The entrapment efficiency of drug loaded pellets was determined and repeated thrice. The high water solubility of 5-FU and the physicochemical proportion of excipients play a key role in achieving high entrapment efficiency in the experimental formulation. Percentage Entrapment efficiency was calculated according to the formula given in section 2.4.6 and was found to be 87.5 ± 0.05% for uncoated pellets and 82 ± 0.05% for coated pellets. A little loss in entrapment efficiency in coated pellets attributed to drug loss associated with coating polymer would adhere to the coating surface during the coating process.

3.3.7. Uniformity of drug content

Drug content was found to homogenous in the formulation and the prepared pellets showed uniformity in drug content and was within the permissible range 99.89 ± 0.74.

3.3.8. In-vitro drug release

The drug release data obtained for plain drug, uncoated and coated formulations are shown in Figure 4. The cumulative percent drug release versus time plot showing the comparison of uncoated and coated pellets, the release of anticancer drug from enteric coated formulation was prolonged over a period of 8 h. Eudragit is of polyanionic nature so it inhibits the burst release of 5-FU in gastric region. This was followed by the approximated zero-order release of the formulated system because that shows a steady drug release due to pH-dependent drug release behavior of Eudragit S 100. Due to the pH-sensitive nature of polymer used to coat the formulation the rate of drug release depends on the swelling of Eudragit S100 in an alkaline pH 7.4 (Pundlikrao P. et al., 2017). As the Eudragit S 100 swells in alkaline pH that will lead to the deterioration of the pores of coating layer. Then ultimately pore size and pore density was influenced the release rate. There was a significant difference in drug release pattern of the coated and uncoated 5-FU loaded anti-cancer pellets.

| Parameters | (F11) | (F10) | Plain drug (5-FU) |
|------------|-------|-------|------------------|
| Cmax (µg/ml) | 27.25 ± 0.35 | 25.60 ± 0.28 | 43.26 ± 0.87 |
| Tmax (hour) | 6.00 ± 0.14 | 6.00 ± 0.11 | 3.00 ± 0.21 |
| T1/2 (hour) | 7.05 ± 0.19 | 7.03 ± 0.21 | 5.60 ± 0.38 |
| AUCtotal (µg/µl/h) | 444.12 ± 11.12 | 389.84 ± 7.89 | 258.16 ± 2.23 |
| MRT | 16.31 ± 0.17 | 16.31 ± 0.21 | 9.43 ± 0.54 |

Abbreviations: AUC= Area under the curve, Cmax= maximum concentration of the drug, Tmax= Peak time when drug achieves Cmax, MRT= Mean residence time.
3.3.9. Compatibility studies and physical state of formulation (XRD and DSC)

Diffractograms of the plain drug, uncoated 5-FU loaded formulation and coated 5-FU formulation had shown peaks at 23° which can be seen in Figure 5 (A), (B) and (C) respectively. Due to the amorphous nature of polymer used for coating, there is a little decrease in the peak intensity was observed in the X-ray diffractogram of enteric-coated polymeric pellets. There was slight disappearance of the 5-FU peak because the encapsulated drug was dispersed at molecular level in the carrier system which indicates the uniform coating of the formulation with the enteric polymer having amorphous nature and also indicates the crystalline state of the encapsulated 5-FU (Lu et al., 2016). Eudragit coated pellets DSC thermogram had shown peaks at 234.7° and 242.4° the area where 5-FU was present as seen in Figure 5 (c). These two compatibility studies further strengthen the evidence that there is chance of encapsulation of drug into the carrier system and the compatibility of polymer and drug.

3.3.10. Cytotoxic study (MTT assay)

The growth medium RPMI 1640 was routinely used for human epithelial colorectal adenocarcinomas cell line EAC cells and the growth medium was supplemented with 10% FBS (fetal bovine serum), 1% antibiotic antimycotic solution, and 1% L-glutamine. For maintenance of the cell lines, they were kept in humidified 5% CO2 incubator and temperature was maintained at 37°C and a good confluence of cells observed. After the incubation for 24 h of EAC cells the cytotoxic activity of the plain drug, drug-loaded uncoated formulation and the drug-loaded coated formulation was determined using MTT assay. Cytotoxicity of drug-loaded uncoated pellets and drug-loaded coated pellets were investigated in comparison with different concentrations of plain anticancer drug to explicate the anticancer activity of developed formulation with respect to dose and time (Elyagoby et al., 2013). A positive correlation was observed. As the concentration of anticancer drug and time of contact with formulation increases the cytotoxic effect also increases. But as in the comparison of the entire formulations, drug loaded coated formulation showed a drastic increase in cytotoxicity at the same conditions. The plain anticancer drug at the highest tested dose that is 800 μg/ml, showed 42.9% cell viability after 24 h. The final formulation showed higher cytotoxicity at corresponding concentration of 5-FU having a percentage cell viability of 14.8% after incubation for 24 h (Figure 6) (Kim et al., 2010).

3.4. In vivo studies

3.4.1. Analytical method development for estimation of 5-FU

A sensitive analytical method for the determination of 5-FU has been developed and validated as per the method described earlier; Figure 7a depicts the overlay spectra of absorbance peaks of drug concentration in plasma. HPLC method was developed in animal plasma for the estimation of 5-FU. A linear response was obtained in the range of 0.1–10 μg/ml with correlation coefficient $r^2 = 0.999$. The reproducibility of the analytical method was measured in terms of linearity, Lower Limit of Quantitation (LLOQ), specificity and precision. The minimum limit of reliable quantification within the range 0.1–10 μg/ml was assessed, and the minimum limit found to be 0.1 μg/ml with a correlation coefficient $r^2 = 0.999$. The precision of developed analytical method for intra and
inter-day was analyzed as the percent variation over the concentration range of LLOQ and (medium quality control) MQC. The accuracy and precision at LLOQ were seemed to be 98.38% and 2.55% respectively. The accuracy under the experimental stress condition for 5-FU was found to be 103.2% as low quality control (LQC) and 102.02% for MQC. The above observations indicated that the developed analytical method was suitable to determine the plasma concentration at different time intervals following administration of 5-FU.

From the given results, it was observed that the developed optimized formulation showed extended plasma concentration with T1/2 7.05 over the plain drug T1/2 5.60 (Table 10). Further, the plasma concentration took a longer time to reach its maximum value in the case of 5-FU-Eudragit S100 formulation followed by plain drug showing sustained release in behavior in the experimental formulation. Observed mean Cmax 27.25 μg/ml and longer half-life of Eudragit S 100 coated formulation indicated lower systemic drug exposure, which may reduce the risk of drug dependent systemic toxicity (Figure 7b), however further in-depth toxicological study is required to support the statement. Pharmacokinetic finding established the controlled drug release behavior of experimental formulation which offered a more effective modality of chemotherapy by providing higher local concentration of the drug at the target sites and minimizing systemic drug absorption.

3.4.2. X-ray study to determine the targeting potential

X-ray studies have shown that the Eudragit S 100 coated formulation successfully targeted to the colon. Polyamionic nature of Eudragit retarded the release of encapsulated material in upper GIT. pH-dependent drug release behavior of Eudragit ensured colon-specific drug delivery as shown in Figure 8B. It can be concluded from the X-ray images that the enteric coated pellets release the drug in the colon. Hence, it proves that the formulation is ideal for colon targeting.

3.4.3. Tumor regression studies

Tumor regression studies were performed on the 21st day with the help of Vernier Caliper. A tumor excised on the 21st day from various groups is shown in Figure 9 (A), (B), (C) and (D). Results obtained from the tumor regression studies indicated that there was a notable difference in tumor volume.

The volume of induced tumors in different groups is shown in Table 11. The decrease in tumor volume in all experimental groups indicated the presence of 5-FU in the active state in the final formulation (He et al., 2008). The group treated with uncoated pellets resulted in not much reduction in tumor volume which might be due to the release of drug in upper GIT. The group treated with coated formulation showed increased therapeutic efficacy against colon cancer as compared to plain drug and uncoated formulation. The coated pellets decreased the tumor size for 5-FU to $3.42 \pm 0.32$ mm$^3$ relative to uncoated pellets having the tumor volume of $3.97 \pm 0.35$ mm$^3$ ($P < 0.01$), whereas the tumor size in plain drug decreased to $3.75 \pm 0.28$ mm$^3$ after 21 days ($P < 0.05$). This might be due to the oral delivery of the formulation and synergistic action of the drug and the chelating agent. The phytic acid might show their action by chelation of manganese and then inhibiting the SOD enzyme. Also, the cell viability studies have shown that the coated formulation had lower cell viability than the formulation with the plain drug.

3.4.4. Histopathological study

The tumor was frozen at -20 °C and then examined under a bright-field microscope at a magnification level of 100x to check the synergistic effect of the prepared formulation in comparison with plain anticancer drug. The test was repeated 3 times for test samples to get more precise results. This study indicates that there is drastic damage to the lamina propria as shown in diseased control specimen (Figure 10 A and B). The targeted release of the anticancer drug with chelating agent shows a better restoration of basement membrane and lamina propria than the plain anticancer drug (Figure 10 C and D). The sample treated with the final formulation shows a lamina propria that regained its original finger-like structure, indicating a synergistic effect of final formulation. Similar observations were reported by Foroushani and co-workers, wherein graphene oxide conjugated polydopamine shows improved localization of pay-load without any sign of tissue abnormality (Foroushani et al., 2019).

4. Conclusion

Metal chelators are found to be an attractive channel to improve the cytotoxic activity of the chemotherapeutic drug. The study further highlights inhibiting redox enzymes, which could be an effective strategy to break the defense mechanism of cancer cells, leading to improve therapeutic activity. Delivery of anticancer drugs using pH-sensitive polymer, ensure high local drug concentration to achieve selective cytotoxic activity and also minimizes systemic exposure of drug, could help to reduce the risk of drug dependent systemic toxicity. The present studies open a new avenue for effective treatment in colon cancer. However, organ-specific toxicity will need to be investigated for further clinical prospects.
Declarations

Author contribution statement

Veerpal Kaur, Amit Goyal, Goutam Ghosh, Sudamshi Chandra, Goutam Rath: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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