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Colon delivery of 5 – Fluoro uracil using cross-linked chitosan microspheres coated with eudragit S 100

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Abstract

5-Fluorouracil though recommended as a chemotherapeutic agent for colorectal cancer, suffers from severe systemic toxicity and so needs site-specific delivery. Objective of present investigation is to design slow release enteric coated solid formulations to avoid drug release in stomach and upper small intestine but slowly to build up required drug concentration in the colon. Chitosan microspheres were prepared by emulsification method using gluteraldehyde as cross linking agent. The microspheres were then coated with Eudragit S – 100 by emulsion solvent evaporation method. The coated microspheres were characterized for particle size, entrapment efficiency and surface characteristics. In-vitro drug release profile was studied by changing pH media as per USP protocol and the data was subjected to kinetic interpretations. The optimized microspheres showed particle size in the range of 62 to 65 µm with 65 ± 2% drug entrapment. Eudragit coated chitosan microspheres showed particle size increase upto 390 ± 2 µm with nearly spherical shape and smooth surface. In vitro drug release profile of uncoated microspheres was typical like conventional dosage forms with 38 %, 62 % and 88 % drug release at the end of 2 hrs, 6 hrs and 10 hrs respectively. Coated microspheres showed no drug release in SGF (2hrs), negligible release (8 %) in 6hrs but substantial release of 95% in 24 hours in simulated colon media. Drug distribution in GI following oral administration of coated microspheres in wistar rats showed 84% of the drug accumulation in colon.

Keywords: 5-FU; colon-targeting; chitosan; microspheres; Eudragit S-100.

Introduction

Colorectal cancer is one of the major causes of mortality and morbidity in industrialized nations. Treatment for colorectal cancer can be by any of the 3 modalities like surgery, radiation therapy or chemotherapy. Chemotherapy, however, is invariably used as an immediate treatment method alone or with surgery or radiotherapy. Out of many chemotherapeutics, 5-fluorouracil (5-FU) has been approved for use against colorectal cancer. IV administration of 5-FU has shown toxicities with complications in gastrointestinal, hematological, neural, cardiac and dermatological areas [1]. Delivery of 5 – FU to colon as site specific delivery in the form of microspheres may reduce the complications of toxicity, thereby, providing drug in therapeutically effective concentrations treating colorectal cancer.

Colon drug delivery has a number of implications in the field of pharmacotherapy. Diseases like inflammatory bowel disease (IBD) are treated effectively by delivering bioactive agents to colon through targeting [2]. Other colonic disorders like amoebiasis, crohn's disease, ulcerative colitis and colorectal cancer are also treated effectively by delivering bioactive agents locally, using coated microspheres which minimize release / absorption of drug in stomach and small intestine but deliver drugs to the large bowel [3]. Colon drug delivery has lot of challenges like minimizing degradation of drug in upper GI tract, protection of peptide drugs from hydrolysis and enzymatic degradation in duodenum and jejunum etc. A carefully designed colon therapeutic system could achieve maximum drug release in colon and elicit high activity with minimum systemic side effects. The specific drug release in colon provides a sufficient time interval between administration and onset of action which proves to be useful in treating chronic diseases such as asthma and arthritis [4].

Some strategies currently available for colon targeting are prodrug formulation, use of coating techniques to pH sensitive polymers, timed release systems, osmotic systems, pressure controlled drug delivery systems and by utilizing colon – specific biodegradable polymers [5]. Among the above methods, colon specific drug delivery systems developed are in majority utilizing the advantage of luminal pH in the ileum and / or microbial enzymes in the colon. Several biodegradable polymers which can be metabolized by colonic bacteria are used to prepare carrier systems like tablets and particulate delivery systems like microparticles / microspheres.

Polysaccharides belong to one such class of biodegradable materials which are normally metabolized in the colon by bacterial enzymes. This approach is exploited to deliver various drugs using polysaccharides such as pectin, alginate, guargum, amylase, inulin, dextran, chitosan, chondroitin sulphate etc. Large number of derivatizable groups present on these natural polymers makes them an ideal carrier system. Varying compositions, wide range of molecular weights, low toxicity, biodegradability and high stability makes them more appealing. Apart from all these, they are also approved by federal authorities for use as pharmaceutical excipients.

In the present investigation, cross linked chitosan microspheres of 5 – fluoro uracil were prepared and studied for their suitability to colon specific delivery. Polysaccharide based systems undergo enzymatic degradation in colon and chitosan is selected here as the matrix material to deliver the drug to colon. Eudragit S-100 is used as an enteric coating material to keep the microspheres intact and not to release the drug in stomach and or upper intestine. In contrast to single unit systems like tablets for oral use, multiple unit systems like microspheres are administered here as they show marked advantages like spreading over a large area in colon and avoiding exposure of high drug concentrations to a confined part of colonic mucosa. This also prolongs drug mean residence time in colon [6-7].

In-vitro drug release experiments in colonic media were conducted and kinetic parameters were calculated to assess the specificity of drug to release in colon [8]. In-vivo drug distribution study in GI tract was also conducted in wister rats model to confirm the suitability of the formulation for site specific delivery of 5-FU to colon.

Materials and Methods

Materials

5- Fluorouracil was kindly provided as a gift sample by Zydus Cadila, Ahmedabad. Chitosan was obtained from Central Institute of Fisheries and Technology, Cochin. Eudragit S 100 was a gift sample from Rohm Pharma, Germany. Hydrochloric acid, Disodium hydrogen phosphate, Potassium dihydrogen phosphate, methanol and dichloromethane were purchased from S.D. Chemicals, Boiser.

Reagents: Simulated Gastric Fluid, pH 1.2 (SGF) and Phosphate Buffered Saline, pH 7.4 (SIF) were prepared as per Indian Pharmacopoeia. Simulated Intestinal Fluid, pH 5.0 was prepared by dissolving specified quantity of KH2PO4 and Na2HPO4.2H2O and then adjusting the pH to 5.0 with 1.0 N NaOH. Simulated Colon Media (SCM) containing 2% rat cecal content was prepared by the method reported by Van den Mooter et al [9].

Methods

Preparation of cross linked chitosan Microspheres

Microspheres of 5 – fluorouracil were prepared with different concentrations of chitosan (Table 1) by emulsion method using gluteraldehyde as cross linking agent as reported by Thanoo et.al. [10]. Chitosan was dissolved in 5 % aqueous acetic acid solution and 5 – FU was dispersed in this solution by stirring. The above mixture was dispersed in liquid paraffin (1:1) containing 2 % span 80 and subjected to stirring using high speed stirrer at 2000 rpm for 4 hours at room temperature. 0.5 ml gluteraldehyde was added after 10 minutes of stirring followed by a second fraction of 0.5 ml gluteraldehyde after 1 hour of stirring. After 4 hours, the formed microspheres were centrifuged and washed several times with n – hexane to remove traces of liquid paraffin. The microspheres were then suspended in 5 % w / v solution of sodium bisulphate and further stirred on a magnetic stirrer for 10 minutes to remove residual gluteraldehyde. The washed microspheres were dried in vacuum dessicator for 48 hours before subjecting to further studies.

Table 1: Preparation of cross linked chitosan Microspheres

Batch No.	Drug: Polymer w/w	Emulsifier conc. (ml)	Particle size (μm)	Entrapment efficiency
CC 1 A	1:2	0.75	72.21 ± 1.93	37.18 ± 0.65
CC 1 B	1:2	1.00	65.22 ± 0.98	36.08 ± 1.17
CC 1 C	1:2	1.25	61.22 ± 1.28	34.22 ± 1.07
CC 2 A	1:3	0.75	80.02 ± 1.80	46.50 ± 0.82
CC 2 B	1:3	1.00	74.15 ± 0.84	44.19 ± 0.97
CC 2 C	1:3	1.25	72.05 ± 1.12	43.29 ± 0.97
CC 3 A	1:4	0.75	98.91 ± 1.20	59.70 ± 1.14
CC 3 B	1:4	1.00	93.41 ± 1.43	58.40 ± 1.34
CC 3 C	1:4	1.25	90.41 ± 1.83	55.44 ± 1.34
CC 4 A	1:5	0.75	110.68 ± 1.41	66.04 ± 0.97
CC 4 B	1:5	1.00	105.51 ± 0.98	65.00 ± 1.15
CC 4 C	1:5	1.25	101.21 ± 1.87	62.87 ± 0.11

Coating of Cross linked Chitosan microspheres

The optimized chitosan microspheres were coated with Eudragit S – 100 by emulsion solvent evaporation method [11]. The microspheres were suspended in 10 ml of organic solvent (acetone:ethanol::2:1) in which different strengths of Eudragit S – 100 was previously dissolved to give core coating ratio between 1:5 and 1:10. The above organic dispersion was then emulsified in 100 ml of liquid paraffin containing 2 % span 80 and stirred at 1000 rpm for 4 hours at room temperature to remove the solvents by evaporation. The eudragit coated microspheres were separated, rinsed with n – Hexane to remove residual traces of liquid paraffin, dried and stored in vacuum desiccators.

Entrapment Efficiency

Drug loaded chitosan microspheres were dispersed in methanol and kept for digestion with continuous stirring upto 24 hours. The microspheres were then centrifuged at 1000 rpm for 10 minutes to remove any insoluble solids, the supernatant layer decanted and filtered. The drug content was determined by using UV – Visible Spectrophotometer at 266 nm in methanol media.

Entrapment efficiency was calculated using the following formula,

Entrapment Efficiency = (Entrapped drug / total drug) X 100 Eq- 1

Yield of Microspheres

Microspheres recovered at the end of the preparation were weighed and the yield was calculated as % of total theoretical weight of the

materials taken for the preparation. The yield of microspheres was calculated as below:

% Yield = (Practical yield / theoretical yield) X 100 Eq- 2

Particle Size Analysis

The mean particle size of prepared microspheres was determined by a laser light scattering technique using Mastersizer (Malvern Instruments, London, UK) operating at a beam length of 2.40 mm and range of lens at 300 mm.

Surface Morphology of microspheres

The microspheres were studied for shape and surface morphology by optical microscopy (Olympus, BX 40, Japan) and SEM (Jeol JSM, 5610 LV, Japan) respectively. Optical microscopy was performed by taking small amount of microspheres dispersed in water on the glass slide and photographs were taken under 40 X resolution. The SEM studies of chitosan and Eudragit coated S 100 coated microspheres were carried out by gently sprinkling the powder previously kept in a dessicator on the double adhesive tape which was fixed on the dies followed by application of vacuum and high voltage for taking the images under high and low resolution.

In vitro Drug Release study from chitosan coated microspheres

In vitro drug release study of coated and uncoated chitosan microspheres were performed in pH progression medium at 37 ^{0} C ± 0.5 ^{0} C as per USP. Release study was further conducted in PBS and SCF from 6 -24 hours for coated formulation to understand the influence of colonic fluid on the release of drug. All dissolution studies were performed in triplicate. The samples withdrawn at regular intervals of time was analyzed using spectrophotometer at 267nm.

Differential Scanning Calorimetry

Microspheres containing drug and polymer as well as individual ingredients were characterized by DSC (Shimadzu, Japan) in the range of 25 – 350 ^{0} C at a heating range of 10^0 C per minute with an average sample weight of 4 mg. The glass transition temperature of polymer as well as the presence of any interaction between the drug and excipients was characterized.

Organ Distribution Study

In-vivo drug Distribution Study in GI Tract

In-vivo drug Distribution Study in gastro-intestinal tract following oral dosage of formulation was conducted in wister rats of either sex after due permission from the institutional animal ethics committee. Wistar rats were kept in well-spaced ventilated cages and maintained on healthy and fixed diet. The animals were divided into 4 groups of 4 animals each. The first group served as control and the other groups were treated with a marketed sample (5-FU capsule), uncoated crosslinked chitosan microspheres and Eudragit S-100 coated microspheres [12]. The doses were administered orally with the help of a feeding tube and the animals were sacrificed humanely after 2, 4, 6, and 8 hour intervals. The stomach, small intestine and the colon were isolated. These organs were homogenized by Micro Tissue Homogenizer (Mac, Mumbai, India) with a small amount of PBS (pH 7.4) followed by addition of 1 ml of acetonitrile and kept for 30 minutes. The contents were centrifuged at 1000 rpm for 20 minutes, the supernatant liquid was separated and diluted appropriately. The drug content in the separated liquid was determined using HPLC system equipped with a C18 (mu) Bondapak column and a UV detector set at 260 nm [13]. The drug content in different parts of the GI tract at different time intervals was calculated.

Results and Discussion

Chitosan is a commonly used polymer for the preparation of microspheres for varied pharmaceutical applications. They are of particular importance as mucoadhesive polymers due to their positive charge. Chitosan is a polysaccharide obtained by the partial alkaline n – acetylation of chitin which is a straight chain homopolymer composed of n acetyl glucosamine units with three dimensional α-helical

configuration. Deacetylation of chitin yields a linear chain copolymer, chitosan, which is comprised of glycosamine and n acetyl glycosamine units linked by β (1,4) glycosidic bonds. Thus, chitosan degrades in presence of colonic enzymes and can act as a suitable carrier for colonic drug delivery.

Oral formulation for colonic drug delivery needs protection from gastric and intestinal media in addition to its degradability in colon. Chitosan microspheres thus need enteric coating to protect the degradation of the microspheres in acidic environment of stomach. In addition, control over the release of the entrapped drug in intestine and colon is also required to achieve maximum availability of the drug in colon. Hence, in the present work, cross linked chitosan microspheres are prepared and then coated with enteric coating polymer eudragit S 100.

Particle Size and Entrapment Efficiency

Several batches of chitosan microspheres prepared using different drug: polymer ratios yielded microspheres in the size range between 61 and 110 μm (Table 1). The percentage entrapment varied between 34 to 66 % depending on drug: polymer ratio and the emulsifier concentration. With increase in drug: polymer ratio, an increase in the entrapment efficiency and particle size was observed in 2 phases. D:P ratio of 1:2 and 1:3 showed marginal change in the entrapment efficiency (34 – 46 %) and particle size (61 – 80) while, D:P ratio of 1:4 and 1:5 recorded entrapment efficiencies in the range between 60 – 66 % and particle size between 90 – 110 microns. These figures demonstrate the requirement of D:P ratio in this range for satisfactory entrapment efficiency. Hence, D: P ratio of 1:5 and emulsifier concentration of 1 ml was optimized.

Cross linking with gluteraldehyde

The above batches of microspheres were subjected to crosslinking using gluteraldehyde for the purpose of modulating the release behavior. Varied concentrations of gluteraldehyde (0.5 – 1.5 ml of 25 % w / w solution) was used to crosslink the optimized batch. Based on the percentage drug release, the volume of gluteraldehye was optimized at 1 ml. The crosslinking process with glutaraldehyde hardens the walls of the microspheres reducing their porosity, resulting slow drug release. The crosslinking bifunctional agents used here bind to different reaction sites in the chitosan. The aldehyde groups of the glutaraldehyde form covalent imine bonds with the amino groups of chitosan, due to the resonance established with adjacent double ethylenic bonds via a Schiff reaction [14] as shown in Figure 1.

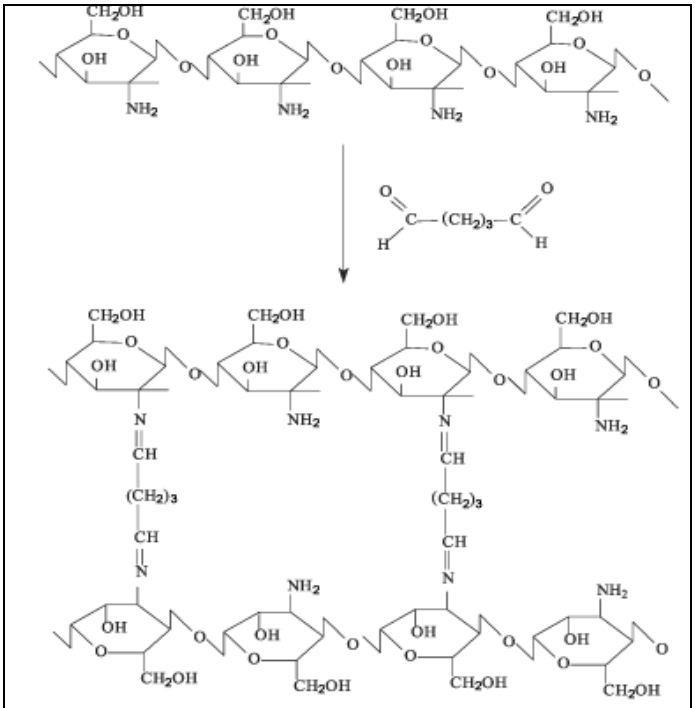

Figure 1. Crosslinking process of chitosan treated with glutaraldehyde.
(Figure reproduced with permission from Ref. [15])

Coating of crosslinked chitosan microspheres

Cross linked chitosan microspheres were coated using Eudragit S 100 to offer protection of the microspheres and to resist release of drug in stomach and upper small intestine. Eudragit S 100 is an anionic co polymer of methacrylic acid and methyl methacrylate with 1:2 carboxylic acid to ester ratio and has pH resistance threshold of 7. It shows lower aqueous solubility than Eudragit L series. Hence, for the purpose of achieving low

release rate in the lower intestine, Eudragit S 100 was selected. The coating was conducted by emulsion solvent evaporation method using core: coat ratio of 1:5 until the weight gain achieved was sufficient for gastric protection. The observed increase in mean particle size after coating was from 105.56 ± 0.98 to 389.13 ± 2.08 for the optimized batch.

In vitro dissolution study

In vitro dissolution study was conducted to understand in-vitro drug release profile of uncoated and coated microspheres. The purpose of this formulation was to avoid release of drug in gastric and upper intestinal region but to release the drug slowly in the lower part of the intestine maximizing drug concentration in the colon. Accordingly, the in-vitro drug release study was conducted in pH change method as per USP protocol (2 hours in SGF, 2-6 hours in SIF and 6-24 hours in SCF) and the results are shown in Table 2. The immediate release marketed formulation showed almost total release in 4 hours. Release profile from the uncoated microspheres was slow but consistent and independent of the pH in the dissolution fluid. This finding corroborates that cross linking has occurred and is responsible in decreasing the drug release rate to a significantly low level in simulated gastric media. It has also resulted in slow drug release throughout the GI tract independent of the pH of the media. Normally, free (not crosslinked) chitosan is more soluble in acidic media (due to protonation of the free amino group) and would preferentially release more drug in gastric pH. Aldehydes like glutaraldehyde cross links by schiffs reaction thus blocking free amino group partially reducing the solubility of the cross linked chitosan in acidic media showing no preferential release in gastric fluid. Drug release kinetics calculated from the release profile of uncoated microspheres show linearity (time Vs percent drug release plot) with R^2 value near to unity (0.9903) indicating the possibility of zero order kinetics. Nearly complete drug release (88 %) in 10 hours indicates the need for enteric coating of the formulation for colon delivery. Looking at the regression equation of the dissolution profile and high value of the intercept (16.984), dose dumping from the uncoated microspheres is evident. Presence of drug on the surface is further evidenced and supported by the DSC thermogram of the microspheres showing endothermic peaks specific to the drug (5 - FU).

Table 2 : Kinetic analysis of 5-FU release profile for coated and uncoated microspheres in various media.

Type of micro-sphere	Media	Time frame	Maxi-mum Release	Regreesion equation	R^2 value
Uncoated	pH change media	0-10 hrs	88 %	y = 7.4597x + 16.984	0.9903
Coated	SGF	0-2 hrs	No release	-	-
	SIF	3-6 hrs	7.9 %	Y= 2.3643X-6.1786	0.9939
	PBS (pH 6.8)	8-24 hrs	95 %	Y= 4.0156X – 0.6406	0.9965
	SCF	8-24 hrs	97 %	Y= 3.9565X + 4.0703	0.9884

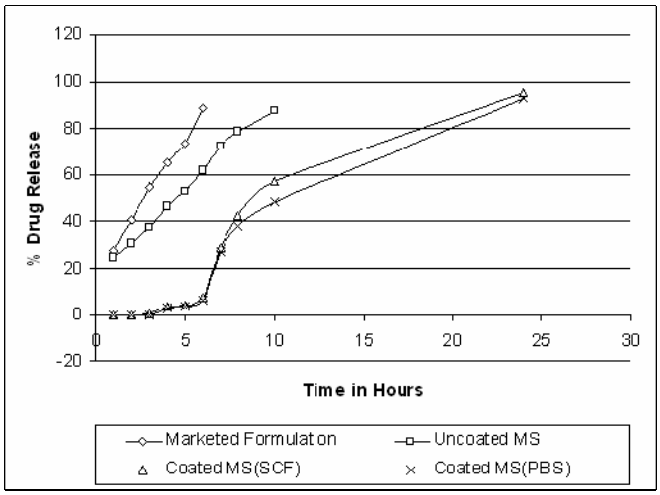

Figure 2: In vitro drug release study of formulations in changing pH media and in simulated colon fluid. Drug release study was also conducted in PBS from 6 -24 hours for coated formulation in order to understand the influence of colonic fluid on the drug release profile.

Table 3: Comparative organ distribution study of Eudragit S-100 coated microspheres with uncoated microspheres and immediate release marketed tablet of 5-FU

Formulation	Organ	Percentage of the administered estimated at varied time after oral administered*			
		2 Hours	4 Hours	6 Hours	8 Hours
Marketed immediate release tablet of 5-FU	Stomach	69.18± 3.45	35.4± 2,53	ND	ND
	Small Intestine	ND	33.3± 3.42	17.5± 4.35	ND
	Colon	ND	ND	41.6± 5.36	29.2± 5.64
Cross linked chitosan microspheres	Stomach	ND	ND	ND	ND
	Small Intestine	ND	64.7± 5.27	67,9± 4.25	22,2± 3.56
	Colon	ND	ND	35.2± 3.21	40.3± 2.58
Eudragit S-100 coated microspheres	Stomach	ND	ND	ND	ND
	Small Intestine	ND	ND	9.4± 1.23	17.8± 2.12
	Colon	ND	ND	12.5± 2,14	84.4± 2.26

*All readings are the mean of three readings; ND: Not detected.

Enteric coated microspheres showed no drug release in simulated gastric fluid upto 2 hours indicating the intactness of the applied coat. Dissolution studies were continued for a further 4 hour period in simulated intestinal media showing negligible drug release (8%). Release of drug beyond 6 hours in simulated colon fluid (SCF) was very substantial with over 95% release in 24 hours. The release profile in this region was linear with R^2 value near to unity (0.9939) indicating zero order kinetics of release. It was interesting to observe negligible difference between the release profiles of coated microspheres in PBS and SCF. This could be due to the cross linking of chitosan which did not disintegrate under the influence of colon enzymes and thus can be stated that the microspheres retained their integrity. The release could be by diffusion and / or by erosion of the microsphere matrix.

Analyzing the overall drug release profile of the coated microspheres, it is evident that the microspheres retained their integrity up to 24 hours releasing the drug slowly and consistently. Considering the average transit time for orally administered solid formulations (particularly small particulates) as approximately 2 hours in stomach, 6 to 8 hours in small intestine and up to 24 hours in colon, Eudragit coated microspheres resisted drug release in stomach, released 8-30 % in small intestine and the remaining 70 to 92% in colon.

In-vivo drug Distribution Study in GI Tract

Drug distribution study in GI tract following oral administration of the optimized formulation was performed in male wistar rats in order to establish the targeting potential to colon. The results of the study conducted with marketed oral tablet of 5-FU, un-coated cross linked chitosan microspheres and Eudragit S-100 coated microspheres are given in Table 3 and graphically represented in Figure 3. The marketed immediate release formulation (Capsule) released maximum (69.18 %) concentration of 5-FU in stomach within 2 hours of oral administration. After four hours, the concentration in stomach and small intestine was almost equal (~ 35%) while the concentration of the drug after 6 hours and 8 hours in colon was

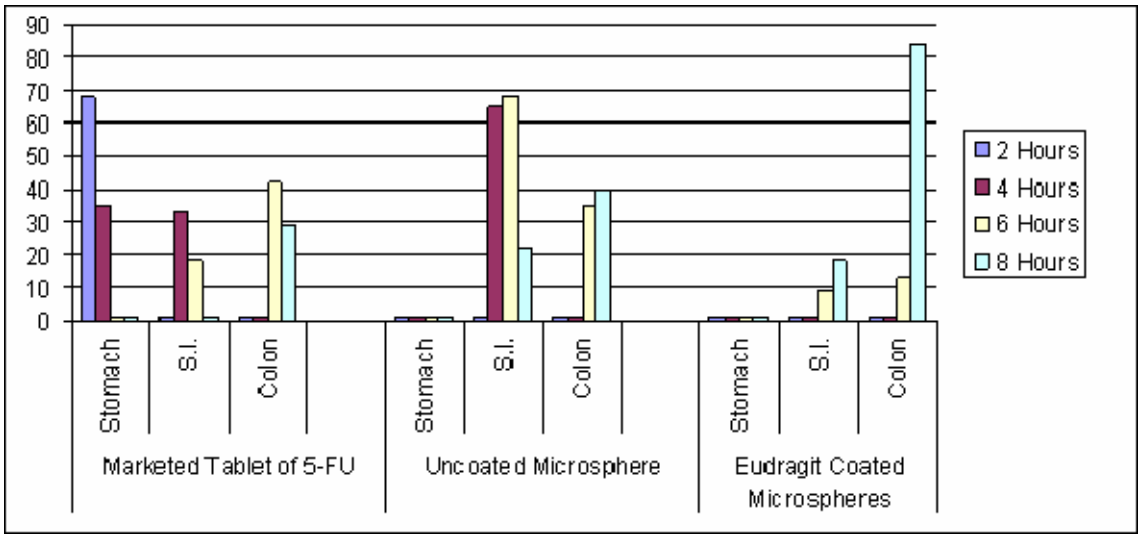

Figure 3: Comparative organ distribution study of Eudragit S-100 coated microspheres, the uncoated microspheres and immediate release marketed tablet of 5-FU conducted on wister rat model. The formulations were orally fed to the groups of animals (12 animals per formulation). After 2, 4, 6 and 8 hours, group of four animals were randomly sacrificed, stomach, Small intestine and colon were isolated, homogenized and the drug content were determined. Average of four readings with SE were recorded and tabulated.

around 40% and 30% respectively. The uncoated cross linked chitosan microspheres failed to release the drug in the first 2 hours in the G I tract. However, it exhibited a substantial drug release in small intestine after 4 hours (64%) and 6 hours (67 %) but released only about 35 and 40 % in colon at the end of 8 hours. Eudragit S-100 coated microspheres showed no drug release up to 6 hours in any part of the gastro-intestinal tract but showed a substantial drug release in colon at 8 hours (~84 %). The results indicate the intactness of the coated microspheres in the upper part of the GI tract followed by negligible drug release (6% to 8%) during its transit through the upper GI tract (2-6 hours) and may possibly be due to the leaching process. After 6 to 8 hours, maximum percentage of drug was observed in colon with no drug present in the stomach and a very small amount found in the small intestine.

Conclusions

The results of the present study indicate the potential of Eudragit S – 100 coated cross linked chitosan microspheres in designing site-specific delivery for treating colon related disorders for 5-FU. The investigations of different parameters involved in preparation of coated system proved that the method is simple and reproducible. The particle size and coat integrity can be controlled and monitored for releasing the drug within the affected / targeted area. In vitro drug release experiments with uncoated cross linked chitosan microspheres showed no drug release in SGF, nearly complete drug release in SIF and very low drug release in SCF. Eudragit S – 100 coated microspheres showed no release in SGF, negligible release in SIF and maximum release in colonic media. The in-vitro observation was found to be in correlation with in-vivo drug distribution studies in GIT conducted in rats wherein about 84% of the drug detected was in colon. Thus, the designed formulation was found suitable for colon delivery which resists drug release in stomach and in upper small intestine but can release maximum amount of drug in colon.

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