

Cytotoxicity and Pharmacokinetic Studies of PLGA Based Capecitabine Loaded Nanoparticles

Goutam Kumar Jena^{1,*}, Chinam Niranjana Patra¹, Prasanna Kumar Dixit²

¹Department of Pharmaceutics, Roland Institute of Pharmaceutical Sciences, Berhampur, BPUT, Rourkela, Odisha, INDIA.

²Department of Zoology, Berhampur University, Berhampur, Ganjam, Odisha, INDIA.

ABSTRACT

Background: Colorectal cancer ranked fourth as devastating cancer globally based on statistical death analysis. The major challenge for treating colorectal cancer is to target the drug to the specific site of the colon. **Purpose:** The main objective of the present research was to develop PLGA based nanoparticles to target and sustain the drug release at the colon for effective treatment of colorectal cancer. The significance of using Eudragit S100 was to prevent drug release in the stomach and small intestine whereas the significance of PLGA is to sustain drug release by its mucoadhesion property. **Methods:** Nanoparticles were prepared by modified nanoprecipitation method and the cytotoxicity study was performed by MTT assay. **Results:** From the cytotoxicity study it was found that Capecitabine loaded PLGA based nanoparticles had more capacity to inhibit HT 29 cell lines than that of the pure drug for all concentrations (10 to 0.0001). Pharmacokinetic (PK) study for the aqueous suspension of Capecitabine and optimized formulation ratified the results of *in vitro* study. The Area under the curve (AUC) for nanoparticles formulation was found to be twice more than the pure drug indicating better bioavailability. **Conclusion:** The PLGA based nanoparticles can be better targeted to colon for effective treatment of colorectal cancer.

Key words: Capecitabine, Colorectal, Cancer, Cytotoxicity, MTT, PLGA.

INTRODUCTION

Colorectal cancer (CRC) is a disease caused due to western life style and changing food habit.¹ Statistically about seven lacs people die every year due to colorectal cancer hence it is regarded as the fourth most devastating cancer in the world.² Capecitabine (Cap) is a prodrug of 5-Fluorouracil which is recommended mainly for the treatment of advanced stage of CRC.³

On the therapeutic front, Cap can extend the average survival time of patient but cannot cure the disease completely. Cap has a very short half-life i.e. 45 to 60 min.⁴ Due to very short half-life it has to be administered frequently to exert required therapeutic effect which leads to severe side effect. In order to reduce recurrence of administration, minimize side effects and to enhance the curative efficacy of the Cap, PLGA based pH sensitive nanoparticles

were developed by nanoprecipitation method. The pH sensitive polymer used in the present research was Eudragit S100. It can ward off drug release in the overhead of the GIT and can target the colon directly with improved therapeutic effectiveness of cap. Poly (L-lactic acid) (PLA) and its copolymers with glycolic acid (PLGA) are the most extensively used biodegradable polymers for achieving sustain release with different drug delivery systems.⁵ Based on the composition and molecular weight of PLGA, different degradation rates from months to years can be attained.

PLGA was used in the present research to sustain drug release. Owing to the Mucoadhesive nature of PLGA it can easily adhere to the mucous layer of colon and prolong the drug release.⁶ The Cap loaded PLGA based nano formulations were optimized

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Correspondence:

Dr. Goutam Kumar Jena,
Associate Professor, Roland
Institute of Pharmaceutical
Sciences, Berhampur-760010,
BPUT, Odisha, INDIA.
Phone: +91 8249097072
E-mail: goutam2902@gmail.
com



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considering mainly four factors i.e. particle size, zeta potential, polydispersity index and percentage entrapment efficiency. Thus, the goal of the present study was to design Eudragit S100 and PLGA based nanoparticles for site specific drug delivery of drug in colon followed by mucoadhesion and sustain release.

MATERIALS AND METHODS

Materials

Capecitabine was obtained as a gift sample from M/s RA CHEM, Hyderabad, India. PLGA (50:50) was purchased from Sigma Chemical CO, USA. Eudragit S100 was purchased from Sigma Chemical CO, USA. Poloxamer 188 and polyvinyl alcohol (PVA) were purchased from M/s Fine chemicals, Bengaluru, India.

Methods

Preparative technique of designing PLGA based nanoparticles

PLGA based nanoparticles were designed by nanoprecipitation method by slight modification.⁷⁻⁹ PLGA and Eudragit S100 (ES 100) polymers with an increasing ratio and a specified quantity of drug (Cap) as per formulation Table 1 were accurately weighed and dissolved in 10 ml acetone which constituted the organic phase. Two different stabilizers Poloxamer 188 and polyvinyl alcohol (PVA) were used at 0.5 % and 1% w/v aqueous solution respectively which constituted the aqueous phase. 19 ml of the aqueous phase was taken in a beaker and 1ml of the organic phase was added to it. It was stirred magnetically with 1000 rpm for 2hr. The nanosuspension was subjected to centrifugation at 10,000 rpm for 25 min. The sediment was collected and dispersed in distilled water and again subjected to lyophilization at -45°C under a vacuum pressure of 0.05 torr using 5% mannitol as cryoprotectant.^{10,11}

Analytical Techniques

Fourier Transform Infrared (FT-IR) Spectroscopy

Potassium bromide pellets were prepared with Capecitabine, PLGA, Eudragit S100 alone and for dried nanoparticles and subjected to FT-IR study using IR affinity (Shimadzu, Japan).

Differential Scanning Calorimetry (DSC)

DSC studies were performed for Capecitabine, Eudragit S100, physical mixture and nanoparticles formulation using DSC-60 (Shimadzu, Japan). Samples were placed in aluminum pan and exposed to a heating rate of 10°C/

min in the range of 25°C to 300°C. The thermograms were observed for fusion and crystallization.

Thermo Gravimetric Analysis (TGA)

In this experiment TGA was carried out in a Perkin-Elmer (SINGAPORE), Model NO Pyris Diamond TG/DTA. Approximately 3 mg of samples were placed in platinum crucibles. Each sample was heated from 60 to 500°C at a constant heating rate of 10°C/min during the measurement process under a dynamic N₂ atmosphere of 50 mL min⁻¹. TG analysis of CAP and PLGA were performed on this research.

XRD analysis

X-ray diffraction (XRD) patterns of Cap, PLGA and Cap loaded nanoparticles were observed using a ULTIMA-III RIGAKU MAKE (JAPAN) Cu target slit 10 mm at 40 kV and 40 mA with Cu K α radiation ($\lambda = 1.5406 \text{ \AA}$). The scanning range was 5–60° (2 θ) and the scanning rate was 4°/min.

Measurement of particle size and Zeta potential

Nanoparticles formulations were evaluated for particle size, zeta potential and polydispersity index after 10 times dilution with distilled water using Malvern zeta sizer. Disposable zeta cells were used for the above tests.

Evaluation of entrapment efficiency

Nanoparticles were ultracentrifuged at 10,000 rpm for 30 min and the free drug content was determined from supernatant by UV spectroscopy at 239 nm.

The drug entrapment efficiency (% EE) of the nanoparticles were determined and calculated as per the following formula.

$$\% \text{ Entrapment efficiency} = \frac{\text{Total amount of drug} - \text{free drug}}{\text{Total amount of drug}} \times 100$$

Transmission electron microscopy (TEM)

Transmission electron microscopy (H 7500, M/s Hitachi, Tokyo, Japan, 120 kv) of optimized nanoparticles was performed by placing one drop of diluted nanosuspension on copper grid with subsequent staining with 0.1% phosphotungstic acid. Magnified image was taken in a photographic film.

Ex vivo drug release study

Ex vivo release study of Cap from PLGA based nanoparticles was investigated by using the dialysis bag.¹² The dialysis membrane was flooded in 0.1 N HCl one night before the experiment. The PLGA based nanosuspension

Table 1: Composition of PLGA based different nano formulations.

Code	Drug (mg)	Polymer (mg)		Drug: polymer	Stabilizer (%)	
		PLGA	ES100	Cap:PLGA:ES100	Poloxamer 188	PVA
F1	10	20	20	1:2:2	0.5	-
F2	10	30	30	1:3:3	0.5	-
F3	10	40	40	1:4:4	0.5	-
F4	10	50	50	1:5:5	0.5	-
F5	10	20	20	1:2:2	-	1
F6	10	30	30	1:3:3	-	1
F7	10	40	40	1:4:4	-	1
F8	10	50	50	1:5:5	-	1

equivalent to 10 mg of Cap was placed in the dialysis bag and both ends were sealed. Three drug diffusion environments, 0.1 N HCl, phosphate buffer pH 6.8 and 7.4 were selected to imitate the actual GIT environment. During the diffusion study the dialysis bag containing PLGA based nanosuspension equivalent to 10 mg of Cap was held on first two solutions for a fixed period of time 2hr and 4hr to substitute different transit times in different parts of GIT before the carrier reached the colon area. The dialysis bag was then placed in the phosphate buffer pH 7.4. The dialysis bag was immersed in 100 ml of diffusion media of the receptor phase, which was stirred at 100 rpm and maintained at $37 \pm 0.5^\circ\text{C}$. The receptor compartment was covered with aluminum foil to prevent the evaporation of the release media. 5 ml of Sample was withdrawn at regular time intervals (0, 1, 2, 4, 6, 8, 10, 12 and 24 hr) and the same volume was replaced by fresh release media. The samples were analyzed by UV spectroscopy at 239 nm.

In vivo Pharmacokinetic study

Animals (White albino rabbits) were acclimatized for a period of 7 days. This experiment was performed with approval number 81 by institutional animal ethical committee having registration number 926/po/Re/5106/CPCSEA of RIPS, Berhampur, India. Ryle's tube was used for the administration of pure drug and optimized formulation.

A reported method of UFLC was adopted for the quantitative estimation of Cap.^{13,14} Solvent extraction method was used for the construction of calibration curve of Cap in rabbit serum. Blood sample of 1 ml were collected from marginal ear vein and allowed to coagulate for 30 min. The blood sample was subjected to centrifugation for 10 min at 5000 revolutions per minute at 4°C . Supernatant (serum) was transferred to six no of micro centrifuge tubes each having 100 μL .

The dilution of drug was carried out with phosphate buffer PH 3 and acetonitrile to make different concentrations (5, 10, 20, 30, 40 and 50 ng/ml). 50 μL of drug solution from each concentration was added and vortexed for 1 min. Then 150 μL of ethyl acetate as extracting solvent was mixed to each sample followed by centrifugation for 5 min at 2500 rpm. Subsequently 75 μL of supernatant was collected. It was subjected to drying at 70°C and the dehydrated sampling was added to 1 ml of mobile phase.

Serum concentration time curve of Cap was constructed and the following parameters like C_{max} , T_{max} , AUC, elimination rate constant, elimination half-life and clearance were determined.

Cytotoxicity study by using HT 29 cell lines by MTT assay

MTT is a chemical substrate having pale yellow color. It is split by living cells only to produce formazon product with dark blue color. Living mitochondria is required for this process of splitting. The quantity of mitochondria split is directly proportional to the number of active cells which is determined colorimetrically. The test was undertaken at Deshpande laboratories Bhopal. In short MTT reagent was dissolved in dimethyl sulfoxide (DMSO) and diluted suitably to obtain desired concentrations range. The concentration of DMSO was limited to less than 0.1 ml or all test samples. HT 29 cell lines were maintained at required conditions and were seeded in 96 well plates and subjected to various concentrations of test samples and incubated at 37°C , 5% CO_2 for 96 hr. The MTT reagent was added to the wells and incubated for 4 hr; product of formazon produced by living cells was dissolved in DMSO under a safety cabinet and measured at 550 nm. The % inhibition and IC_{50} values were calculated.

RESULTS AND DISCUSSION

FT-IR investigation

The chemical congeniality between Cap, PLGA and Eudragit S100 was evaluated by performing FT-IR study as given in Figure 1. The sharp peaks observed for cap at 1757 cm^{-1} indicates C=O stretching vibrations, at 1333 cm^{-1} indicates C-N bending vibrations, at 1242 cm^{-1} indicates N-H bending vibrations and at 1117 cm^{-1} indicates C-F stretching vibrations respectively almost same as previously reported.¹⁵ The nanoparticles loaded with cap also exhibits same peaks as that of the pure cap. The matching of peaks between nanoparticles and the pure cap revealed cap, PLGA and Eudragit S100 were quite compatible with each other.^{16,17} The FT-IR Spectra of Cap, PLGA, Eudragit S100 and Capecitabine loaded nanoparticles were presented in Figure 1.

DSC study

DSC study for Cap exhibited a sharp endothermic peak at 128.08°C . It had on-set temperature of 122.71°C but end set temperature of 134.04°C as represented Figure 2. A sharp endothermic peak was observed for Cap and its physical mixture with PLGA and Eudragit S100 at 127.95°C but onset and end set temperatures of 123.25°C and 133.35°C as given in Figure 2. Nearly similar endothermic peaks were observed for Cap and its physical mixture with PLGA and Eudragit S100.

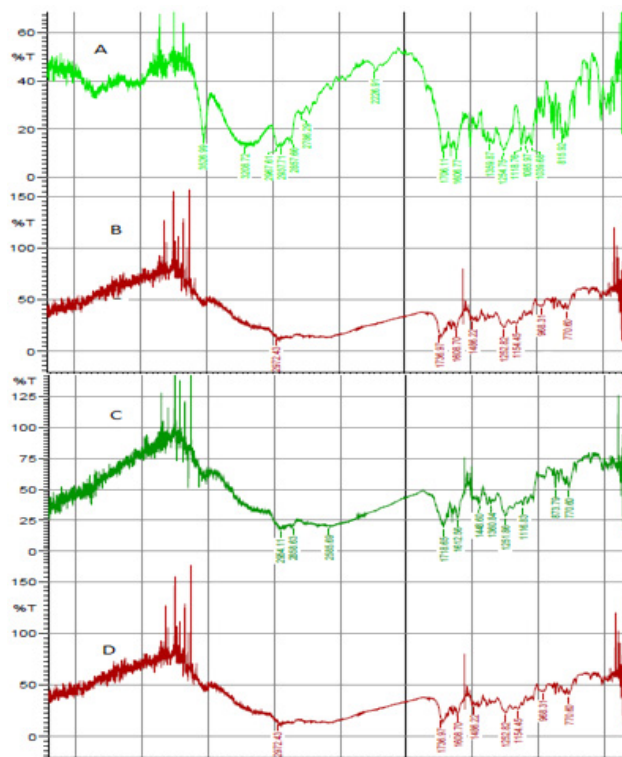


Figure 1: FT-IR Spectra of (A) Capecitabine (B) PLGA (C) Eudragit S100 and (D) Capecitabine loaded nanoparticles

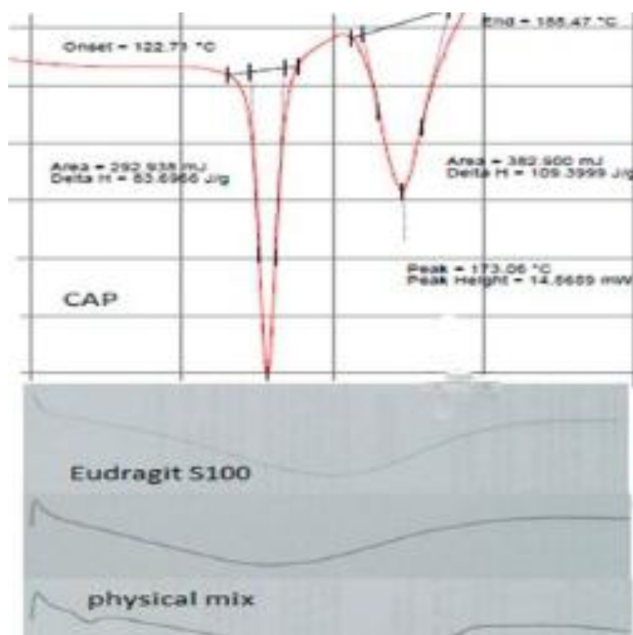


Figure 2: DSC thermograms of Cap (A), Eudragit S100 (B), physical mixture (C) and Capecitabine loaded PLGA NPs (D).

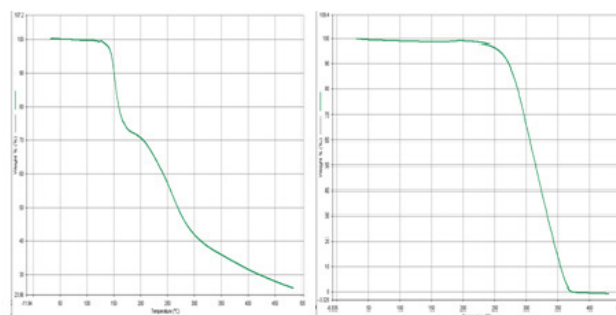


Figure 3: TGA thermograms of PLGA and Capecitabine.

Hence Cap was found to be compatible with both PLGA and Eudragit S100. DSC study for PLGA based Capecitabine loaded nanoparticles did not exhibit any peak. The absence of endothermic peaks can be attributed to complete entrapment of drug in PLGA based nanoparticles.^{18,19}

TGA study

Thermo Gravimetric analysis (TGA) analyzes substances which show loss of mass or gain of mass as a result of decomposition, oxidation, loss of moisture as volatiles. In the present study, TGA analysis of Cap and PLGA showed constant weight over a wide range of temperature indicating thermal stability of the product as portrayed in Figure 3.

X-RD Diffraction

X-RD study of Cap with different polymers and nanoparticles revealed no crystallinity on nanoparticles.

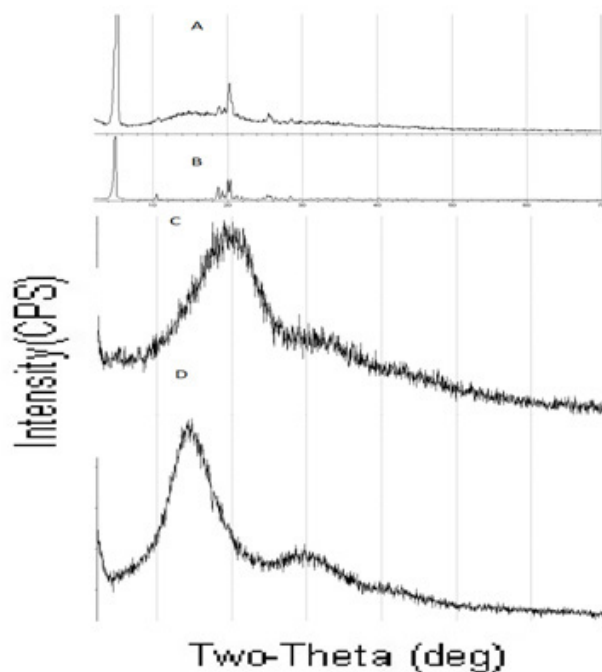


Figure 4: XRD spectra of A. Cap, B. Nanoparticles C. PLGA and D. Eudragit S100.

The patterns of X-RD diffraction of Cap revealed that intensity (counts) at different 2θ angles of 8.15, 9.53, 20.06, 30.05, 31.06 and 33.29 which indicate that the drug is crystalline in nature. The X-RD diffraction pattern of optimized formulation also showed similar counts i.e. 2θ values as that of pure drug indicating that drug loaded nanoparticles are also crystalline in nature and showing same lattice arrangement as represented in Figure 4. However, the difference in crystal sizes and their outer appearances may lead to slight variations in the relative intensities of their peaks.

Percentage Entrapment Efficiency (%EE)

The drug entrapment efficiency of the formulations (F1-F4) was found to be 45.4 ± 1.11 , 64.6 ± 1.12 , 66.5 ± 1.13 and $67.5 \pm 1.45\%$ and formulations (F5-F8) was found to be 45.8 ± 1.11 , 52.3 ± 1.33 , 54.6 ± 1.21 and $55.8 \pm 1.56\%$ as portrayed in Table 2. Thus, there was a steady increase in the entrapment efficiency on increasing PLGA concentration in the formulation. The formulations F3 and F8 registered highest entrapment of $67.5 \pm 1.45\%$ and $55.8 \pm 1.56\%$ respectively.

Zeta potential

Zeta potential, particle size and polydispersity index (PDI) were determined by photon correlation spectroscopy (PCS) using zeta sizer 5000 (Malvern Instrument Ltd, U.K.). Average particle size and polydispersity index were measured three times for each formulation by adopting suitable dilution. The zeta potential

Formulations	Particle size	PDI	Zeta potential	% EE
F1	195.1	0.398	-6.90 ± 3.04	45.4 ± 1.11
F2	219.9	0.368	-10.5 ± 3.55	64.6 ± 1.12
F3	215.7	0.138	-15 ± 5.07	66.5 ± 1.13
F4	263.9	0.293	-11.2 ± 4.88	67.5 ± 1.45
F5	345.1	1.000	-8.65 ± 2.99	45.8 ± 1.11
F6	363.4	0.891	-8.98 ± 3.11	52.3 ± 1.33
F7	379.8	1.000	-9.09 ± 4.07	54.6 ± 1.21
F8	315	0.480	-7.42 ± 4.22	55.8 ± 1.56

Mean \pm SD, $n = 3$

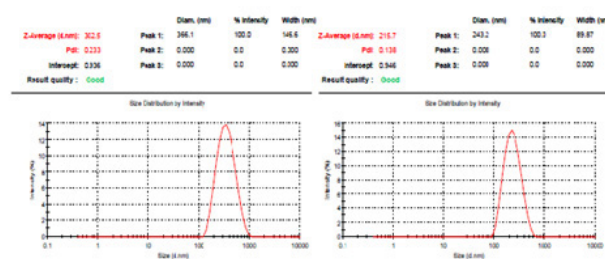


Figure 5: Average size of nanoparticles of formulation F4 and F5.

and the standard deviations were obtained directly from the instrument.

The average particle size of all formulations using poloxamer 188 as stabilizer were in the range of 195.1 nm to 263.9 nm whereas formulations with PVA as stabilizer were in the range of 315 nm to 379.8 nm as portrayed in Figure 5.

The zeta potential of all formulations using poloxamer 188 as stabilizer were in the range of -6.90 ± 3.04 to -15 ± 5.07 as presented in Figure 6. The PDI values of all formulations using poloxamer 188 as stabilizer were in the range of 0.138 to 0.398 and whereas PVA as stabilizer were in the range of 0.480 to 1.00. Considering particle size, zeta potential, polydispersity index (PDI) values and percentage entrapment efficiency F3 and F8 were selected as optimized formulations.

In-vitro drug diffusion study

In-vitro drug diffusion study was performed for all nanoparticulate formulations. The drug diffusion study was performed using gradient pH simulating GI tract. The drug diffusion was studied initially in 0.1 N HCl for 2 hr followed by a change of medium to phosphate buffer pH 6.8 for 4 hr and subsequently for 24 hr in phosphate buffer pH 7.4. All formulations (F1-F8) showed negligible drug release in both in 0.1 N HCl and phosphate buffer pH 6.8.²⁰ This can be

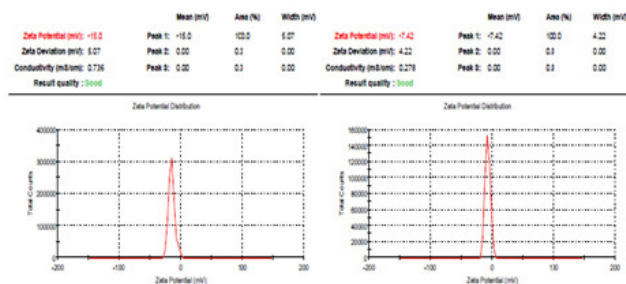


Figure 6: Zeta potential of nanoparticles of formulation F4 and F5.

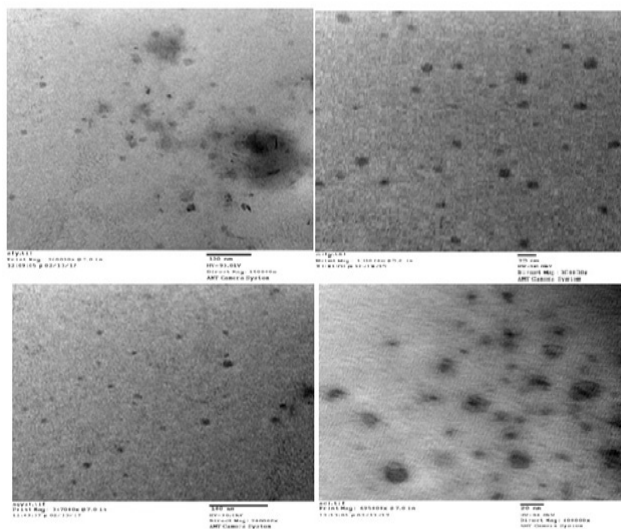


Figure 7: TEM images of nanoparticles.

attributed to the pH dependent solubility of Eudragit S100 (Soluble at pH > 7). It was observed that as the proportion of PLGA increased (F1-F4), the drug release rate decreased.

Diffusion of drug, erosion of polymers and swelling of polymers were considered to be the main mechanisms for drug release. This sustained release behavior can be attributed to slow degradation rate of PLGA.

TEM analysis

The TEM analysis performed for nanoparticles of optimized formulation (F3) were found to be discrete with mean particle size of about 100 nm (Figure 7).

Cytotoxicity study

Cytotoxicity study using HT 29 cell lines by MTT assay indicated that Cap loaded nanoparticles were more cytotoxic than the pure drug solution as compared to previously reported²¹ and IC₅₀ value of all formulations were found to be reduced to half than the pure drug solutions indicating more effective than pure drug (Figure 8)

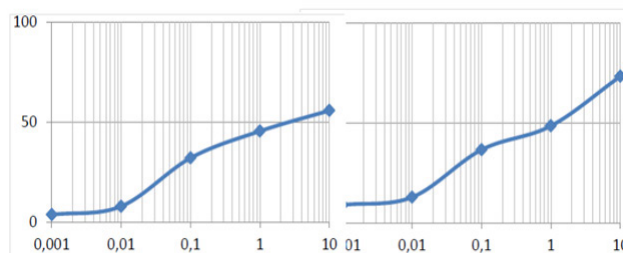


Figure 8: Percentage inhibition of pure drug of Cap and Cap loaded PLGA based Nanoparticles.

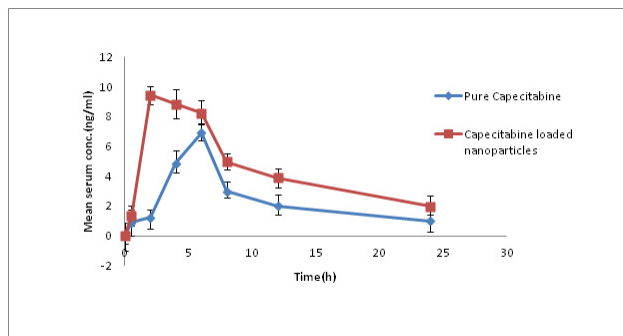


Figure 9: Comparative plots of cap and cap loaded PLGA based nanoparticles.

Pharmacokinetic study

UFLC (ultrafast liquid chromatographic) technique was adopted for analysis of Cap. This technique was very simple and rapid and here reverse phase method was followed. Chromatography was performed on a 25 cm×4.6 mm i.e. 5µm particle, C₁₈ column with 55: 45 (% v/v) acetonitrile: phosphate buffer pH 3 as a mobile phase at a flow rate of 10 ml/min, PDA detection at 239 nm. Cap was eluted with a retention time of 4.292 min. The method was found to be linear in the concentration range of 5-50 ng/ml of Cap with coefficient of correlation 0.999.

The serum sample of rabbit were analyzed by HPLC method with acetonitrile and phosphate buffer pH 3 in the ratio of 55:45, retention time of 4.292 min, column C₁₈. The standard curve of pure drug in serum was prepared in the concentration range of 5 to 50 ng/ml. Ethyl acetate was used as extracting solvent. Pharmacokinetic study for the aqueous suspension of pure drug Capecitabine and optimized formulation (F3) ratified the results of *in vitro* study. C_{max} of optimized formulation F3 was found to be higher as compared to pure drug indicating better absorption of drug from nanoparticles and the T_{max} for pure drug was found to be of less (2 hr) as compared to formulation (2.5 h) as given in Figure 9. The AUC for nanoparticles formulation was found to be approximately two times

Table 3: Pharmacokinetic parameters utilizing Microsoft Excel 2007.

parameters	(Cap) (n = 6)	Cap loaded PLGA based nanoparticles (n = 6)
C _{max} (ngmL ⁻¹)	6.9345	9.4569
T _{max} (h)	6	2.5
AUC _{0-t} (ng-h/mL)	95.6489	185.6783
AUC _{0-∞} (ng-h/mL)	96.8556	200.7855
t _{1/2} (h)	8.599	26.86
CL(L/h)	13.068	16.488
VD(L)	3.413	6.835

more than the pure drug indicating better bioavailability (Table 3).

CONCLUSION

Capecitabine loaded PLGA in combination with Eudragit S100 can be developed by nanoprecipitation method and can be selectively targeted to colon with sustained release for the effective treatment of colorectal cancer. The cytotoxic effect of Capecitabine loaded nanoparticles was found to be more cytotoxic to tumor cells than pure Cap. Hence our developed nano formulations would be highly toxic to tumor cells and less toxic to normal cells which indicated it would produce fewer side effects.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

ABBREVIATIONS

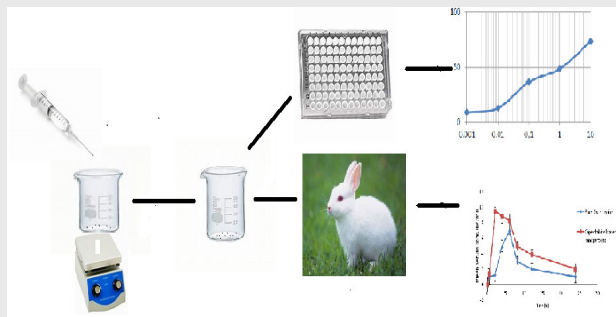
CRC: Colorectal cancer; **Cap:** Capecitabine; **PLGA:** Poly (lactic-co-glycolic acid); **FT-IR:** Fourier Transform Infrared; **DSC:** Differential Scanning Calorimetry; **TGA:** Thermo Gravimetric Analysis; **XRD:** X-ray diffraction; **TEM:** Transmission electron microscopy; **%EE:** Percentage entrapment efficiency; **DMSO:** Di Methyl Sulfoxide; **PDI:** Polydispersity index; **PCS:**

Photon Correlation Spectroscopy; **PVA:** Poly Vinyl Alcohol.

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PICTORIAL ABSTRACT



About Authors



Goutam Kumar Jena: Roland Institute of Pharmaceutical Sciences, Berhampur, BPUT, Odisha, INDIA.



Ch. Niranjana Patra: Roland Institute of Pharmaceutical Sciences, Berhampur, BPUT, Odisha, INDIA.



Prasanna Kumar Dixit: Post Graduate Department of Zoology, Berhampur University, Berhampur, Odisha, INDIA.

SUMMARY

Colorectal cancer is completely curable if diagnosed at early stage and provided proper treatment with right approach. Capecitabine loaded PLGA based nanoparticles would be the best approach for targeting colon for effective treatment of colorectal cancer. Nanoprecipitation technique with little modification was adapted for developing PLGA based nanoparticles and subjected to characterization such as determination of particle size, Zeta potential, % entrapment efficiency, *in vitro* cytotoxicity studies using HT 29 cell lines by MTT assay and *in vivo* pharmacokinetic studies using rabbit model. The results obtained from cytotoxicity studies revealed that Capecitabine loaded PLGA based nanoparticles with magnified cytotoxicity to cancer cells as compared to pure Capecitabine and *in vivo* study exhibited enhanced oral bioavailability. Hence PLGA based nanoparticles can be employed as suitable drug carrier for effective treatment of colorectal cancer.

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