Section A-Research paper

Development of PLGA Based Anticancer Drug Loaded Nanoparticles for treating Colorectal cancer: Cytotoxicity and Pharmacokinetic Studies

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ABSTRACT

Based on statistical analyses of death, colorectal cancer was the fourth most fatal cancer in the world. Targeting the medicine to the specific area of the colon is the main problem in the treatment of colorectal cancer. The development of PLGA-based nanoparticles to target and maintain medication release at the colon for the successful treatment of colorectal cancer was the primary goal of the current study. While PLGA's role is to maintain medication release due to its mucoadhesion property, Eudragit S100's purpose was to stop drug release in the stomach and small intestine. Modified nanoprecipitation was used to create the nanoparticles, and the MTT assay was used to test for cytotoxicity. According to the results of the cytotoxicity investigation, Capecitabine-loaded PLGA-based nanoparticles were more effective at inhibiting HT 29 cell lines than the pure medication did at all concentrations (10 to 0.0001). The outcomes of the in vitro investigation were validated by the pharmacokinetic (PK) study for the aqueous solution of Capecitabine and the optimised formulation. The area under the curve (AUC) for the formulation containing nanoparticles was discovered to be twice as high as that for the pure medication, demonstrating improved bioavailability. For more effective therapy of colorectal cancer, the colon can be more effectively targeted with PLGA-based nanoparticles.

Key Words: Colorectal cancer, cytotoxicity, MTT test, PLGA and Capecitabine.

INTRODUCTION: Colorectal cancer (CRC) is a condition brought on by the western way of life and evolving dietary practises [1]. According to statistics, colorectal cancer causes about seven lac deaths

annually, making it the fourth most deadly cancer in the world [2]. A prodrug of 5-fluorouracil called capecitabine (Cap) is primarily advised for the treatment of advanced CRC stages [3].

On the treatment front, capecitabine can increase the typical patient survival time but cannot fully treat the illness. The half-life of capecitabine is only 45 to 60 minutes [4]. Due to the medication's extremely short half-life, regular administration is necessary for it to have the desired therapeutic impact, which causes serious side effects. PLGA-based pH sensitive nanoparticles were created by the nanoprecipitation process in order to decrease the frequency of administration, eliminate adverse effects, and increase the curative efficacy of Capecitabine. Eudragit S100 was the pH-sensitive polymer employed in the current study. With increased Capecitabine therapeutic effectiveness, it can prevent medication release in the GIT's overhead and directly target the colon. For establishing sustained release using various drug delivery systems, poly (I-lactic acid) (PLA) and its copolymers with glycolic acid (PLGA) are the most commonly utilised biodegradable polymers [5]. Depending on the molecular weight and content of PLGA, a range of degradation rates from months to years can be achieved.

In the current study, PLGA was employed to maintain drug release. Due to PLGA's mucoadhesive properties, it can easily cling to the colon's mucous layer and delay the release of the medicine [6]. The particle size, zeta potential, polydispersity index, and percentage entrapment efficiency were the key four criteria that were taken into consideration when optimising the capecitabine loaded PLGA based nano formulations. Therefore, the objective of the current study was to develop PLGA and Eudragit S100-based nanoparticles for mucoadhesion- and sustain-release medication delivery to specific sites in the colon.

MATERIALS AND METHODS

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Materials:

Capecitabine was received from M/s Reddy's laboratory in Hyderabad, India as a gift sample. Sigma Chemical CO, a US-based company, provided PLGA (50:50). We bought Eudragit S100 from SD Fine Chemicals in Mumbai. Polyvinyl alcohol (PVA) and Poloxamer 188 were bought from M/s Fine Chemicals in Bengaluru, India.

Methods:

Preparative technique of designing PLGA based nanoparticles

By slightly modifying the nanoprecipitation process, PLGA-based nanoparticles were created [7, 8]. According to formulation table 1, an exact amount of the medicine (Capecitabine) and PLGA and Eudragit S100 (ES 100) polymers in an increasing ratio were weighed out and dissolved in 10 ml of acetone, which made up the organic phase. The aqueous phase was made up of two distinct stabilisers, Poloxamer 188 and polyvinyl alcohol (PVA), at concentrations of 0.5% and 1% w/v, respectively. 1 ml of the organic phase was added to 19 ml of the aqueous phase in a beaker. For two hours, it was magnetically agitated at 1000 rpm. [9]

Table No.1 Composition of PLGA based different nano formulations

Formulation Code	Capecitabine (mg)	Polymer (mg)		Drug:Polymer	Stabilizer (%)	
		PLGA	Eudragit S100	Capecitabine:PLGA:Eudragit S100	Poloxamer 188	PVA
CNF1	20	40	40	1:2:2	0.5	-
CNF2	20	60	60	1:3:3	0.5	-
CNF3	20	80	80	1:4:4	0.75	-
CNF4	20	100	100	1:5:5	0.75	-
CNF5	20	40	40	1:2:2	-	0.75
CNF6	20	60	60	1:3:3	-	0.75
CNF7	20	80	80	1:4:4	-	1
CNF8	20	100	100	1:5:5	-	1

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ANALYTICAL TECHNIQUES

Fourier Transform Infrared (FT-IR) Spectroscopy:

With the use of capecitabine, PLGA, Eudragit S100 by itself, and dried nanoparticles, potassium bromide pellets were created and then put through an FTIR analysis employing IR affinity (Shimadzu, Japan).

Differential Scanning Calorimetry (DSC):

Using the DSC-60 (Shimadzu, Japan) for DSC research, formulations of nanoparticles, Eudragit S100, physical mixtures, and capecitabine were all examined. Samples were heated between 25 and 300 degrees Celsius at a rate of 10 degrees per minute in an aluminium pan. We looked at the thermograms for fusion and crystallisation. [11]

Thermo Gravimetric Analysis (TGA):

This experiment used a Perkin-Elmer (SINGAPORE) Model NO Pyris Diamond TG/DTA to conduct TGA. Samples weighing around 3 mg were put in platinum crucibles. Throughout the measurement procedure, each sample was heated from 60 to 500 °C at a constant rate of 10 °C/min while being exposed to a dynamic N2 atmosphere of 50 mL min1. On this study, TG analyses of capecitabine and PLGA were conducted. [12]

XRD analysis:

Using a ULTIMA-III RIGAKU MAKE (JAPAN) Cu target slit 10 mm at 40 kV and 40 mA with Cu k radiation (= 1.5406), X-ray diffraction (XRD) patterns of Capecitabine, PLGA, and Capecitabine loaded nanoparticles were detected. The scanning range was 5° to 60° and the scan speed was 4° per minute. [13]

Measurement of particle size and Zeta potential:

After being diluted with distilled water 10 times, Malvern zeta sizer was used to assess the nanoparticle formulations for particle size, zeta potential, and polydispersity index. The tests mentioned above used disposable zeta cells. [14]

Evaluation of entrapment efficiency:

For 30 minutes, nanoparticles were ultracentrifuged at 10,000 rpm to determine the amount of free medicine in the supernatant using UV spectroscopy at 239 nm. [15]

The formula below was used to determine and calculate the nanoparticles' drug entrapment efficiency (% EE).

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

Transmission electron microscopy (TEM):

Optimised nanoparticles were analysed using transmission electron microscopy (H 7500, M/s Hitachi, Tokyo, Japan, 120 kv) by depositing one drop of diluted nanosuspension on copper grid and then staining the sample with 0.1% phosphotungetic acid. A photograph was taken of a magnified image. [16]

Ex vivo drug release study:

Utilising a dialysis bag, ex vivo releases of capecitabine from PLGA-based nanoparticles were studied [17]. The night before the experiment, 0.1 N HCl was flooded onto the dialysis membrane. The dialysis bag was filled with the PLGA-based nanosuspension, which is equal to 10 mg of Capecitabine, and both ends were sealed. To simulate the actual GIT environment, three drug diffusion conditions were chosen: phosphate buffer pH 6.8 and 7.4 and 0.1 N HCl. through order to simulate varying transit periods through various portions of the GIT before the carrier reached the colon area, the dialysis bag containing PLGA-based nanosuspension equivalent to 10 mg of Capecitabine was held on the first two

solutions for a predetermined period of time (2 hours) and 4 hours. The phosphate buffer pH 7.4 was then used to contain the dialysis bag. The dialysis bag was submerged in 100 ml of the receptor phase's diffusion medium while being swirled at 100 rpm and kept at 370.50C. To stop the release material from evaporating, aluminium foil was placed over the receptor compartment. At certain intervals (0, 1, 2, 4, 6, 8, 10, 12 and 24 h), 5 ml of the Sample were taken out and replaced with brand-new release media. UV spectroscopy was used to examine the materials at 239 nm. [18]

***In vivo* Pharmacokinetic study:**

For a week, animals (White albino rabbits) were acclimated. The institutional animal ethical committee gave its clearance for the conduct of this experiment. For the administration of pure medication and improved formulation, Ryle's tube was utilised.

For the quantitative estimation of Capecitabine, a previously reported ultrafast liquid chromatographic technique was used [19]. The creation of Capecitabine's calibration curve in rabbit serum involved the solvent extraction approach. One millilitre of blood was drawn from a vein in the outer ear and allowed to coagulate for 30 minutes. The blood sample was centrifuged at 5000 revolutions per minute for 10 minutes at 40C. Serum supernatant was divided among six micro centrifuge tubes, each holding 100 l. Acetonitrile and phosphate buffer pH 3 were used to dilute the medication to create a range of concentrations (5, 10, 20, 30, 40, and 50 ng/ml). Each medication solution was added in 50 L portions, vortexed for one minute, and then removed. Each sample was then combined with 150 L of ethyl acetate as the extracting solvent, then centrifuged for 5 min. at 2500 rpm. 75 litres of supernatant were subsequently collected. The sample was dehydrated and added to 1 cc of mobile phase after drying at 700 C. [20]

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Following the construction of the serum concentration time curve for capecitabine, the following parameters were calculated: Cmax, Tmax, AUC, elimination rate constant, elimination half-life, and clearance.

Cytotoxicity study by using HT 29 cell lines by MTT assay

Cell viability is evaluated using MTT as a function of redox potential. The water-soluble MTT is changed into an insoluble purple formazan by actively respiring cells. The formazan is then dissolved, and optical density is used to calculate its concentration. In viability, proliferation, and cytotoxicity tests, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a tetrazolium salt that is frequently used to identify reductive metabolism in cells. Chemical substrate MTT has a light yellow tint. Living cells only split it to create the dark blue formazon product. [21] This splitting process requires living mitochondria. The number of mitochondria split directly relates to the colorimetrically determined number of active cells. In order to achieve the desired concentration range, the MTT reagent was first dissolved in dimethyl sulfoxide (DMSO) and then appropriately diluted. For all test samples, the DMSO concentration was restricted to less than 0.1 ml. The HT 29 cell lines were kept in the appropriate conditions, seeded in 96-well plates, exposed to varying test sample concentrations, and incubated at 370°C, 5% CO2 for 96 hours. After 4 hours of incubation with the MTT reagent in the wells, the formazon generated by living cells was dissolved in DMSO in a safety cabinet and quantified at 550 nm. Calculated were the IC50 values and the percent inhibition. [22]

RESULTS AND DISCUSSIONS

FTIR investigation:

By conducting an FT-IR investigation as shown in Fig.1, the chemical congeniality between Capecitabine, PLGA, and Eudragit S100 was assessed. Sharp peaks were seen for Capecitabine at 1756.85 cm-1, 1332.96 cm-1, 1241.56 cm-1, and 1116.58 cm-1, which are nearly identical to those previously reported [23]. These sharp peaks indicate C=O stretching vibrations, C-N bending vibrations, N-H bending vibrations, and C-F stretching vibrations, respectively. The peaks of the Capecitabine-loaded nanoparticles are identical to those of the pure cap. When the peaks of nanoparticles and pure Capecitabine were compared, it was discovered that cap, PLGA, and Eudragit S100 were quite compatible with one another [24]. In Fig. 1, the FTIR spectra of nanoparticles loaded with Capecitabine, PLGA, Eudragit S100, and other drugs were displayed.

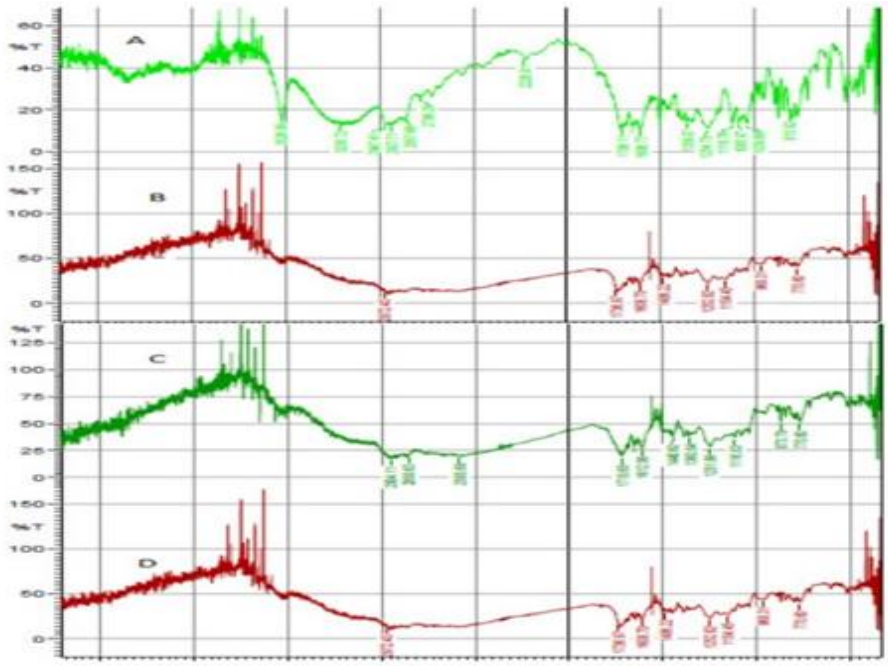

Fig 1: FTIR spectra of Capecitabine, PLGA, Eudragit S100, Capecitabine loaded with nanoparticle

DSC study:

A clear endothermic peak was detected in the DSC testing for capecitabine at 128.35^{0}C. As shown in Fig. 2, it had an on-set temperature of 122.45^{0}C and an end set temperature of 134.28^{0}C. For Capecitabine and its physical mixture with PLGA and Eudragit S100, a pronounced endothermic peak was detected at 127.68^{0}C, with onset and end set temperatures of 123.85^0 C and 133.72^0 C as shown in Fig.2. For Capecitabine and its physical mixture with PLGA and Eudragit S100, identical endothermic peaks were seen. As a result, it was discovered that capecitabine works well with PLGA and Eudragit S100. There was no peak in the DSC testing for the PLGA-based Capecitabine-loaded nanoparticles. The total entrapment of the medication in PLGA-based nanoparticles is the cause of the absence of endothermic peaks.

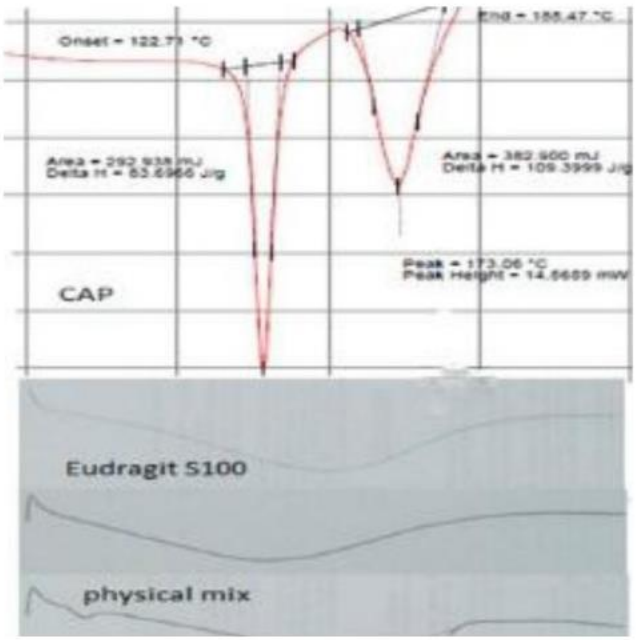

Fig. 2: DSC thermogram of Capecitabine. Eudragit S100, Physical mixture, Capecitabine loaded nanoparticles

TGA study:

Thermo Gravimetric Analysis (TGA) examines materials that exhibit mass loss or gain due to oxidation, breakdown, or volatile moisture loss. As shown in Fig. 3, the TGA analysis of Cap and PLGA in the current study revealed stable weight over a broad temperature range, showing thermal stability of the product.

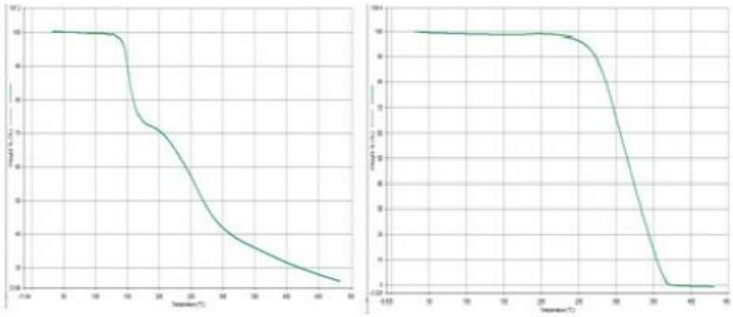

Fig. 3: TGA thermogram of PLGA and Capecitabine

X-RD Diffraction: No crystallinity was found on the nanoparticles in an X-RD analysis of Capecitabine with various polymers and nanoparticles. The patterns of Capecitabine's X-RD diffraction showed intensity (counts) at various 2 angles of 8.23, 9.46, 20.23, 30.40, and 31.52 and 33.56, indicating that the medicine is crystalline in nature. The optimised formulation's X-RD diffraction pattern revealed comparable counts, or 2 values, to that of the pure drug, demonstrating that the drug-loaded nanoparticles are also crystalline in structure and exhibit the same lattice arrangement as shown in Fig. 4. The relative intensities of their peaks may, however, vary slightly due to the differences in crystal sizes and their outward appearances.

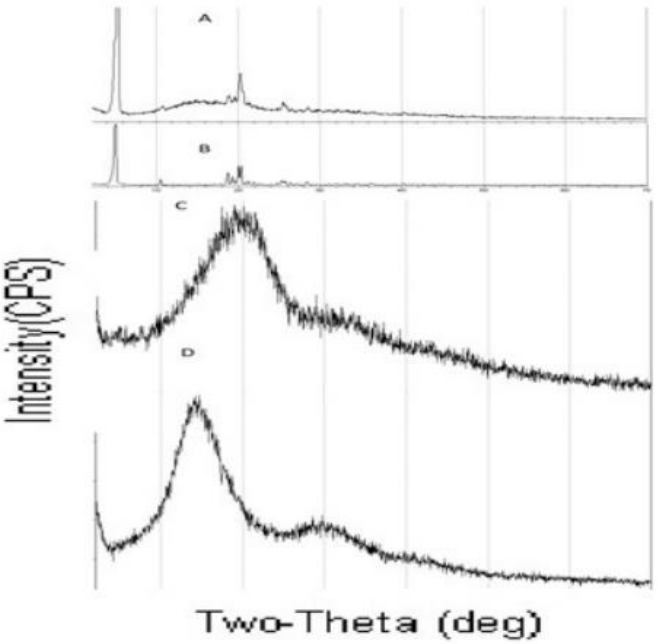

Fig. 4: XRD Spectra of Capecitabine, Nanoparticle, PLGA, Eudragit S100

Percentage Entrapment Efficiency (%EE):

According to Table 2, the formulations (CNF1-CNF4) had a drug entrapment efficiency of 52.4±1.23, 68.6±1.34, 69.5±1.42, and 72.5±1.15%, whereas formulations (CNF5-CNF8) had a drug entrapment efficiency of 62.8±1.20, 65.3±1.16, 69.6±1.18, and 72.8±1.24%. As the concentration of PLGA in the formulation increased, the entrapment efficiency increased steadily. The maximum entrapment was recorded by the formulations CNF4 and CNF8, with 72.5±1.15% and 72.8±1.24%, respectively.

Zeta potential:

Using the zeta sizer 5000 (Malvern Instrument Ltd., U.K.), photon correlation spectroscopy (PCS) was used to quantify the zeta potential, particle size, and polydispersity index (PDI). By using the

appropriate dilution, the average particle size and polydispersity index were measured three times for each formulation. The instrument's zeta potential and standard deviations were acquired directly.

As shown in Fig. 5, the average particle size of all formulations employing poloxamer 188 as a stabiliser ranged from 184.1 nm to 234.2 nm, while formulations utilising PVA as a stabiliser ranged from -8.35 nm to 295.2 nm.

All formulations employing poloxamer 188 as a stabiliser had zeta potentials between -7.90 ± 2.14 and -11.56 ± 3.15, while formulations utilising PVA as a stabiliser had zeta potentials between 286.1 ± 4.26 and -11.54 ± 3.45, as shown in Fig. 6. All formulations utilising poloxamer 188 as a stabiliser had PDI values between 0.273 ± 0.46 and 0.352 ± 0.38, while formulations using PVA as a stabiliser had values between 0.654 ± 0.72 and 0.956 ± 0.51. F3 and F8 exhibited the best values compared to other formulations when taking into account particle size, zeta potential, polydispersity index (PDI) values, and % entrapment efficiency.

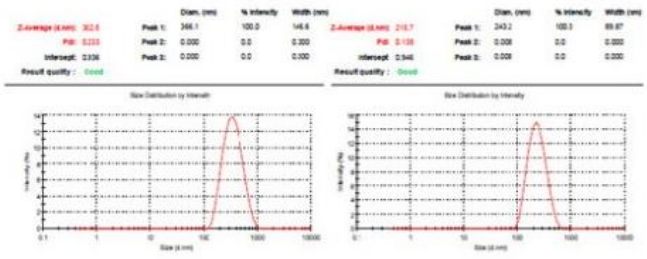

Fig. 5: Average particle size of nanoparticle of formulation CNF4 and CNF5

Table No. 2: Result showing Characterization of Capecitabine loaded PLGA based nanoparticles

Formulations	Particle Size	Polydispersity index (PDI)	Zeta Potential	% EE
CNF1	184.1± 6.34	0.324±0.42	-7.90 ± 2.14	52.4 ±1.23
CNF2	202.4± 5.57	0.352±0.38	-9.50 ± 3.46	68.6 ±1.34

CNF3	209.3± 7.62	0.273±0.46	-11.56 ± 3.15	69.5 ±1.42
CNF4	234.2± 5.39	0.285±0.65	-10.25 ± 3.37	72.5 ±1.15
CNF5	286.1± 4.26	0.921±0.28	-11.54 ± 3.45	62.8 ±1.20
CNF6	275.5± 5.71	0.764±0.69	-9.45 ± 4.25	65.3 ±1.16
CNF7	289.6± 6.65	0.956±0.51	-8.67 ± 3.35	69.6 ±1.18
CNF8	295.2± 5.48	0.654±0.72	-8.35± 3.20	72.8 ±1.24

All values are expressed in Mean ± SD, n = 3

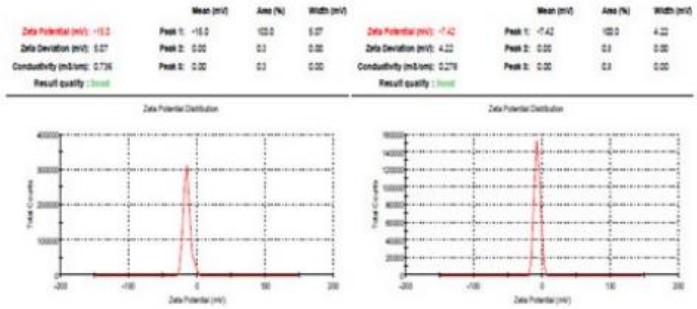

Fig. 6: Zeta Potential of nanoparticle of formulation CNF4 and CNF5

***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 h followed by a change of medium to phosphate buffer pH 6.8 for 4 h and subsequently for 24 h 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 [15]. This can be 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 investigation for the optimised formulation (CNF3) of nanoparticles revealed that they were discrete, with a mean particle size of roughly 100 nm (Fig. 7).

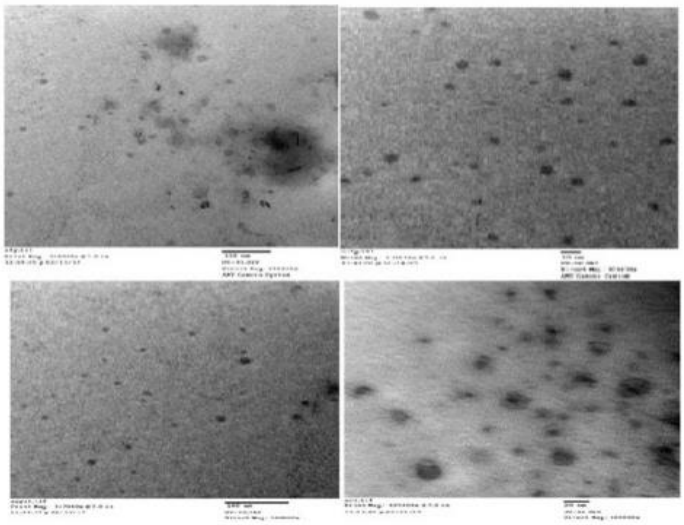

Fig. 7: TEM images of Nanoparticles of formulations CNF5

Cytotoxicity study:

The IC50 value of all formulations was found to be reduced to half than the pure drug solutions, indicating more effective than pure drug (Fig. 8), and a cytotoxicity study using HT 29 cell lines by MTT assay revealed that Capecitabine loaded nanoparticles were more cytotoxic than the pure drug solution.

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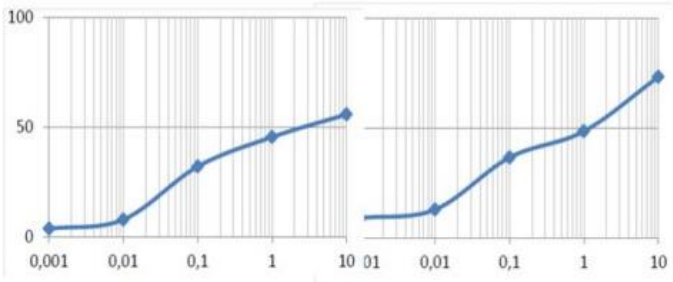

Fig. 8: Percentage inhibition of pure drug Capecitabine, Capecitabine loaded PLGA nanoparticle

Pharmacokinetic study:

For the analysis of Capecitabine, the UFLC (ultrafast liquid chromatographic) technique was used. This technology, which used the reverse phase, was incredibly quick and easy to use. Chromatography was carried out using a 25 cm x 4.6 mm (5 m) C18 column, a mobile phase of 55:45 (% v/v) acetonitrile: phosphate buffer pH 3, with PDA detection at 239 nm. The retention time of capecitabine during elution was 4.292 minutes. With a coefficient of correlation of 0.999, it was discovered that the approach is linear for Capecitabine concentrations between 5 and 50 ng/ml.

With acetonitrile and phosphate buffer pH 3 in the ratio of 55:45, retention period of 4.292 min, and column C-18, the serum sample from rabbits was examined using the HPLC method. The concentration range of 5 to 50 ng/ml was used to produce the standard curve of the pure medication in serum. The extraction solvent employed was ethyl acetate.

The outcomes of the in vitro investigation were validated by the pharmacokinetic study for the aqueous suspension of the pure medication Capecitabine and optimised formulation (CNF3). The Tmax for pure drug was found to be of 6.5 h as opposed to formulation 3 h as shown in Fig. 8, and the Cmax of

optimised formulation CNF3 was found to be greater as compared to pure drug, showing better absorption of medication from nanoparticles. The AUC for the formulation containing nanoparticles was discovered to be around two times higher than that for the pure medication, demonstrating improved bioavailability (Table 3).

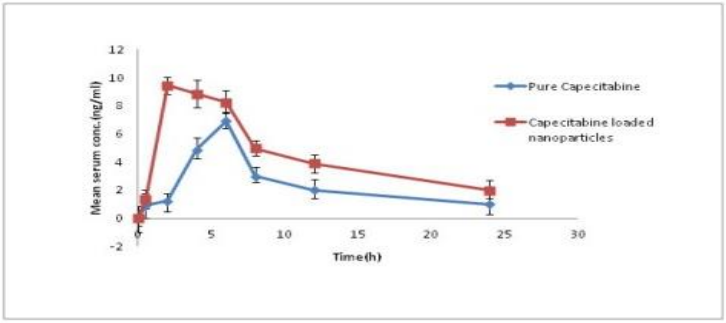

Fig. 9: Comparative plasma level study of Capecitabine and Capecitabine loaded nanoparticle

Table No.3: Pharmacokinetic parameters Capecitabine and Capecitabine loaded nanoparticles

Parameters	Capecitabine (n = 6)	Capecitabine loaded PLGA based nanoparticles (n = 6)
C_{max} (ng/mL)	6.4523	9.3948
T_{max} (h)	6.5	3
AUC $_{0-t}$ (ng-h/mL)	94.2854	182.5792
AUC $_{0-\infty}$ (ng-h/mL)	98.2854	199.5423
$t_{1/2}$(h)	9.25	24.75
Cl_T(L/h)	14.352	17.355
V_d(L)	4.246	7.578

CONCLUSION: To effectively treat colorectal cancer, a sustained-release formulation of capecitabine-loaded PLGA in conjunction with Eudragit S100 can be created using the

nanoprecipitation process. It was discovered that the cytotoxic effects of Capecitabine-loaded nanoparticles were more lethal to tumour cells than those of pure Capecitabine. Inferring that it would have less side effects, our created nano formulations would be extremely toxic to tumour cells while being less hazardous to normal cells.

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