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Enhanced Activity of Camptothecin Hydrogel by Using HP-β-Cyclodextrin

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Abstract : The use of a novel injectable biocompatible and biodegradable camptothecin formulation for controlled intra-tumoral release of camptothecin is described. The drug delivery vehicle is an *in-situ* pH gelling formulation, which is based on the natural biopolymer chitosan. The pH sensitive hydrogel based on chitosan/Glyceryl monooleate (GMO)/HP-β-Cyclodextrin was prepared by Crosslinking methods. The formulations were characterized by Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), gelation time and viscosities (Brookfield DV-II + Pro viscometer) were investigated for controlled release hydrogel formulation. The hydrogel formulation of camptothecin (CPT) showed good release profile with polymer (chitosan/GMO/HP-β-CD) compare to without polymer. The optimized formulation PF18 having CPT 0.5%, chitosan 3% w/v, GMO 3% w/v and HP-β-CD 1.5% w/v, showed best release compared to other formulation. These formulations showed good properties in terms of pH, gelation, viscosity and in-vitro release. The formulation, containing homogeneously dispersed camptothecin, was studied on tumor cell MCF-7. The effectiveness of treatment was measured in terms of percentage control tumor growth inhibition (TGI). The tumor cell treated with the polymer containing CPT showed 17.3% TGI which is significantly more compared to CPT which showed 10.2% without polymer. The results indicate that this novel biodegradable polymer hydrogel is an effective for the controlled intra-tumoral delivery of CPT. Hydrogel is a promising safe and more effective delivery system that can be developed to serve as an alternative to currently used system for anticancer drug delivery.
Keywords: Chitosan; Hydrogel; HP-β-Cyclodextrin; MCF-7; Camptothecin.

INTRODUCTION:

Camptothecin (CPT), a plant alkaloid isolated from an oriental tree, *Camptotheca acuminata*, was first identified in the 1950s [1]. CPT has shown significant antitumor activity against various cancers, including lung, ovarian, breast, pancreas and stomach, by inhibiting the activity of DNA topoisomerase-I, which is required for replication and transcription of the cell cycle [2-4]. DNA topoisomerase-I is believed to stabilize the DNA topoisomerase complex, and this complex causes the apoptosis of cancer cells [5-7]. CPT has low aqueous solubility in its therapeutically active lactone form. Once placed in an aqueous solution at physiological pH, the lactone form of CPT is quickly transformed to its carboxylate form, which is highly toxic and therapeutically inactive [8-10]. These pharmacological properties of CPT result in rapid deactivation and fast clearance of CPT

from the circulation after it is intravenously administered. To overcome these drawbacks, CPT has been conjugated to various polymeric carriers for improved solubility, enhanced stability of its lactone form and reduced renal clearance[11-14].

Hydroxy propyl-β-cyclodextrin (HP-β-CD) is a hydroxyl alkylated-β-CD derivative that combines relatively high water solubility with low toxicity and satisfactory inclusion ability. Several commercial formulations are composed of cyclodextrin inclusion complexes, illustrating the usefulness of this approach [15]. Pharmacokinetic studies of CPT formulations in rats indicated that the complex had higher bioavailability and ratio of active lactone form in plasma compared to free CPT, which suggested that the complex may exhibit better therapeutic efficacy [16].

Chitosan is soluble, mucoadhesive and active as an absorption enhancer in its protonated form because the pKa of the amine groups of chitosan is 6.2, chitosan at neutral pH hardly carries a charge, has a low solubility and is therefore essentially inactive. Because of the presence of functional groups (amine and hydroxyl) various chemical chitosan derivatives have been synthesized and studied for different applications. Thiolated chitosans, obtained by modification of the primary amine groups with cysteine, thioglycolic acid and 2-iminothiolane, are a class of derivatives that showed improved mucoadhesive properties and have been applied in mucoadhesive oral and nasal drug delivery systems. These thiolated chitosans have shown in situ gelling properties due to the formation of inter- and intramolecular disulfide bonds at physiological pH [17].

Controlled drug-delivery systems are designed to deliver the drugs at desirable times and/or specific sites to achieve the therapeutic objective [18, 19]. Hydrogels are hydrophilic polymer networks that may retain a large amount of water and exhibit a semi-solid morphology. The hydrophilic three-dimension net-work formed by chemical or physical crosslinking can be considered as an ideal candidate for the controlled drug release matrix [20].

During the last decade, injectable *in situ* gel-forming systems have received increased interest in drug delivery and tissue engineering. These devices can overcome many of the problems associated with polymers or microspheres in that they are both injectable and produce solid biodegradable implants with a range of mechanical characteristics in terms of rigidity and load bearing making them compatible with both soft and hard tissues. In the present work, we have used a chitosan polymer to formulate a biodegradable and biocompatible formulation for controlled delivery of camptothecin in a slow-release manner directly into a tumor cell. In this paper, we report the *in vitro* release characteristics of the camptothecin polymer hydrogel and the *in vitro* effect of delivering camptothecin in to a MFC7 tumor cell. The delivery vehicle used is pH sensitive chitosan solutions. The polymeric matrix used in this study consists of chitosan polymer and GMO. Addition of GMO to chitosan solution produces a hydrogel which undergoes sol to gel transition at a pH 7.4, making the formulation a suitable vehicle for drug administration since the hydrogel when implanted into the body, flows to fill voids or cavities and becomes solid at body pH. These hydrogels are suitable carriers for water-insoluble drugs and they are non-toxic and highly biocompatible. Chitosan is an important natural polymer widely used for medical and pharmaceutical applications.

MATERIALS AND METHODS:

Chemical and Reagents

Chitosan (Deacetylation degree DDA = 80%), HP-β-Cyclodextrin (HP-β-CD) were obtained from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Camptothecin (CPT) obtained from Coral Drugs, New Delhi and Glyceryl monooleate (GMO) was obtained from Estelle Chemicals Pvt. Ltd., Ahmednagar, India. Demineralized and double distilled water was used. All chemicals and reagents used were of analytical grade.

Preparation of pH sensitive an autogelling chitosan solution

Preparation of an autogelling chitosan/ GMO solution

Chitosan/GMO solutions were prepared in 0.33 M citric acid. The solutions which consisted of 3% w/v chitosan and 3% & 4% w/v GMO in 0.33 M citric acid. The mixture was stirred for a further 3 h. sterile formulations were obtained by autoclaving (121^0C, 20 min).

Preparation of chitosan/ GMO/HP-β-CD solution

Homogeneous clear chitosan/GMO solutions were prepared then added HP-β-CD at room temperature by homogenously dispersing the powered cyclodextrin in chitosan solution under aseptic condition.

Preparation of chitosan/GMO/HP-β-CD loaded with camptothecin

Chitosan/GMO/HP-β-CD/CPT formulations were prepared at room temperature by homogeneously dispersing the powdered camptothecin in chitosan solutions under aseptic conditions. The final formulations were prepared according to Table 1.

Table 1: Formulation table for hydrogel

Formulation code	CPT (% w/v)	Chitosan (%w/v)	GMO (%w/v)	HP- β -CD (%w/v)	Citric acid (M)
PF	0.5	3.0	3.0	-	0.33
PF16	0.5	3.0	3.0	0.5	0.33
PF17	0.5	3.0	3.0	1.0	0.33
PF18	0.5	3.0	3.0	1.5	0.33
PF22	0.5	3.0	4.0	0.5	0.33
PF23	0.5	3.0	4.0	1.0	0.33
PF24	0.5	3.0	4.0	1.5	0.33

Physicochemical characterization

Detection of CPT by HPLC

Quantitative analysis was performed on a Shimadzu LC 2010C HT HPLC chromatographic system equipped with an Auto sampler, a solvent module, Detector and a System HP ChemStations system. The column was a reverse-phase RP18 column. The HPLC system was eluted isocratically with methanol: water (63:37; v/v) at room temperature. The flow rate of the mobile phase was 1.0 ml/min and samples were measured at a wavelength of 370 nm. A standard curve was constructed by plotting peak area against concentration. The assay was found to be 98.20 %.

Fourier Transform Infrared spectroscopy (FTIR)

It was performed, using a Perkin Elmer Spectrum Two spectrophotometer, to understand if there exists some interaction between drug and exipients. The spectra were obtained in the region from 4000 $cm^{-1}$to 650 cm^{-1}

X-ray Diffractometry (XRD)

The crystal X-ray scattering measurements for the obtained sample of Camptothecin and its formulation were performed to determine the solid structure of Drug. XRD Patterns were obtained with a Seifert Germany ISO debyeflex 2002 apparatus (Japan) using Cu-K α radiation (λ = 1.541841A*), a voltage of 40 kV and a 100 mA current. Samples were scanned from 0–60^0 2θ for qualitative studies and the scanning rate was 4^0/ min.

In-vitro Gelation and Viscosity Studies

The two main prerequisites of an *in situ* gelling system are viscosity and gelling capacity (speed and extent of gelation). The formulation should have an optimum viscosity that will allow easy injectable into the body as a liquid (drops), which would undergo a rapid sol-to-gel transition. Additionally, to facilitate sustained release of drug to the tumoral tissue, the gel formed *in situ* should preserve its integrity without dissolving or eroding for a prolonged period of time. Viscosity of injected formulation is an important factor in determining residence time of drug in the injected area. The developed formulations were poured into the small sample adaptor of the Brookfield DV-II + Pro viscometer, RV spindle 6 and the angular velocity increased gradually from 0.5 to 50 rpm. The hierarchy of the angular velocity was reversed. The average of the three readings was used to calculate the viscosity.

Standard calibration curve of Camptothecin

Accurately weighed 10 mg Camptothecin was dissolved in 100 ml of Phosphate buffer pH 7.4 to get the stock solution of 100µg/ml. From this stock solution aliquots of 1 ml was withdrawn and get the stock solution of 10 µg/ml. from this stock solution aliquots of 1, 2, 3, 4, 5,6 & 7 ml were withdrawn and further diluted up to 10 ml with buffer to obtain a concentrations range of 1, 2, 3, 4, 5, 6 & 7 µg/ml. The absorbance of the solutions was measured at 285 nm by using U V spectrophotometer.

In vitro release of CPT from Hydrogel formulations

The release profile of a drug predicts how a delivery system might function and gives valuable insight into its *in vivo* behavior. All the pH sensitive *in situ* gelling formulations of CPT were subjected to *in vitro* release studies. These *in vitro* release studies were carried out using potassium phosphate buffer of pH 7.4 as the dissolution medium. Approx 1.2 inch length of the dialysis tube was taken and then soaked overnight in the phosphate buffer 7.4 pH. Now the amount of CPT equivalent to 10 mg of drug was calculated and placed in the dialysis tube whose ends were tied with a thread to prevent leakage. The dialysis tube bags were then placed in 100 ml of phosphate buffer 7.4 pH placed in the shaking water bath and maintained at 37^0C with a frequency of 50 shakings per minute. Aliquots of 2 ml were withdrawn and filtered and sink condition maintained using phosphate buffer. The filtrate obtained was then suitably diluted 10 times (1 ml filtrate up to 10 ml) and the absorbance taken after scanning. The experiment was carried out in triplicate.

In vitro Cytotoxicity study of CPT formulations

The *in vitro* cytotoxicity of the CPT formulations was performed on the human breast cancer cell line MCF-7. The concentration of drug was 10 µg/ml used for in vitro studied. Sensitivity of MCF-7 cells to formulations was determined individually by the MTT colorimetric assay. Cells were seeded in a flat-bottomed 96-well plate and incubated for 24 h at 37°C and in 5% CO_2. The cell line was exposed to all formulations mentioned above. The solvent DMSO treated cells served as control. Cells were then treated with MTT reagent (20µl/well) for 4 h at 37°C and then DMSO (200µl) was added to each well to dissolve the formazan crystals. The optical density was recorded at 492 nm in a microplate reader (21). Percentage of residual cell viability was determined as (1-(OD of treated cells/OD of control cells)) x100.

RESULTS AND DISCUSSION:

X-ray Diffractometry (XRD)

The XRD pattern for the pure CPT is shown in Figure 1a and XRD pattern for physical mixture of CPT shown in figure 1b. In the X-ray diffraction spectrum, CPT exhibited several strong characteristic

crystalline peaks between $2\theta = 23.324^0$ to 29.45^0 confirming the highly crystalline nature of drug. The XRD spectrum of physical mixture of CPT exhibited several strong characteristic crystalline peaks at $2\theta = 21.15^0$, 21.7^0, 24^0, 24.75^0, 26^0 and 27.4^0, confirming that the drug was present as a crystalline material.

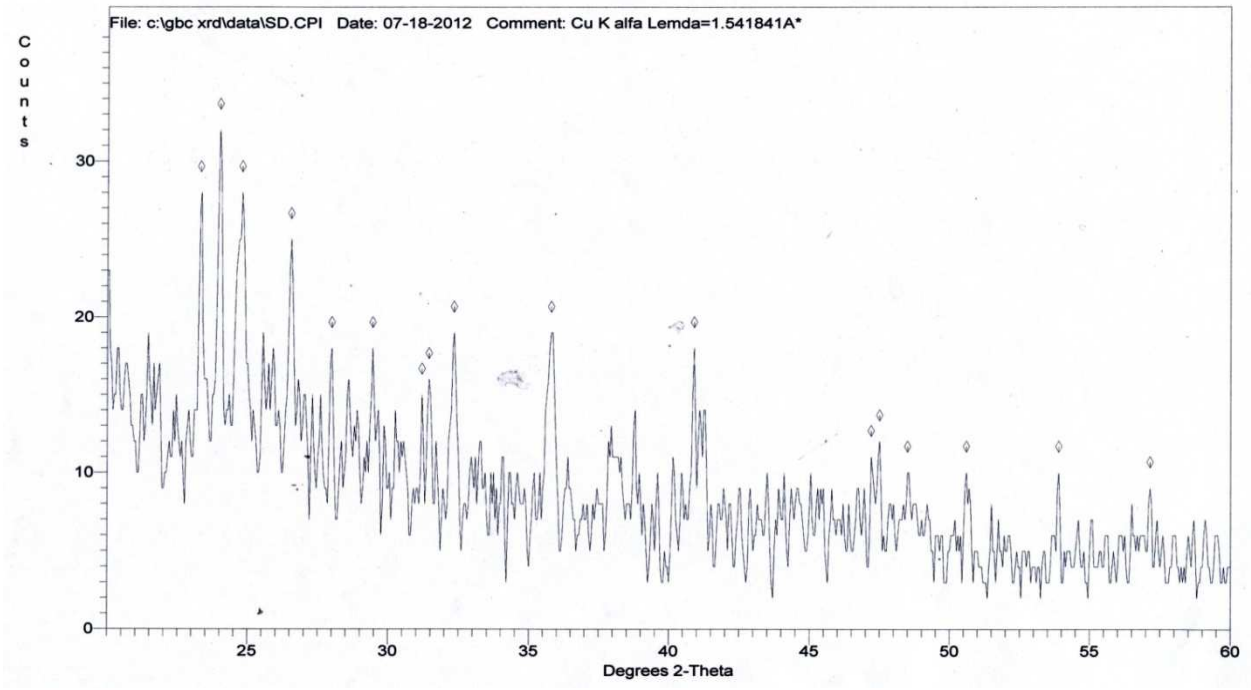

Figure 1a: XRD pattern of pure CPT powder

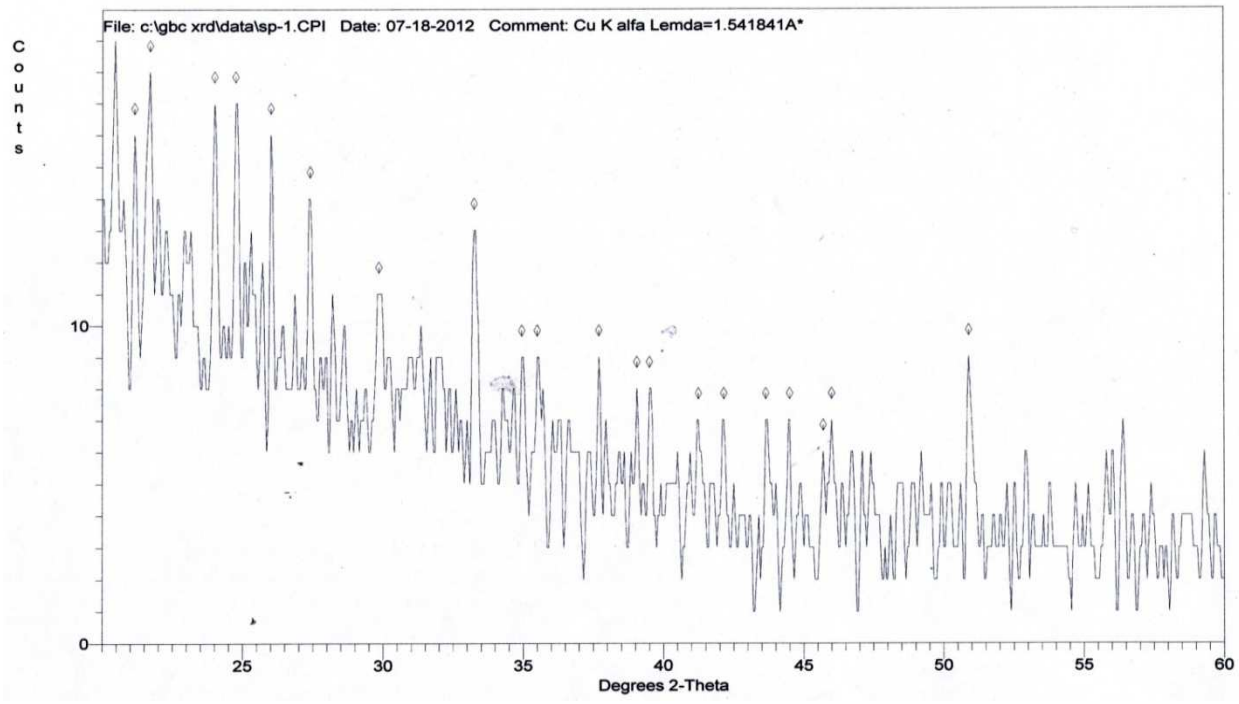

Figure 1b: XRD pattern of physical mixture of CPT, Chitosan, GMO and HP- β -CD

Fourier Transform Infrared spectroscopy (FTIR) studies

The FTIR spectra of drug CPT, Chitosan, GMO, HP-β-CD and Physical Mixture of CPT are shown in Figure 2. The FTIR studies showed that there no interactions between CPT and Exipients. The main characteristic peaks of CPT are at around 1750, 1460-1600, 1270-1290 cm^{-1}. We can see from the FTIR spectra between mixture of CPT, Chitosan, GMO and HP-β-CD that no significant differences were shown.

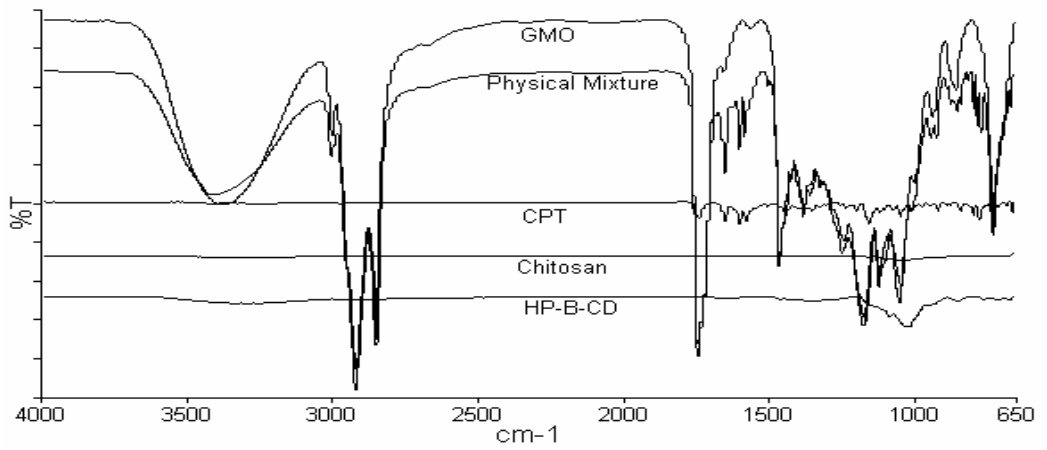

Figure 2: FTIR Spectra of drug & excipients

In-vitro Gelation and Viscosity Studies

The gelation property and the viscosity of the formulated hydrogel are shown in table 2. The gelation pH of the hydrogel formulation was in range of pH 6.5 to 7.5 and the viscosity of the hydrogels was in range of 3413.3 cP to 7298.6 cP at 20 rpm and 25^{0}C temperature which is effective for the syringeable of the formulation.

Table 2: Gelation time & viscosity of formulation

Formulation code	pH	Gelation	Viscosity (cP) (20 rpm, 25^{0}C)
PF	6.58	++	3413.3±12.39
PF16	7.38	+++	5642.1±15.63
PF17	6.50	+++	6062.8±18.99
PF18	7.32	+++	6933.3±17.81
PF22	7.26	++++	5516.3±23.33
PF23	6.92	++++	6831.2±21.20
PF24	7.50	++++	7298.6±26.73

The gelation and viscosity data is (Mean ± SD, n=3) for formulation.

In vitro release of CPT from Hydrogel formulations

Chitosan/GMO/HP-β-CD was loaded with camptothecin 0.5% (w/v) and triplicate samples of polymer hydrogels were incubated in phosphate-buffered saline solutions pH 7.4, 37^{0}C. At intervals, the supernatant fractions were removed and the medium replenished to maintain the sink conditions. The amount of drug in the supernatant samples was quantified by UV spectrophotometer and the cumulative percentage of the loaded drug released in the supernatant fractions was studied versus time. The amount of drug loaded initially in the polymer was confirmed by extraction of the polymer with methanol to release the residual camptothecin.

The graphical representation between percentage cumulative releases of camptothecin versus time is shown in Figure 3. The formulation code PF, PF16, PF17, PF18, PF22, PF23 and PF24 were released 48.48%, 79.74%, 96.41%, 98.46%, 89.87%, 92.56% and 66.92 % respectively of the drug after 10 hrs. The percentage cumulative release of formulation code PF18 showed maximum release of drug and the formulation PF 24 showed minimum percentage cumulative release compare to all formulation having cyclodextrin. The formulation PF which was without cyclodextrin showed less percentage cumulative release and approximately constant at 9.5 and 10.00 hrs than the formulations having cyclodextrin

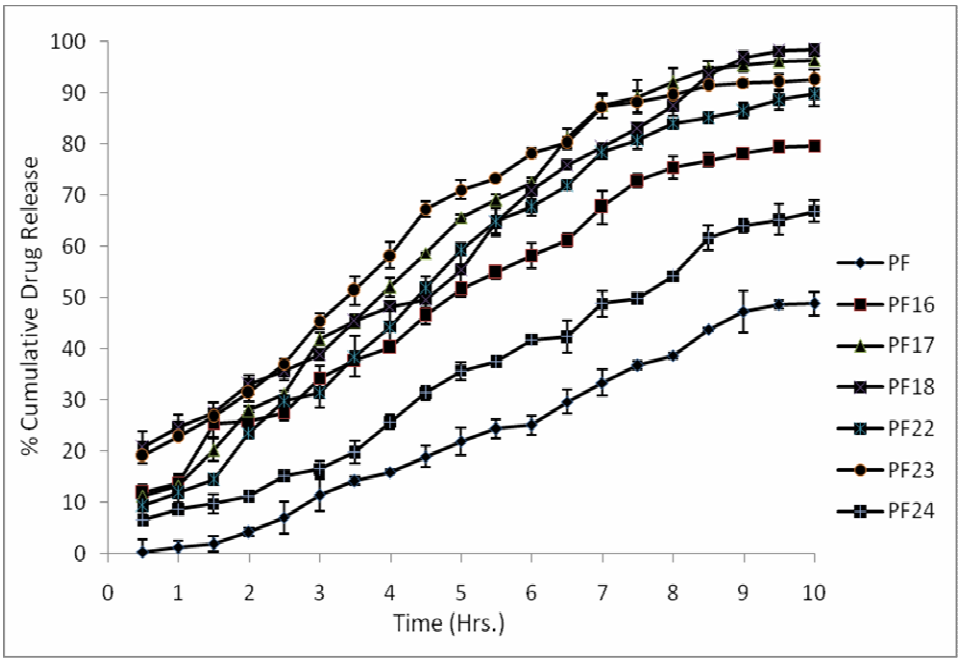

Figure 3: In-vitro drug release profile of the designed formulation.
Values are expressed as (Mean ± SD, n=3) formulations.

The models fitting for the release profile of formulations by using various models shown in table 3. The transport mechanism of formulation PF was found to be super case II transport and the best fit model was zero order. The transport mechanism of formulation PF16, PF17, PF22 and PF23 was found to be Non-Fickian diffusion and the best fit model was Hixson-Crowell. The transport mechanism of formulation PF18 was found to be Fickian diffusion and the best fit model was Zero order. The transport mechanism of formulation PF24 was found to be Non-Fickian diffusion and the best fit model was Zero order.

Table 3: Model fitting for the release profile of formulations by using 5 different models

Code	Zero Order	First Order	Higuchi Matrix	Hixson-Crowell	Korsmeyer-Peppas		Best Fit Model	Transport Mechanism
	R^2	R^2	R^2	R^2	n	R^2		
PF	0.992	0.976	0.946	0.983	1.662	0.985	Zero Order	Super case II transport
PF16	0.981	0.978	0.979	0.986	0.668	0.966	Hixson-Crowell	Non-Fickian
PF17	0.966	0.951	0.981	0.983	0.806	0.963	Hixson-Crowell	Non-Fickian
PF18	0.990	0.837	0.957	0.932	0.437	0.960	Zero Order	Fickian diffusion
PF22	0.970	0.981	0.978	0.990	0.849	0.954	Hixson-Crowell	Non-Fickian
PF23	0.940	0.981	0.972	0.982	0.545	0.930	Hixson-Crowell	Non-Fickian
PF24	0.989	0.965	0.944	0.977	0.854	0.941	Zero Order	Non-Fickian

Statistical Analysis

All observations were presented as Mean ± SD (standard deviation). The data was analyzed by student's t-test. $P < 0.05$ was considered as significant.

In vitro Cytotoxicity study of CPT formulations

To evaluate its antitumor efficacy camptothecin formulated in chitosan/GMO/HP-β-CD was intratumorally using a MCF-7 breast tumor cell model. The MCF-7 tumor has proven to be a useful model for preliminary screening of various compounds for efficacy because of its reproducible growth, non-immunogenicity in the syngeneic host and low frequency of spontaneous metastases. The effect of the camptothecin containing biodegradable polymer on tumor percentage growth inhibition was examined. The results of these studies are shown in Figure 4. The hydrogels containing camptothecin was found to be more effective than without hydrogel delivered camptothecin. The formulation PF18 was found to be more effective compare to the other formulations. Tumors injected with blank chitosan/GMO/HP-β-CD showed no inhibition of growth as untreated tumors, it confirming that the hydrogel alone has no effect on the growth of this tumor.

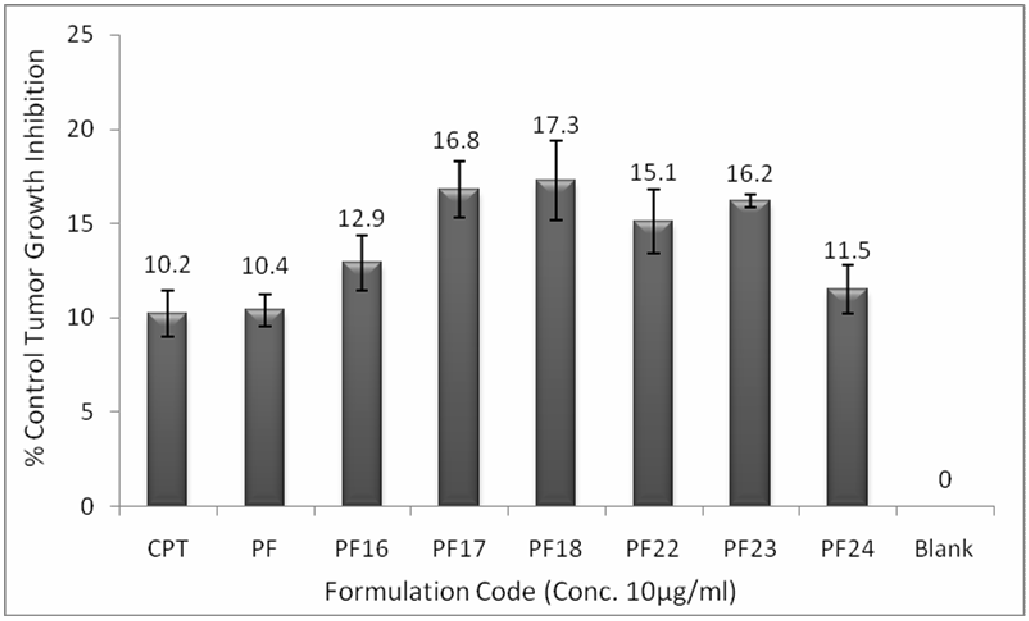

Figure 4: In-vitro study on MCF-7 Tumor Cell line, % control growth inhibition data (Mean ± SD, n=3) for pH sensitive hydrogel formulations. *$P < 0.05$ was considered as significant

The greater effectiveness of the hydrogel formulation code PF18 is due to maximum release of the drug in the tumor and the exposure of tumor cells to drug concentrations for a period of time this causes more cell death. The formulation CPT, PF, PF16, PF17, PF18, PF22, PF23 and PF24 were showed the percentage control tumor growth inhibition 10.2%, 10.4%, 12.9%, 16.8%, 17.3%, 15.1%, 16.2% and 11.5 % respectively.

We selected camptothecin as a model drug for this study, because its insolubility in water makes it difficult to administer systemically by other means and because of the potential applications of camptothecin and the insoluble camptothecin analogues in chemotherapy. Additionally, the pharmacologically important lactone ring of camptothecin and its analogs is unstable in the presence of human serum albumin which results in the conversion of the active drug to the inactive carboxylate form bound to albumin [22]. This imposes a severe pharmacokinetic limitation on the systemic use of camptothecin and related compounds. An approach to overcoming this and other shortcomings of camptothecin and its analogs, especially their high systemic toxicity is to load it into a delivery system such as a chitosan based formulations which will protect the drug from hydrolysis and control its release over a prolonged period. In this study estimation of % tumor growth inhibition of CPT or chitosan/GMO/HP-β-CD/CPT was based on changes in tumor cell line with hydrogel formulation.

In the present study the formulated hydrogel was injected into the tumor cell. In the case of the MCF-7 tumor CPT without exipients appears to have less tumoricidal effect. CPT with Chitosan/GMO/HP-β-CD has been shown to activate macrophages for tumoricidal activity in MCF-7. Again, since we found the difference in the % tumor growth inhibition between the CPT and with chitosan/GMO/β-CD/CPT. The effectiveness of the polymer hydrogel in delaying tumor growth clearly demonstrates the importance of this delivery system in maintaining an inhibitory level of drug over a long period of time. The main advantages of the biodegradable polymer implant such as chitosan/GMO/HP-β-CD used for the delivery of camptothecin to the tumor cell are the high intra-tumoral concentrations of drug attainable, low systemic toxicity and the extended period of time over which the drug can be released in the tumor. The dose of camptothecin delivered using the hydrogel was 10μg/ml, which is 3 times the mean dose for MCF-7 cell, for the hydrogel the delayed release of the drug and localization in the tumor prevents toxic systemic levels being reached.

CONCLUSION:

Local deliveries of chemotherapeutic agent by controlled release polymers are a new strategy with the potential to maximize the anti-tumor effect of a drug and reduce systemic toxicity. In this study, we have demonstrated the effectiveness of using the biodegradable chitosan polymer to deliver high doses of camptothecin locally to a tumor cell model. Growth of tumors treated in this fashion was retarded for significantly longer periods than were tumors treated with systemically administered camptothecin.

The system formulated with camptothecin was found to be stable and the release profiles of a formulation with chitosan, GMO and HP-β-CD showed all most effective release kinetics. These findings show chitosan/GMO/HP-β-CD hydrogel to be a safe, effective, homogeneous, injectable and stable formulation for delivery of camptothecin and this approach represents an attractive technology platform for the delivery of other clinically important hydrophobic drugs. The mechanism of gelation, which does not involve covalent cross-linkers, organic solvent or detergents, combined with a controllable residence time, renders this injectable biomaterial uniquely compatible with sensitive chemotherapeutic agents. Drug release of CPT from the hydrogel was found to be too rapid due to the hydrophilic nature of the drug and the small size of the molecules compared to that of the pore size in the hydrogel.

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