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In-vitro Characterization and Cytotoxicity Analysis of 5-Fluorouracil loaded Chitosan Microspheres for Targeting Colon Cancer

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Abstract

The objectives of the present investigation were to prepare the 5-Fluorouracil (5-FU) loaded chitosan microspheres for colon targeting and its in vitro cytotoxicity analysis on HT-29 human colon cancer cell lines. Chitosan microspheres prepared by the emulsion polymerization method were analyzed for morphology, mean particle size, drug polymer interaction, entrapment efficiency, in vitro drug release and cytotoxicity on HT-29 colon cancer cell lines. The mean particle size of unloaded microspheres underwent significant change with increase in concentration of chitosan solution. The stirring speed had a significant effect only at the lower level (i.e. 1000 to 4000 rpm). Entrapment efficiency increased with increase in drug concentration. The formation of chitosan microspheres was heeled by the use of differential stirring. With an increase in the concentration of water-soluble drug, there was an increase in entrapment efficiency and drug load over a large concentration range. Cytotoxicity study indicated that 5-FU loaded chitosan microspheres prolonged the cytotoxic effect on HT-29 colon cancer cell lines in comparison to free 5-FU

Keywords: *Chitosan microspheres, 5- Fluorouracil, Cytotoxicity*.

INTRODUCTION

Chitosan microspheres have been widely accepted for drug delivery, fabrication of biosensors as well as delivery of both hydrophilic and lipophillic drugs [1-3]. Chitosan, a polysaccharide comprising copolymers of glucosamine and N-acetyl glucosamine, being biodegradable and biocompatiable, is widely used in the formulation of particulate drug delivery systems to achieve controlled drug delivery [4]. Chitosan microspheres are prepared by chemical denaturation, of chitosan present in the inner phase of water/oil (w/o) emulsion. Denaturation is usually carried out using glutaraldehyde with continuous stirring. Various process parameters affecting characteristics of chitosan microspheres have been identified, along with their significance[5]. It has been reported that irrespective of molecular weight, chitosan microspheres are formed only at the minimum concentration of the chitosan solution at 1% w/v. However, no reason was offered for this observation. This finding has led the workers in this filed to restrict the minimum level of chitosan concentration to 1% w/v. In addition to concentration of chitosan solution, several other process parameters have been identified and optimized [6]. However, no attempt has been made to study the effect of the physical properties of the drug on the attributes of microspheres. The degree of stirring (ie, time and speed of stirring during emulsification) determines the size of droplets, which can be changed to obtain the product (i.e. chitosan microspheres) in the desired size range. However, no further division of quasi-solid or solid particles, formed during the process of cross-linking, are desired in order to protect the structural integrity of the microspheres [7]. Based on this hypothesis, it was planned to carry out stirring at a higher rate of agitation during initial emulsification followed by lower rate during the cross – linking stage. The present study was carried out with two objectives. The first objective was to change the method of preparation based on the above hypothesis to see whether microspheres could be obtained by the modified method using a chitosan solution of lower concentration.

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The second objective was planned to compare the basic characteristics of the prepared micro spheres with the microspheres obtained with a higher concentration of chitosan solution. The study was conducted in two different stages. In the first stage, optimization of the concentration of chitosan solution was carried out. In the second phase, in vitro cytotoxicity analysis of 5-FU solution and 5-FU loaded chitosan microspheres was carried out on HT-29 human colon cancer cell lines.

MATERIALS AND METHODS

Chitosan (85% deacetylated) was purchased from Sigma-Aldrich, USA. 5-FU was obtained as a gift sample from Shalaks Pharmaceutical Private Limited, New Delhi, India. Light paraffin oil and hard paraffin oil were purchased from Merck Chemicals, Mumbai, India. Glutaraldehyde, 25% in water, was purchased from S.D Fine chemicals, Mumbai, India.

Analytical estimation of 5-Fluorouracil

The estimation of 5-Fluorouracil was done by UV-Visible Spectrophotometric method. Aqueous solution of 5-Fluorouracil was prepared in distilled water and the absorbance was measured at 266 nm spectrophotometrically from 2.5 to 20 g mL^{-1} concentration (R^2=0.994).

Preparation of 5-Fluorouracil loaded chitosan microsphers

5-FU loaded chitosan microspheres were prepared by chemical crosslinking method.[8] 75 ml of light liquid paraffin and 75 ml of hard liquid paraffin oil were placed in a 250-ml plastic beaker. 1% w/w of span 80 was mixed with the oil with stirring and heated up to 80 ^{0}C. To this, 10 mL of chitosan solution of different concentration (prepared by dissolving chitosan in 2% v/v glacial acetic acid) was added drop wise using a 22-gauge hypodermic syringe. This addition was accompanied by stirring of paraffin oil at different speeds (1000 to 4000 rpm) with the help of a high – speed stirrer with propellers (Remi Motors, India). Stirring was continued for 1 h after the complete addition of chitosan solution into oil. After 1 h stirring, 10 mL of glutaraldehyde solution saturated with 30 ml of toluene was added dropwise to the mixture with continuous stirring at 500 rpm for next 1 h at the temperature 50-55 ^{0}C. Stirring was stopped after 1 hour of the final addition of glutaraldehyde. Suspension of chitosan microspheres in paraffin oil thus obtained was allowed to stand for 1 hour to settledown the microspheres under gravity. Clear supernatant was decanted and microspheres were washed three times with hexane. After the final wash, microspheres were allowed to dry in air and stored in desiccators at room temperature.

Determination of Mean Particle Size and its Particle Size Distribution

Particle size analysis of unloaded and drug-loaded chitosan microspheres was performed by optical microscopy using a compound microscope. A small amount of dry microspheres was suspended in purified waster. The suspension was sonicated for 5 seconds. A small drop of suspension, thus obtained, was placed on a clean glass slide. The slide containing chitosan microspheres was mounted on the stage of the microscope and diameter of at least 500 particles was measured using a calibrated ocular micrometer.

Morphological Study of Microspheres

The shape and surface morphology of the microspheres was investigated using scanning electron microscopy (SEM; Jeol, JSM – 6100). The microspheres were fixed on supports with carbon-glue, and coated with gold using a gold sputter module (JFC-1100) in a high vacuum evaporator. Samples were observed by SEM at 15kV.

Determination of Percent Drug Entrapment

5-Fluorouracil loaded chitosan microspheres (200mg) were digested in 50 ml of distilled water. The suspension was then warmed for few min, filtered with 0.2m membrane filter (MDI, India) and an aliquot of the filtrate was diluted appropriately with respective solvent system. Absorbance was measured at 266 nm and the concentration was calculated according to the standard regression.

Fourier transform infrared spectroscopy (FTIR)

Drug polymer interactions were studied by FTIR spectroscopy. The spectrum was recorded for 5-fluorouracil, blank chitosan microspheres, physical mixture of blank chitosan microspheres and 5-fluorouracil and 5-fluorouracil loaded chitosan microspheres using Spectrum BX (Perkin Elmer) infrared spectrophotometer. Samples were prepared in KBr disk (2 mg sample in 200 mg KBr) with a hydrostatic press at a force of 40psi for 4 min. The scanning range was 400-4400cm^{-1} and the resolution was 4 cm^{-1}.

Differential scanning calorimetry (DSC)

The thermal behavior of 5-fluorouracil, blank chitosan microspheres, physical mixture and 5-fluorouracil loaded chitosan microspheres was examined with a DSC 7 (Perkin-Elmer) Thermal analyzer. Argon was used as carrier gas and the DSC analysis was carried out at a heating rate of 10 ^{0}C/min and an argon flow rate of 35cc/min. The sample size was 5 mg and curves were recorded at a temperature range of 60-300 ^{0}C.

Powder X-ray diffraction analysis (PXRD)

PXRD was carried out to investigate the effect of microencapsulation process on crystallinity of the drug. PXRD patterns were recorded on a RIGAKU, Rotaflex , RV 200 (Rigaku Corporation, Japan) powder XRD using Ni-filtered, CuKradiation, a voltage of 60 kV, and a current of 50 mA. The scanning rate employed was 1^0 /min over the 10^0 to 40^0 diffraction angle (2) range. The XRD patterns of 5-fluorouracil crystals, blank chitosan microspheres and 5-fluorouracil loaded chitosan microspheres were recorded. Microspheres were triturated to get fine powder before taking the scan.

In vitro release of 5-FU from chitosan microspheres

The *in vitro* drug release studies were performed using USP dissolution rate rest apparatus (paddle apparatus, 100 rpm, 37 0.1C).[9] Chitosan microspheres bearing 5-fluorouracil was suspended in simulated gastric fluid (SGF, pH 1.2, 900 ml) for 2 h. The dissolution media was then replaced with simulated intestinal fluid (SIF) pH 7.5 and the releases study was carried out for a further 3 h, which corresponds to the average small intestinal transit time. Aliquots of the dissolution medium were withdrawn at the pre-determined time interval and the amount of drug was quantified at 266 nm.

In vitro release of 5-FU from chitosan microspheres in the presence of rat cecal contents

In vitro drug release studies were also investigated in the presence of rat cecal contents. The animal experimentation protocols were conducted as per the guidelines of CPCSEA approved by Institutional Animal Ethics Committee. Male Albino rats weighing 15030g, maintained on standard normal diet and water *ad* libitum were selected for the present investigation. Rats were sacrificed and cecal were removed and transferred into simulated colonic fluid (SCF), pH 7.0 previously bubbled with CO_2. The contents of the cecum were weighed and transferred into SCF, pH 7.0, to produce 2% w/v cecal dilution. The release rate studies were carried out using USP type II dissolution rate test apparatus. The study was carried out with 100ml of dissolution medium at on 37 0.1C and rotated at a speed of 100 rpm. A 250 ml beaker containing 100 ml dissolution medium was immersed in the water contained in a 900 ml vessel, which was kept in the water bath of the dissolution rate test apparatus. The formulations, which were previously subjected to *in vitro drug* release studies in 0.1N Hydrochloric acid and SIF, pH 7.4, were kept in an empty gelatin capsule and immersed in the dissolution medium. At pre-specified time intervals, 5 ml of the dissolution media was withdrawn and compensated with the same amount of fresh SCF, pH 7.0, bubbled with CO_2. Samples were filtered through a 0.22m membrane filter and the amount of drug was quantified at 266 nm by UV Visible spectophotometer (Shimadzu, Japan). The experiment was also carried out with 4% w/v cecal contents in the dissolution media.

In vitro cytotoxicity analysis of non-embedded and embedded on HT-29 human colon cancer cell lines

The HT-29 human colon cancer cell lines were purchased from National cell lines facility, Pune and cultured in DMEM (Dulbecco's Modified Eagle Medium) medium supplemented with 10% fetal bovine serum (FBS) at 37 ^{0}C in a 5% CO_2 atmosphere. To examine the effects of non-embedded 5-FU and embedded (chitosan microspheres bearing 5-FU), the cells were treated with 150 mM, 100 mM, 50mM of 5-FU and similar concentrations of embedded 5-FU.

MTT assay

The MTT [3, (4, 5-Demethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay was performed as per standard protocol. In brief, HT-29 human colon cancer cells were cultured in 24 well plates at a density of 5x 10^4 cells per well**.** The cells were treated with varying concentrations of 5-FU and embedded 5-FU. After 48 h, the cells were washed and treated with MTT. Plates were incubated in dark for 4 h, and the absorbance was measured at 570 nm using a microtitre plate reader. To determine the cell viability, percent viability was calculated [(absorbance of drug-treated) sample) / (control absorbance)] x 100.

RESULTS AND DISCUSSION

Effect of various process variables on particle size and entrapment efficiency

The development of a drug carrier made of a bioadhesive and biodegaradable polymer is receiving increasing attention in the field of pharmaceutical technology. [10] These systems offer a number of advantages over the classical drug delivery systems. eg.(i) by selecting the appropriate drug/polymer combination it is possible to achieve the encapsulation hydrophilic and hydrophocbic drugs simultaneously; (ii) the bioactive molecule can be conveniently isolated and protected in the microcavity and (iii) the desired release rate of drug can be easily achieved by selecting a suitable polymer. In the present investigation, a microparticulate system consisting of a microcore of a chitosan (hydrophilic swellable polymer) was used to microencapsulate a water-soluble drug, 5-Fluorouracil. This design was used for colon delivery of 5-Fluorouracil combining two approaches of colon drug delivery: timed release and biodegradation in the colon environment.

The results of entrapment efficiency and particle size measurement due to various core to coat ratio are shown in table 1. The two-way analysis of variance (ANOVA) has shown the concentration of drug had significant effect on the entrapment efficiency and particle size ($p<0.05$). The results show that the increase in drug concentration increases the entrapment efficiency and particle size respectively. There was a linear increase in entrapment efficiency of the drug in chitosan microspheres as a function of concentration. However, the stirring speed also affected the particle size. Increase in stirring speed decreased the particle size significantly.

The cross-linked microspheres of chitosan were subjected *in vitro* drug release rate studies in SGF (pH 1.2) for 2 h and SIF (pH 7.5) for 3 h in order to investigate the capability of the formulation to withstand the physiological environment of the stomach and small intestine. The amount of the 5-FU released during 5 h studies was found to be 15.27 0.56%, which attests the ability of the chitosan to remain intact in the physiological environment of stomach and small intestine. A little amount of the drug, released during 5 h release rate studies was due to the presence of un-entrapped drug on the surface of the microspheres. It is a well established fact that as the chitosan comes in contact with the dissolution medium it creates viscous gel layer around it which controls the release of the entrapped drug. The initials release of the drug present on the surface was higher during 2 h study, as there was no viscous gel layer around the particles and might have been formed after 2 or 3 h which controlled the further releases of drug. After 5 h of testing in 0.1 M HC1 and pH 7.4 Sorensen's phosphate buffer, 20.96 0.58% of the drug was released due to strong shielding effect of compression coat of chitosan. The *in vitro* drug release studies were performed in SCF (pH 7.0) with and without using rat cecal contents. A significant difference ($p <0.005$) was observed in the amount of the 5-FU released at the end of the 24 h from the dissolution medium containing rat cecal content in comparison to the study conducted without rat cecal content. The amount of the drug released from formulations was found to be 47.72 2.39% with 2% w/v cecal matter after 24 h whereas in control study, (without rat cecal contents in dissolution medium) only 31.23 1.49% of drug was released. In case of dissolution medium with 4% cecal matter, 61.65 2.96% of drug release was observed which is considerably higher in comparison to the study involving no cecal matter. The study reveals that the release of the drug in the physiological environment of the colon is due to the degradation of chitosan by colonic bacteria released from rat cecal content. The release of drug from cross-linked chitosan microspheres was supposed to take place after swelling which resulted in the formation of gel followed by the dissolution of 5-FU and diffusion through the gel.

The gel strength of the chitosan microspheres swelled in the dissolution media may be too high, preventing the release of drug from formulation. The colonic bacteria action of rat cecal content medium (2 and 4% w/v) might not be sufficient to degrade the high strength gel barrier of the swollen microspheres. As a result, only 47.72 2.39 and 61.65 2.96% of drug was released after 24 h with 2 and 4% w/v rat cecal content medium, respectively. Hence, one set of animals were administered with 1 ml of 1% w/v aqueous solution of chitosan for 2 days to induce the enzymes that specifically act on chitosan during passage through the colon. The amount of the drug released from the formulation after 24 h with 2 and 4 %

w/v rat cecal contents medium after 2 days induction was found to be 59.35 2.81 and 76.72 3.52% respectively (Fig. 2). Induction of enzymes for 2 days resulted in improved activity of colonic enzymes, as reflected from the release of higher amount of drug in comparison to those, which involved rat cecal content without induction. The release of the drug was much faster during the 18-24 h study period. It is due to the fact that during the initial period (0-18h), the gel strength of the barrier was too high to be broken and during 18-24 h period the network was somewhat loosened which facilitated the release of drug.

In spite of the release of higher percent of drug after 2 days of induction as compared to those without induction, there was a considerable amount of drug to be released and hence, the rats were treated with 1 ml of 1% w/v aqueous solution of chitosan for 4 and 6 days and the release rate study was repeated with 2 and 4 % w/v of cecal matter. The release of the drug was considerably improved with cecal content obtained after 6 days of enzyme induction in comparison to those without enzyme induction or 2 days induction. In the 12-18 h period after 6 days of induction, there was a relatively faster release of the drug due to reduction in the viscosity of the gel network of swollen chitosan around the particles that was susceptible to attack by colonic enzymes. The percent drug release after 24 h release rate study period was observed to be 67.44 3.15 and 88.75 4.15% respectively, with 2 and 4% w/v rat cecal matter obtained after 4 days of enzyme induction and 75.56 3.75 and 96.24 4.77% after 6 days of enzyme induction (Fig. 3). The release of higher amount of drug in case of microspheres with 4% w/v rat cecal mater obtained after 6 days of enzyme induction is due to the larger surface area of microspheres as compared to matrix tablet, which facilitated the release of the drug. The release of the drug is the combined effect of the swelling behavior of chitosan as well as by the biodegradability of guar gum under the influence of colonic enzymes.

FTIR

As mentioned in fig. 4, there was no significant difference in the FTIR spectra of physical mixtures of 5-FU and blank chitosan microspheres as well as 5-FU, when compared to the spectra of individual components.

DSC

Curves of DSC as shown in Fig. 5, one can conclude that drug-loaded microsphere was not a physical mixture, but the formation of real microsphere. The characteristic exothermic peak of 5-FU at 292 ^{0}C and 290 ^{0}C of blank chitosan microspheres, respectively, disappeared in 5-FU loaded chitosan microsphres curve, in which a new characteristic peak at 294 ^{0}C appeared. The DSC curve of the physical mixture also different from that of 5-FU loaded microspheres.

PXRD

PXRD technique was used to define the nature of drug in the microparticles. The X-ray powder diffraction patterns of 5-FU, blank chtosan microspheres, Physical mixture of 5-Fluorouracil and blank chitosan microspheres and 5-FU loaded chitosan microspheres are shown in Fig. 6. The XRD pattern of 5-FU showed peaks, which were intense and sharp indicating its crystalline nature, whereas blank chitosan microspheres showed few sharp peaks. However, 5-FU loaded chitosan microspheres presented the peaks of diminished intensity, suggesting the amorphous nature of drug present in the chitosan microspheres.

Cell cytotoxicity

The cytotoxicity of 5-FU loaded chitosan microspheres and 5-FU-solution was investigated using HT-29 human colon cancer cell lines by MTT assay studying their effect on cell survival and cell cytotoxicity (Table 2). For survival studies, cells were incubated with 5-FU-solution and 5-FU loaded chitosan microspheres continuously and then washed to remove the drug (Fig. 7). Cell survival was determined following the addition of 150, 100, 50 M equivalent of 5-FU. However, equivalent amount of 5-FU embedded in chitosan microspheres crosslinked with different concentrations of GLA exhibited lower cytotoxicity in comparison with 5-FU-solution. There was 42.22 % cell viability after 48 h with free 100 mM 5-FU whereas encapsulated form showed 48.31% cell viability after 72 h with 5omM 5-FU.

CONCLUSION

Use of differential stirring speed during the preparation of chitosan microspheres by the chemical cross-linking method may help to prepare chitosan microspheres using a chitosan solution by less than 1% wt/vol concentration. The pharmaceutical attributes of microspheres were significantly affected by stirring speed, chitosan

concentration and their interaction. Effect of change in drug concentration on the pharmaceutical characteristics of drug-loaded chitosan microspheres is more prominent for water-soluble drug. Therefore, the present investigation showed the promising results of chitosan microspheres as a matrix for drug delivery and also warrants for in vivo study for scale up the technology.

Table 1. Various process parameters used in optimization of 5-Fluorouracil loaded chitosan microspheres

Formulation Code	Composition (Polymer:Drug) (mg)	Entrapment efficiency (%)	Particle size (μm)
CHF-1	200:50	2.12±1.14	10.32±4.32
CHF-2	200:100	5.20±2.49	12.81±5.64
CHF-3	200:200	12.45±1.98	16.28±6.13
CHF-4	200:300	14.66±4.83	18.64±4.29
CHF-5	200:400	18.23±3.56	22.38±5.21

Table 2. Percent viability of 5-FU in free and encapsulated form at different time intervals

		24h		48h		72h	
Sr. No.	Concentration	Free 5-FU	Encap. 5-FU	Free 5-FU	Encap. 5-FU	Free 5-FU	Encap. 5-FU
1.	150 mM	49.87%	89.12%	26.78%	60.24%	15.70%	42.35%
2.	100 mM	94.99%	98.99%	42.22%	80.14%	18.38%	45.26%
3.	50 mM	96.52%	99.10%	79.15%	89.23%	22.68%	48.31%

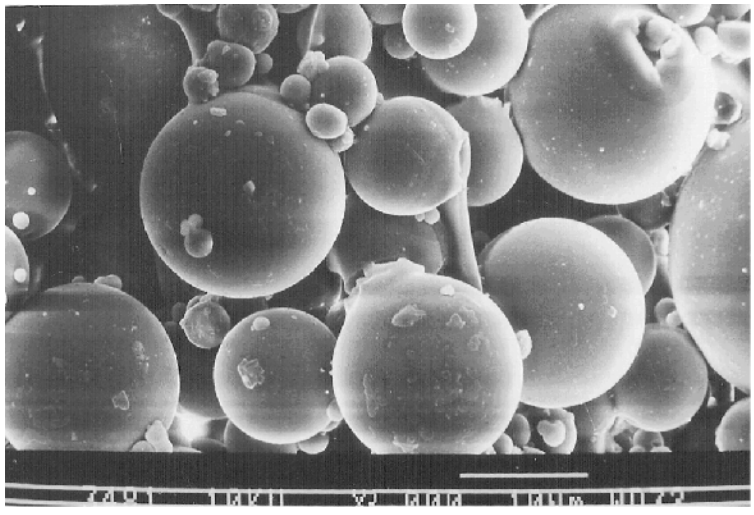

Fig. 1: Scanning electron micrpscopy of drug loaded chitosan microspheres, which indicated the smooth shaped microspheres are formed after loading of 5-fluorouracil

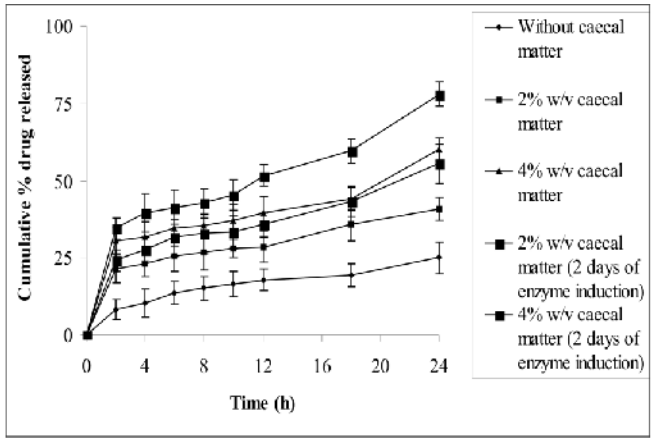

Fig. 2: Cumulative percent drug release in SCF (pH 7.0) with rat caecal matter, without rat caecal matter and after 2 days of enzyme induction. Results indicated that in presence of rat caecal contents, chitosn amicrospheres releases significantly higher amount of 5-FU in comparison of without caecal matter.

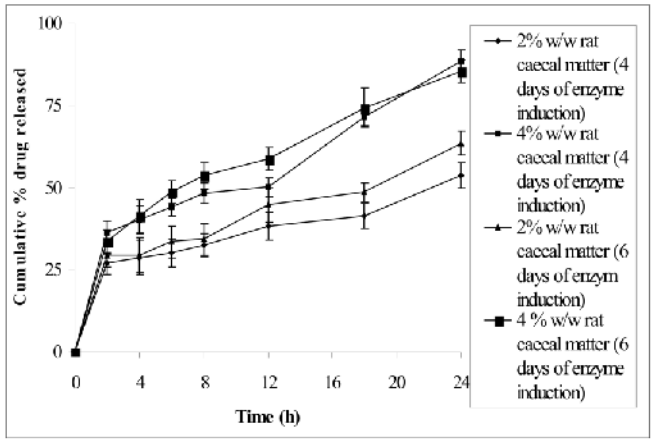

Fig 3: In vitro drug release in presence of rat caecal contents after 4 and 6 days of enzyme induction, which indicated that 6 days of enzyme induction significantly enhanced the release rate.

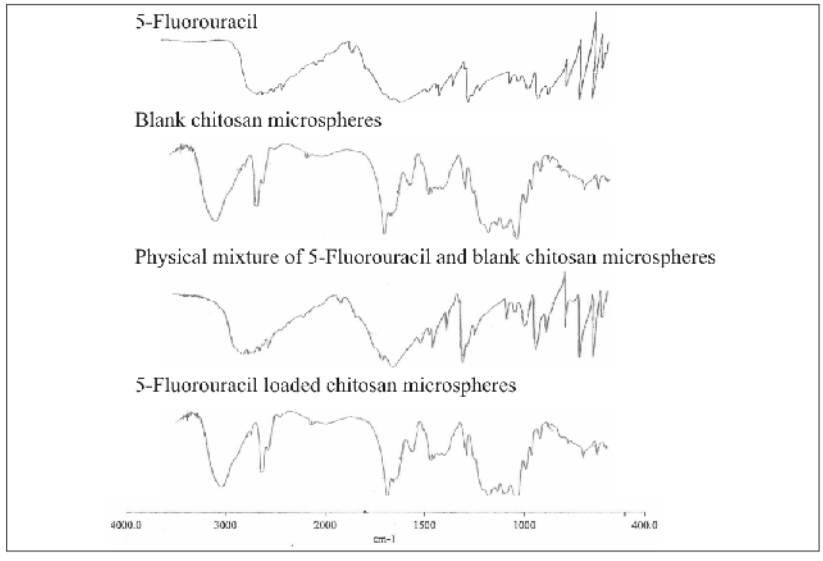

Fig. 4: FTIR spectra of 5-fluorouracil, blank chirosan microspheres, physical mixture of 5-fluorouracil and blank chitosan microspheres and 5-fluorouracil loaded chitosan microspheres

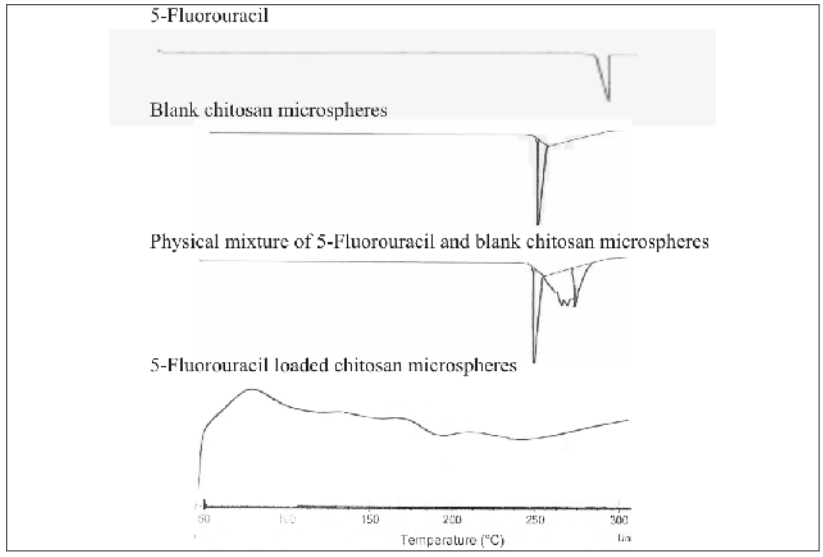

Fig. 5: DSC spectra of 5-fluorouracil, blank chirosan microspheres, physical mixture of 5-fluorouracil and blank chitosan microspheres and 5-fluorouracil loaded chitosan microspheres

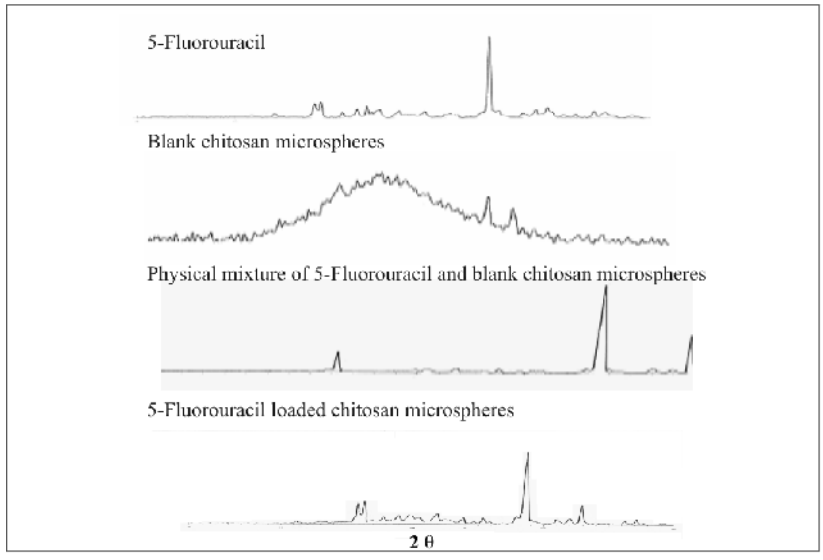

Fig. 6: X-ray diffraction pattern spectra of 5-fluorouracil, blank chirosan microspheres, physical mixture of 5-fluorouracil and blank chitosan microspheres and 5-fluorouracil loaded chitosan microspheres

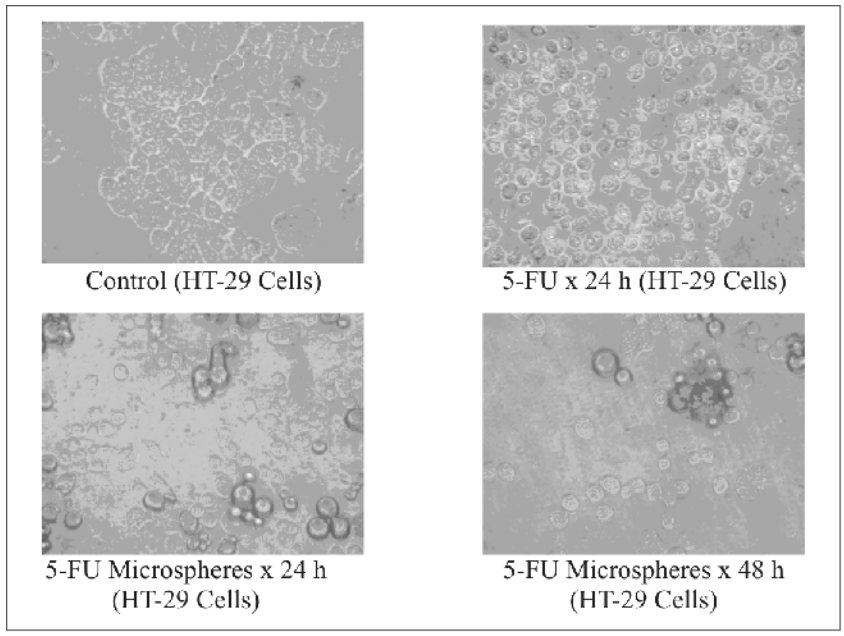

Fig. 7: Photomicrographs of cytotoxicity analysis of 5-fluorouracil and 5-fluorouracil loaded chitosan microspheres

REFERENCES

1. Shi P, Zuo Y, Zou Q, Shen J, Zhang L, Li Y, Morsi YS. Improved properties of incorporated chitosan film with ethyl cellulose microspheres for controlled release. Int J Pharm. 2009; 375 (1-2): 67-74.
2. Nagda C, Chotai NP, Patel U, Patel S, Soni T, Patel P, Hingorani L. Preparation and characterization of spray dried mucoadhesive microspheres of aceclofenac. Drug Dev Ind Pharm. 2009 [in press].
3. Ell chi S, Marzoulci MN, Korri-Youssoufi H. Direct monitoring of pollutants based on an electrochemical biosensor with novel peroxidase (POX1B). Biosens Bioelectron 2009; 24 (10): 3084-90.
4. Thanoo BC, Sunny MC and Jayakrishnan A. Cross-linked chitosan microspheres: preparation and evaluation as a matrix for the controlled release of pharmaceuticals. J Pharm Pharmacol 1992; 44: 283-286.
5. Ko JA, Park HJ, Hwang SJ, Park JB and Less JS. Preparation and characterization of chitosan microparticles intended for controlled drug delivery. Int J Pharm 2000; 249: 165-174.
6. Kumbar SG, Kulkarni AR and Aminabhavi TM. Crosslinked chitosan microspheres for encapsulation of diclofenac sodium: effect of crosslinking agent. J Microencapsul 2002; 19: 173-180.
7. Yoshino T, Machida Y, Onishi H and Nagai T. Preparation and characterization of chitosan microspheres containing foxifluridine. Drug Dev Ind Pharm 2003; 29: 417-427.
8. Berthold A, Cremer K and Kreuter J. Preparation and characterization of chitosan microspheres as drug carrier for prednisolone sodium phosphate as model for anti-inflammatory drugs. J Control Release 1996; 39: 17-25.
9. Denkbas EB, Seyyal, M and Piskins E. 5-Flourouracil loaded chitosan microspheres for chemoembolization J Microencapsul 1999; 16: 741-749.
10. Tomlinson E. Passive and active vectoring with microparticles: localization and drug release. J Control Release 1985; 2: 385-391