International Journal of Pharmaceutics 359 (2008) 182–189

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Con-A conjugated mucoadhesive microspheres for the colonic delivery of diloxanide furoate

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ARTICLE INFO

Article history:
Received 8 February 2008
Received in revised form 28 March 2008
Accepted 4 April 2008
Available online 12 April 2008

Keywords:
Colon targeting
Microspheres
Gamma scintigraphy
Diloxanide furoate
Concanavalin-A

ABSTRACT

The aim of the research work was to develop cyst-targeted novel concanavalin-A (Con-A) conjugated mucoadhesive microspheres of diloxanide furoate (DF) for the effective treatment of amoebiasis. Eudragit microspheres of DF were prepared using emulsification–solvent evaporation method. Formulations were characterized for particle size and size distribution, % drug entrapment, surface morphology and *in vitro* drug release in simulated gastrointestinal (GI) fluids. Eudragit microspheres of DF were conjugated with Con-A. IR spectroscopy and DSC were used to confirm successful conjugation of Con-A to Eudragit microspheres while Con-A conjugated microspheres were further characterized using the parameters of zeta potential, mucoadhesiveness to colonic mucosa and Con-A conjugation efficiency with microspheres. IR studies confirmed the attachment of Con-A with Eudragit microspheres. All the microsphere formulations showed good % drug entrapment (78 ± 5%). Zeta potential of Eudragit microspheres and Con-A conjugated Eudragit microspheres were found to be 3.12 ± 0.7 mV and 16.12 ± 0.5 mV, respectively. Attachment of lectin to the Eudragit microspheres significantly increases the mucoadhesiveness and also controls the release of DF in simulated GI fluids. Gamma scintigraphy study suggested that Eudragit S100 coated gelatin capsule retarded the release of Con-A conjugated microspheres at low pH and released microspheres slowly at pH 7.4 in the colon.

1. Introduction

A number of essential aspects that should be considered in the design of drug delivery systems to achieve controlled and targeted drug delivery include target, carrier, ligand(s) and physically modulated components. Targeted drug delivery implies a selective and effective localization of pharmacologically active moiety at pre-identified (pre-selected) target(s) in therapeutic concentration, while restricting its access to non-target normal cellular linings, thus minimizing toxic effects and maximizing therapeutic index (Gregoriadis and Florence, 1993). In the last 15 years, selective drug delivery to the colon has been the focus of increasing interest in research. The site specific drug delivery to the colon would be beneficial not only for the oral delivery of proteins and peptide drugs, which are degraded by digestive enzymes of stomach and small intestine, but also for the delivery of low molecular weight compounds used in the treatment of diseases associated with colon or large intestine.

Amoebiasis is an infection caused by the protozoal organism *Entamoeba histolytica*, an organism that feeds on cells in the human colon. It is the cause of amebic colitis and liver abscess. Amoebiasis probably is second next to malaria as a protozoal cause of death. *E. histolytica* infection results in 50 million cases of invasive amoebiasis and 100,000 deaths annually (Swords and Cantey, 2002). Umamaheshwari and Jain (2003) suggested the potential for using lectin-conjugated gliadin nanoparticles bearing acetohydroxamic acid as a means of locating and anchoring a drug delivery system on the carbohydrate receptors of *Helicobacter pylori*. Sharma et al. (2004) prepared and evaluated lectin-functionalized poly lactide-co-glycolide (PLG) nanoparticles for treatment of tuberculosis.

The aim of the present work was to develop cyst-targeted novel mucoadhesive microspheres of diloxanide furoate (DF) for the effective treatment of amoebiasis. Concanavalin-A (Con-A) is a ligand, used as targeting material, because cyst wall of *E. histolytica* contains glycoproteins having specific affinity toward Con-A. Also Con-A acts as a mucoadhesive and binds to the glycoproteins of mucus layer of colon (Kalb and Levitzki, 1968). These mucoadhesive microspheres will be delivered to the colon by means of enteric-coated capsules. Diloxanide furoate is a safer and more effective, direct luminal amoebicidal drug, than other luminal amoebicidal

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doi:10.1016/j.ijpharm.2008.04.009

drugs (Dubey et al., 1965). In the light of the above facts, it was proposed that Con-A conjugated microspheres could be effective in the treatment of amoebiasis. This system could plug and seal the carbohydrate receptor on the surface of cyst of *E. histolytica* and target the drug at the site of infection.

2. Materials and methods

2.1. Materials

Diloxanide furoate was supplied as a gift sample by M/s Adani Pharmaceutical Pvt. Ltd. (Rajkot, India) and M/s Chemsales Corporation (Calcutta, India). Eudragit S100 was obtained as gift sample from M/s S. Zhaveri & Company (Mumbai, India). Concanavalin-A was supplied as a gift sample by Bio-Research Products, Inc. (323 W. Cherry St., North library, IA 52317). *N*-Hydroxysuccinimide (NHS) was supplied as gift sample from Shivam Enterprises (Pune, India). 1-Ethyl-3,3-(dimethylaminopropyl) carbodiimide (EDC), liquid paraffin, acetone, magnesium stearate, stannous chloride, and hexane were purchased from HiMedia Laboratory (Mumbai, India). Cyst of *E. histolytica* was generously supplied as gift by Mr. Arun Dani, Director, Dani Institute of Paramedical Sciences (Champa, India). Technetium-99m (as pertechnetate) (^{99m}Tc O^{-4}) was obtained from the Nuclear Medicine Department, Jawaharlal Nehru Cancer Hospital and Research Center (Bhopal, India). All other chemicals were of analytical reagent grade and were used as received. The *in vivo* and *ex vivo* studies were performed following the guidelines approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Institutional Animal Ethical Committee of Guru Ghasidas University, Bilaspur (India) granted permission for the study.

2.2. Preparation of Eudragit microspheres (EM)

Microspheres were prepared by solvent evaporation method using an acetone/liquid paraffin system (Sahoo et al., 2005). Eudragit S100 (ES) (300 mg) was dissolved in 20 ml of acetone and pure DF (300 mg) was dissolved in polymer solution. The solution was filtered and magnesium stearate (50 mg) was dispersed in solution uniformly by ultrasonication (Soniweld, Imeco Ultrasonics, Mumbai, India). This resulting dispersion was then added drop-wise to 500 ml beaker containing a mixture of 100 ml liquid paraffin (heavy:light, 1:1) and 20 ml *n*-hexane with continuous stirring at 1000 rpm in a mechanical stirrer (Remi, Mumbai, India). The stirring was continued for 1.5 h at room temperature ($\leq$25 °C), until acetone evaporated completely. After evaporation of acetone, the microspheres were collected by vacuum filtration. The microspheres were washed 4–5 times in 40 ml *n*-hexane and dried at room temperature in a desiccator for 24 h.

2.3. Particle size determination

Particle size and size distribution of different microsphere formulations were measured using an optical microscope, and the mean particle size was calculated by measuring 600 particles with the help of a calibrated ocular micrometer.

2.4. Drug entrapment efficiency and percent yield

The % drug entrapment of ES microspheres containing DF was determined by dispersing 50 mg formulation in 20 ml ethanol followed by agitation with a magnetic stirrer for 12 h to dissolve the polymer and to extract the drug. After filtration through a 5-μm membrane filter (Millipore, USA), the drug concentration in the ethanol phase was analyzed spectrophotometrically at 263 nm using UV–vis spectrophotometer (Systronics, Mumbai, India). ES, EDC, NHS and Con-A did not interfere under these conditions. Each determination was made in triplicate. The percentage drug entrapment and yield were calculated as follows:

$$\%\ \text{drug entrapment} = \frac{\text{calculated drug content}}{\text{theoretical drug content}} \times 100$$

$$\%\ \text{yield} = \frac{\text{total weight of microparticles}}{\text{total weight of drug, polymer and other non-volatile solids (/if added)}} \times 100$$

2.5. Conjugation of Con-A with microspheres

Activation of carboxyl group of ES microspheres (50 mg) was carried out by addition of 1 ml of 0.1 M EDC and 1 ml of 0.11 M of NHS in phosphate buffer (pH 5.8) (Olde Damink et al., 1996). After 3 h incubation at room temperature, excess coupling agent was removed by washing of microspheres with phosphate buffer (pH 5.8). The latter were suspended in 1 ml of Con-A solution in phosphate buffer (pH 5.8) and after incubation overnight Con-A conjugated microspheres were obtained by centrifugation (CFC-FREE, C-24, cooling centrifuge, REMI, India) at 8000 rpm, followed by three to four washings with distilled water. The conjugated microspheres thus obtained were dried at room temperature.

2.6. IR study

IR spectroscopy was carried out to confirm the conjugation of Con-A with Eudragit microspheres. The KBr discs of EM (without drug) and Con-A conjugated Eudragit microspheres (CCEM) (without drug) were prepared and scanned in an IR spectrophotometer (PerkinElmer, Spectrum RX-I, Lambda, USA).

2.7. Particle morphology

The shape and surface morphology of EM and CCEM were investigated using scanning electron microscopy (SEM). The samples for SEM study were prepared by lightly sprinkling the formulation on a double adhesive tape stuck to an aluminum stub. The stubs were then coated with gold to a thickness of about 300 Å under an argon atmosphere using a gold sputter module in a high-vacuum evaporator. The coated samples were then randomly scanned and photomicrographs were taken with a scanning electron microscope (Jeol JSM-1600, Tokyo, Japan).

2.8. In vitro drug release study

The *in vitro* drug release from EM, CCEM and marketed tablet formulation of DF (Amicline, Franco Indian Pharmceuticals Pvt. Ltd., Mumbai, India) were studied by using rotating six paddle apparatus (dissolution rate test apparatus USP/IP/BP STD, Jyoti, Gwalior, India) in simulated GI fluids. Microspheres (100 mg) were weighed accurately and gently spread over the surface of 900 ml of dissolution medium (simulated GI fluid, SGF). The content was rotated at 100 rpm at 37 ± 0.5 °C. Perfect sink conditions prevailed during the drug dissolution study period. The simulation of GI transit condition

was achieved by altering the pH of dissolution medium at different time intervals. The pH of the dissolution medium was maintained at 1.2 for 2 h using 0.1N HCl. Then KH_2PO_4 (1.7 g) and $Na_2HPO_4{\cdot}2H_2O$ (2.2 g) were added to the dissolution medium, adjusting the pH to 4.5 with 1.0 M NaOH and the release rate study was continued for further 2 h. After 4 h, the pH of dissolution medium was adjusted to 7.4 with 0.1N NaOH and maintained up to 24 h (Rai et al., 2005; Paharia et al., 2007). A 5 ml sample was withdrawn from the dissolution medium at various time intervals using a pipette fitted with a micro-filter and analyzed DF release using a UV–vis spectrophotometer (Systronics, Mumbai, India) at 263 nm. The receptor volume was maintained constant by replacing with equivalent volume of SGF after each withdrawal. The concentration of DF in the samples was calculated based on average calibration curves ($n = 6$). All dissolution studies were performed in triplicate.

2.9. Differential scanning calorimetry

In order to determine the physical state of drug, i.e. amorphous or crystalline, before and after final formulation and to evaluate any possible drug–polymer, drug–other components interaction (Kawashia et al., 1991), DSC examination was conducted for the optimized formulation, pure drug, the polymer, Con-A, EDC and NHS (EDC and NHS are coupling agents) using a DSC instrument (Mettler 305, Switzerland). Samples of 2–6 mg were placed in aluminum pans (Al-Crucibles, 40 Al) and sealed. The probes were heated from 25 °C to 400 °C at a rate of 10 °C/min under nitrogen atmosphere.

2.10. Con-A conjugation efficiency

The amount of Con-A bound to the Eudragit microspheres containing DF was calculated as the difference between the Con-A added initially and the Con-A recovered after incubation with the microspheres (Yin et al., 2006). The amount of lectin in the supernatant was estimated by using Folin-Ciocalteu phenol reagent. Reagent C (10 ml) was added in suspension containing 100 mg of conjugated microspheres in 100 ml distilled water, mixed thoroughly and allowed to stand for 20 min. Then 1 ml of reagent D was added rapidly with immediate mixing to above solution. After 30 min, solution was filtered with Whatman filter paper (# 41), volume was made up to 10 ml and absorbance was measured against blank using a UV–vis spectrophotometer (Systronics, Mumbai, India) at 750 nm. The experiment was performed in triplicate.

Reagent A and reagent B were prepared by dissolving Na_2CO_3 (2 g) in 0.1N NaOH and $CuSO_4{\cdot}5H_2O$ (0.5 g) in 1% sodium/potassium tartrate to make 100 ml solutions each, respectively. Reagent C was prepared by mixing 50 ml reagent A with 1 ml of reagent B. Reagent D was prepared by diluting Folin-Ciocalteu phenol reagent (2.0N) with distilled water to make it 1.0N.

2.11. Percent mucoadhesion

Everted sac experiment, reported by Santos et al. (1999), was performed for the determination of % mucoadhesion using viable segment of rat colon on EM and CCEM. Albino rats (200 ± 10 g, male) were sacrificed and intestinal tissue was excised and flushed with 10 ml of ice-cold phosphate buffered saline (pH 7.2) containing 200 mg/dl glucose (PBSG). Two segments (6 cm, length) of colon were everted using a stainless steel rod and lightly washed with PBSG to remove the contents for each study. Weight of colon segment was taken separately. Ligatures were placed at both ends of the segment and the sac was filled with 1–1.5 ml of PBSG. Tissue was maintained at 4 °C prior to incubation. The sac was introduced into a 15 ml test tube containing 50 mg of CCEM microspheres in 10 ml phosphate buffer, pH 5.8, in two separate test tubes. The sac was incubated at 37 °C and agitated end-over-end. After 30 min, the sac was removed and the solution of phosphate buffer, pH 5.8 and CCEM microspheres were centrifuged for 30 min. The supernatant was discarded. The sedimented microspheres were washed three times with 5 ml distilled water and centrifuged. The supernatant fluid was discarded again and microspheres were dried for 24 h. The weight of the bound microspheres is determined by subtraction of the weight of tissue and dried microspheres from the initial weight of tissue. The same procedure was followed for EM. The experiment was performed in triplicate. The following formula was used for the determination of % binding,

$$\%\ \text{binding} = \frac{\text{initial weight of microspheres} - \text{weight of unbound microspheres}}{\text{initial weight of microspheres}} \times 100$$

2.12. Zeta potential measurement

Zeta potential was measured by electrophoresis, which was performed with a Malvern Zetasizer instrument (UK). The microspheres were suspended in distilled water by ultrasonication for 30 min. The concentration of the suspension was 2% (w/v). The cell was filled with a measured amount of sample and inserted with its integral gold electrodes close to the lid.

2.13. Uptake of sodium pertechnetate (^{99m}Tc)

Weighed quantity (150 mg) of the microspheres loaded with $SnCl_2$ (3%, w/v) was placed in a test tube and soaked in 10 ml of normal saline (0.9% NaCl) for 15 min. A small amount of ^{99m}Tc solution equivalent to radioactivity of 40 mBq in a sterile vial obtained from a technetium generator (column generator, Monrol, Mon-tek, $M_o{}^{99}T_c{}^{99}$, Turkey) was added to the test tube. The suspension was mixed intermittently for 15 min using a vortex shaker (Superfit, India) and microspheres were allowed to equilibrate. The supernatant was removed and the labeled microspheres were recovered by filtration through a Whatmann filter paper (# 41) followed by washing thoroughly with deionized water. The formulation was then allowed to dry in air for 15 min. Dried microspheres were filled in gelatin capsule (# 4), and the capsule was coated with optimized coating of Eudragit S100 polymer (4%, three coatings), and allowed to dry in air for 30 min. The radioactivity in the supernatant and filtrate was counted in an auto gamma counter (Cobra II, Germany) to determine the remaining unbound pertechnetate. The radioactivity of the coated capsule was also counted for bound pertechnetate (Atyabi et al., 1996).

Marketed uncoated dosage form of DF (Amicline—500 mg, tablet) was powdered; about 100 mg powder was mixed with 3 mg $SnCl_2$ and compressed. The compressed tablet was then placed in test tube. A small amount of ^{99m}Tc solution equivalent to radioactivity of 40 mBq in a sterile vial obtained from a technetium generator was added to test tube, mixed for 15 min, followed by drying in air for 15 min. The radioactivity of the formulation was counted in an auto gamma counter (Cobra II, Germany) for bound pertechnetate.

2.14. Organ distribution study

Organ distribution study was performed in three adult male albino rats (Jain et al., 2006). ^{99m}Tc-labeled microspheres (CCEM) in gelatin capsule (# 5) were orally administered with the help of feeding tube to rats followed by 20 ml drinking water. Anterior position of the rat was scanned under gamma camera 2 h post-dosing. The rats were sacrificed and thyroid gland, stomach, small intestine and colon were isolated. These organs were placed separately in petriplates with 10 ml of normal saline. Gamma images and radioactive counts of organs were recorded using E-Cam Single Head gamma camera (Siemen's, Germany).

2.15. Gamma scintigraphy

Nine 1-year-old male albino rabbits (New Zealand) were used to monitor the *in vivo* transit behavior of Amicline (Tablet), EM and CCEM (Jain et al., 2006). None of them had symptoms or a past history of GI disease. Animals were divided into three groups of three animals each. In order to standardize the condition of GI motility, the animals were fasted for 12 h prior to the commencement of each experiment. One coated capsule of CCEM was orally administered to animals of the first group, one coated capsule of EM to the second group and Amicline (tablet) to the third group with the help of feeding tube, followed by sufficient volume of drinking water. All four legs of the rabbit were tied over a piece of plywood (20 in. × 20 in.) and the location of the formulation in GI tract was monitored every 1 h by keeping the subject in front of gamma camera. The gamma camera had a field view of 40 cm and was fitted with a medium energy parallel hole collimator. The 140 keV gamma rays emitted by ^{99m}Tc were imaged. Specific GI tract sites (anterior) were imaged by E-Cam Single Head gamma camera (Siemen's, Germany) after definite time intervals. The gamma images were recorded for 9 h study period using an online computer system and stored on magnetic disk and analyzed to determine the distribution of activity in the GI tract. During the gamma scanning, the animals were freed and allowed to move and carry out normal activities.

3. Results and discussion

Eudragit microspheres were prepared by solvent evaporation method. The concept of preparing delivery system for cyst targeting is explained in Fig. 1. Microspheres were prepared by using different drug:polymer ratios and at different stirring rates. Rate of evaporation of solvent from dispersion is a key factor in preparing desirable microspheres. Magnesium stearate was added to avoid aggregation of polymer rich droplets during preparation.

3.1. Particle size determination, drug entrapment efficiency and percent yield

Size of microspheres affects the release rate of drug. Increase in size, decreases the effective surface area which ultimately decreases the drug release rate (Muramatsu and Kondo, 1995). The average particle size of the microspheres was found to be 60.12 ± 1.50 μm, 76.28 ± 2.84 μm, and 110 ± 3.24 μm for 1:1, 1:2 and 1:3 polymer concentrations, respectively. The average particle size of Eudragit microspheres increased as the amount of polymer was increased, which may be attributed to the increase in solution viscosity with increasing polymer concentration, resulting in large particles. Thus, average particle size also increased. The average particle size of Eudragit microspheres decreased with increasing agitation speed of mechanical stirrer from 500 rpm to 1500 rpm. This was expected because high turbulence caused frothing and adhesion to the container wall. Similar results were obtained by

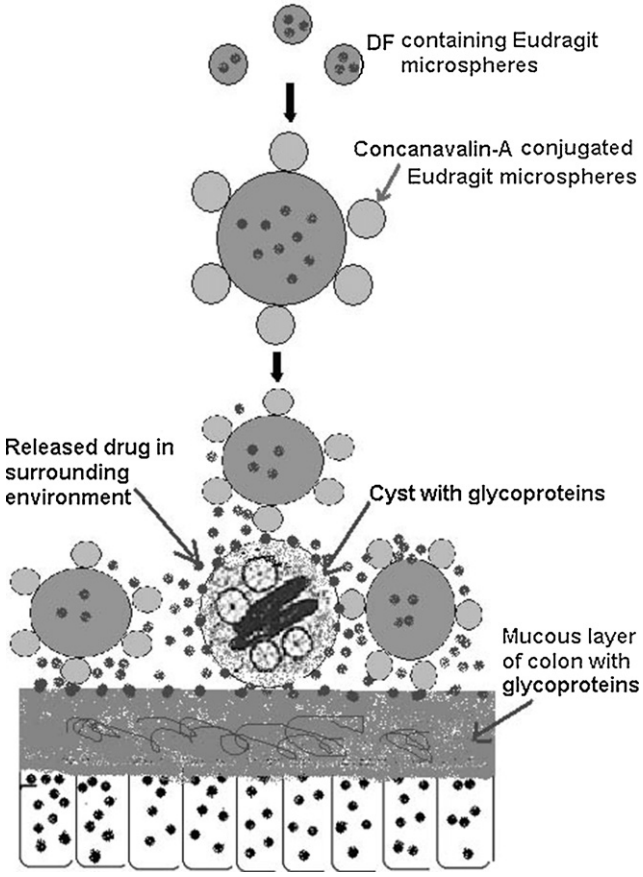

Fig. 1. Concept of cyst-targeted mucoadhesive drug delivery system.

Sahoo et al. (2005) who demonstrated that an increase in stirring rate resulted in a decrease in the mean particle size of stavudine microspheres because of high turbulence. The % yield of microspheres was found to be 79.56 ± 2.1% to 90.00 ± 0.9% and % drug entrapment was found to be 68.34 ± 3.8% to 78.99 ± 2.0% with varying polymer concentrations from 1% (w/v) to 3% (w/v) and stirring rates from 500 rpm to 1500 rpm.

Formulation EM (drug polymer ratio 1:1; and stirring rate 1000 rpm) showed highest % yield (90.00 ± 0.9), % drug entrapment (78.99 ± 2.0) and small average particle size (60.12 ± 1.24), as well as regular and spherical in shape thus it was selected as optimized formulation for coupling with Con-A.

3.2. Con-A coupling to Eudragit microspheres

The conjugation procedure is usually performed by covalent attachment. Depending on functional groups available on the carrier surface, the lectin coupling was carried out (Olde Damink et al., 1996). The EDC and NHS were used to activate the carboxylic group of Eudragit prior to coupling of Con-A. Optimized Eudragit microspheres containing DF were used for the conjugation of Con-A. A formulation conjugated with Con-A was assigned a batch code of CCEM. IR spectra of CCEM and EM microspheres (without drug) were taken and compared (Figs. 2 and 3). IR studies confirmed the attachment of Con-A to Eudragit microspheres, because coupling of Con-A to Eudragit S100 microspheres depends on the formation of amide bond between NH_2 group of Con-A and COOH group of Eudragit S100. Peaks (3430.0, 1702.2, 1650, 1541.1, etc.) for amide groups in CCEM suggested the presence of amide group in the formulation but peaks for amide groups were absent in the IR

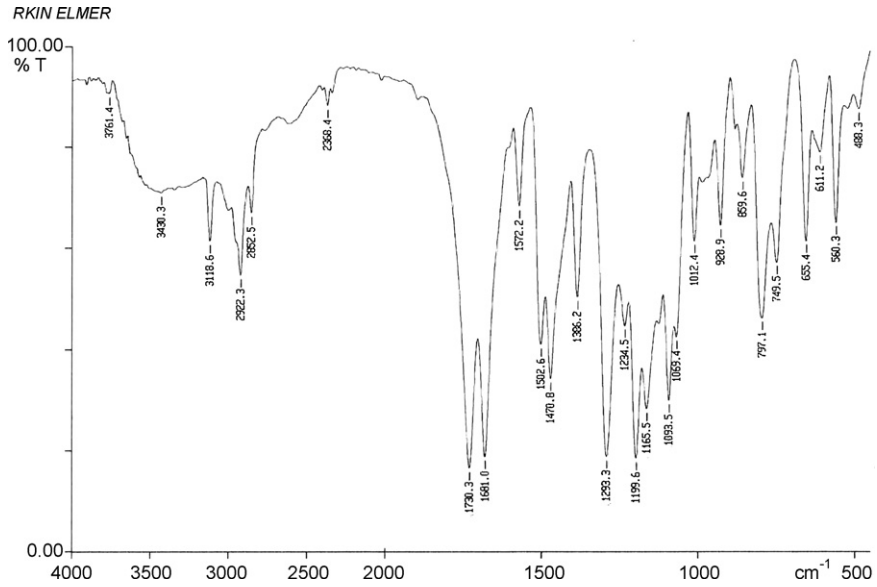

Fig. 2. IR of Con-A conjugated Eudragit microspheres (CCEM).

spectrum of EM microspheres. It was also confirmed due to presence of two amide bonds (I and II) at 1650 cm^{-1} and 1541.1 cm^{-1}, which are characteristics of amides mainly in proteins. IR spectra of both the formulations were taken without drug loading in the microspheres; because presence of amide group in the DF may create confusion while interpreting the spectrum of CCEM.

The size of Con-A conjugated formulation (63.15 ± 2.20) was found to be slightly higher than non-conjugated microspheres (60.12 ± 1.24), which may be due to the presence of lectin coating on formulation.

3.3. Particle morphology

Surface morphology and shape of developed formulation were determined using scanning electron microscopy. Photomicrographs show that microparticles were regular and spherical in shape (Fig. 4A and B). The SEM picture (Fig. 4B) suggested that the Con-A conjugated microspheres were regular and spherical in shape indicating that Con-A attachment did not affect the structural integrity of the microspheres.

3.4. In vitro drug release

Release of DF from different formulations, i.e. CCEM, EM and Amicline was evaluated in pH progression medium at $37 \pm 0.5\,^{\circ}C$. *In vitro* DF release from CCEM and EM was found to be significantly different as compared with release profile of Amicline. The cumulative % release curve from EM and CCEM showed the desired rate as there was no measurable drug release up to 2 h in pH 1.2 while at pH 4.5 the drug release was quite insignificant up to 4 h. As shown in Fig. 5, 71% of DF was released from Amicline, while >5% of DF was released from EM and CCEM after 2 h at pH 1.2. At pH 4.5, 87% of DF was released from Amicline, while DF released from EM and CCEM was 11% and 10%, respectively after 4 h. Since the acrylic polymer used is not soluble in acidic pH and starts to dissolve above pH 7, no significant amount of drug was released

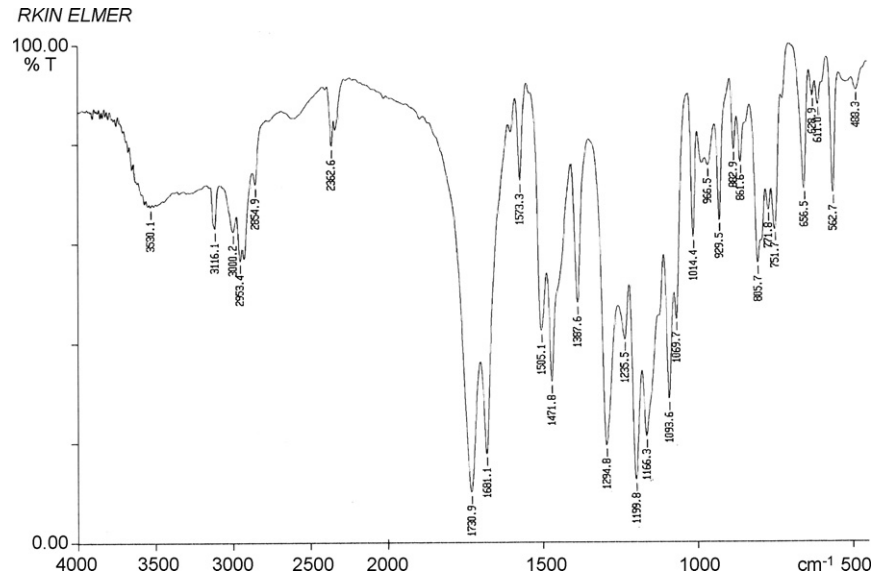

Fig. 3. IR of Eudragit microspheres (EM).

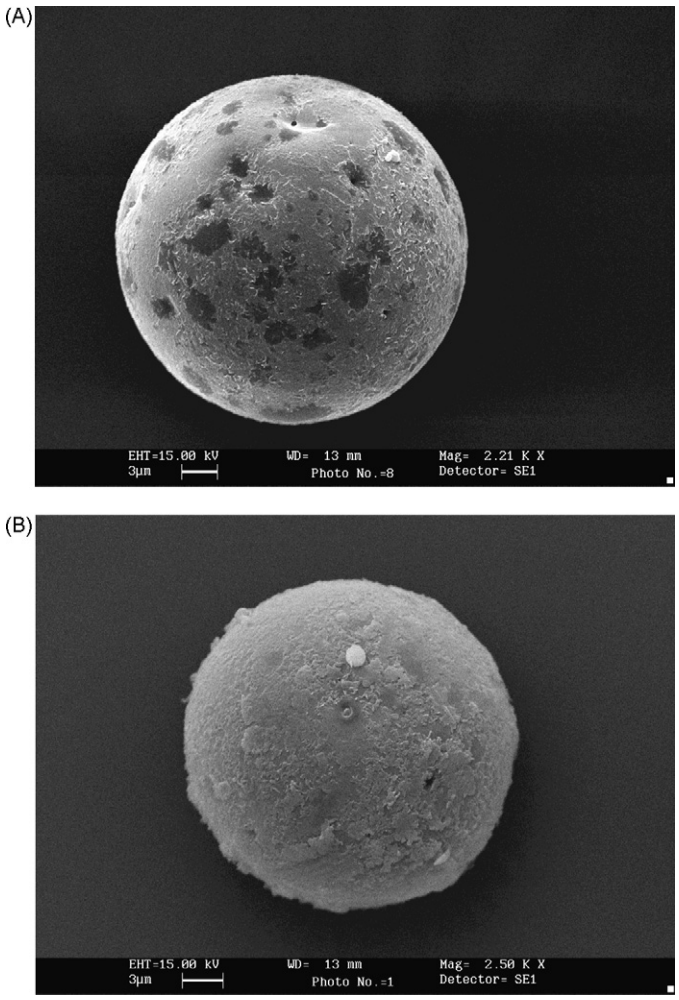

Fig. 4. Scanning electron photomicrographs of (A) Eudragit microsphere of DF and (B) Con-A conjugated Eudragit microsphere of DF.

from microspheres in pH 1.2 and 4.5. The microspheres released some drug in pH 1.2 and pH 4.5, which may be due to the diffusion process but release in pH 7.4 could follow both diffusion and erosion mechanisms. CCEM showed comparatively better controlled release behavior as compared to EM, which may be due to lectin coating on microspheres.

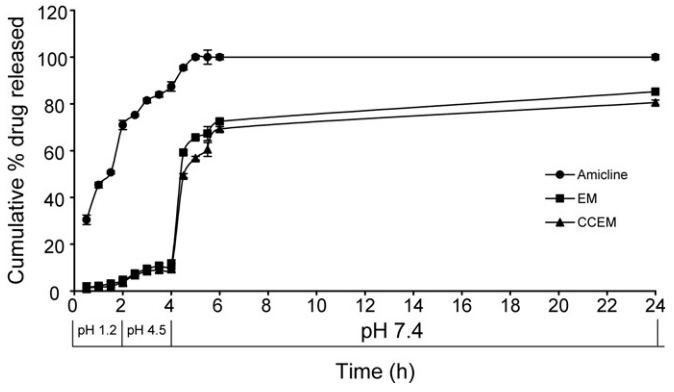

Fig. 5. *In vitro* drug release profile of Eudragit microspheres of DF, Con-A conjugated Eudragit microspheres of DF and marketed product of DF (Amicline). The values are mean ± S.D. ($n = 3$).

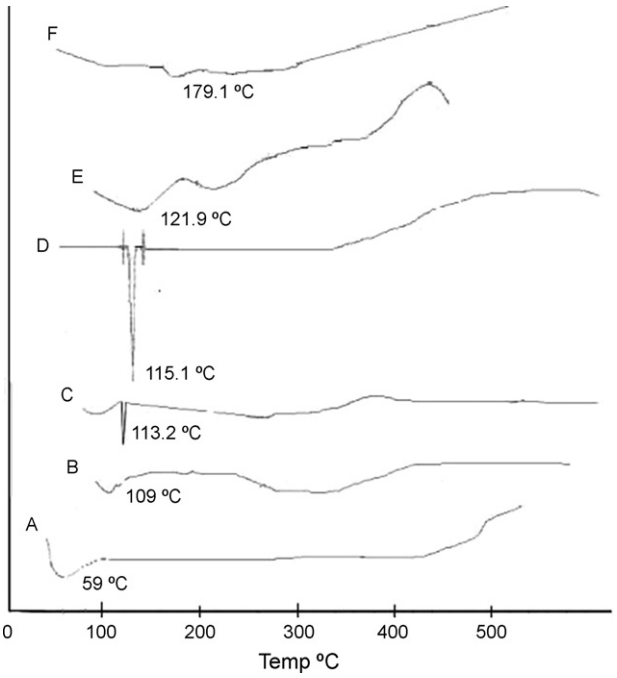

Fig. 6. DSC Thermogram of (A) Con-A, (B) 1-ethyl-3,3-(dimethylaminopropyl) carbodiimide, (C) Con-A conjugated microspheres, (D) diloxanide furoate, (E) *N*-hydroxysuccinimide, and (F) Eudragit S100.

3.5. Differential scanning colorimetry

A sharp melting transition of pure DF was observed at 115.1 °C. Eudragit S100 showed a broad transition from 170 °C to 225 °C. Con-A, EDC and NHS showed endothermic peaks at 59 °C, 109 °C and 121.9 °C, respectively. A DSC thermogram of CCEM showed the DF peak at 113.2 °C, ruled out any interaction between drug and other components of optimized formulation as well as any change in physical state of DF (Fig. 6). One slightly broader peak from 40 °C to 60 °C, which might be the peak of Con-A, suggests some interaction between Con-A and Eudragit S100. Absence of EDC and NHS peaks in DSC thermogram of optimized formulation confirms the absence of EDC and NHS in the formulation.

3.6. Con-A conjugation efficiency and mucoadhesion

The amount of Con-A bound to the Eudragit microspheres containing DF was determined. The amount of lectin was estimated by using Folin-Ciocalteu reagent. The percent conjugation efficiency of CCEM containing DF was calculated as 89.12 ± 1.9%. Mucoadhesion studies were carried out to ensure the adhesion of the formulation to the mucosa for a prolonged period of time. Everted sac method was used to test mucoadhesive property of EM and CCEM. Maximum mucoadhesion (86 ± 1.2%) was shown by Con-A conjugated Eudragit microspheres as compared with non-conjugated Eudragit microspheres (5.0 ± 1.4%). This significant difference ($p < 0.05$) in the mucoadhesion may be due to affinity of the Con-A towards glycoproteins of mucus membrane of colon (Fig. 7).

3.7. Zeta potential measurement

Zeta potential of EM and CCEM were found to be 3.12 ± 0.7 mV and 16.12 ± 0.5 mV, respectively (Fig. 7). Lectin coating caused a positive increase in zeta potential of formulation. It is also reported by Umamaheshwari and Jain (2003) that lectin coating on gliadin nanoparticles increased zeta potential positively. This property is

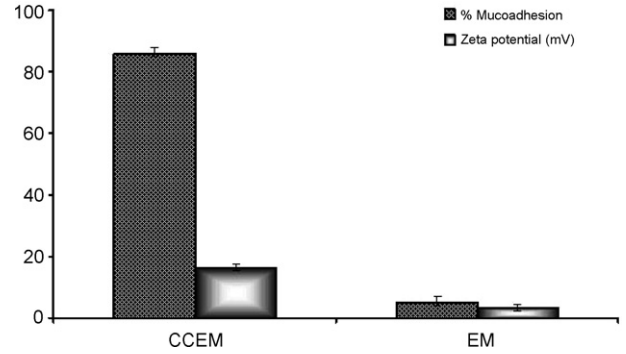

Fig. 7. Mucoadhesion and zeta potential study of Eudragit microspheres of DF and Con-A conjugated Eudragit microspheres of DF. The values are mean ± S.D. ($n = 3$).

beneficial for interaction of formulation with the gastric mucosa. The positively charged drug loaded microspheres are expected to interact with the negatively charged sialic acid and fucose residue of mucin in the intestine by electrostatic interactions and prolonged the residence time.

3.8. Gamma scintigraphy

The most useful technique, to date, to evaluate *in vivo* behavior of dosage forms in animals and humans is external scintigraphy or gamma scintigraphy. Since first employed to investigate the functionality of tablets and capsules *in vivo* (Casey et al., 1976), gamma scintigraphy has become an established technique and is extensively used to monitor the performance of novel drug delivery systems within human GI tract. After testing the *in vitro* colon targeting behavior of the developed formulation, it was worthwhile to assess the *in vivo* behavior of formulation in terms of residence time of formulation in different parts of GI tract. The gamma scintigraphy of the optimized formulation and marketed product of DF were performed in rabbits in order to establish the colon targeting potential of optimized formulation. Marketed uncoated product of DF, i.e. Amicline was used for comparison with optimized formulation. The uptake of pertechnetate anions by CCEM, EM and Amicline was determined using auto gamma counter. The uptake values were 93 ± 0.7%, 90 ± 0.2% and 92 ± 0.5% for CCEM, EM and amicline, respectively. The organ distribution study was performed in rats in order to measure the labeling efficiency of the formulation (CCEM) with ^{99m}Tc. Measurable radioactive counts in stomach, small intestine and colon while zero counts in thyroid gland where observed. The counts per minute (CPM) were 156.8 ± 4.2, 88.4 ± 4.0 and 65 ± 3.2, in stomach, small intestine and colon, respectively. Thyroid gland is the target organ of free ^{99m}Tc and if the formulation is not properly tagged with ^{99m}Tc, it will go to thyroid gland. Therefore zero counts in thyroid gland confirmed the proper tagging of ^{99m}Tc with the formulation (Jain et al., 2006).

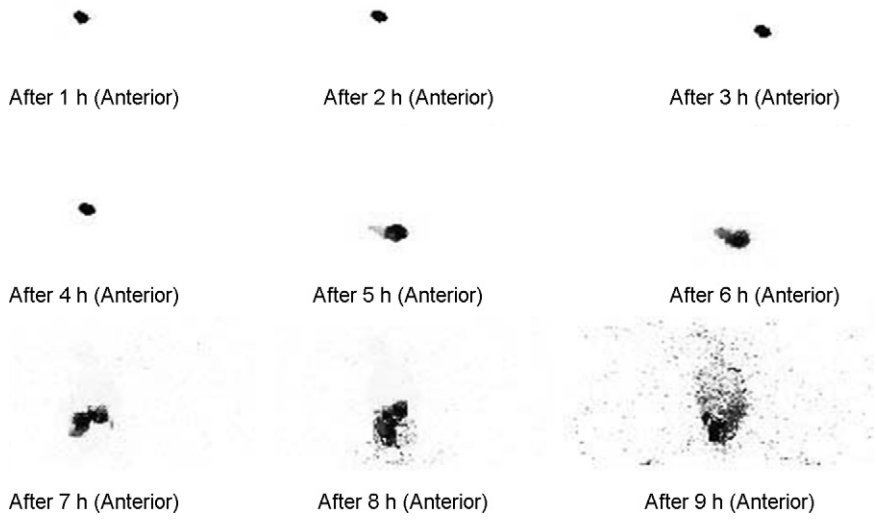

Fig. 8. Gamma scintigraphic images of Con-A conjugated Eudragit microspheres of DF in rabbit at different time intervals.

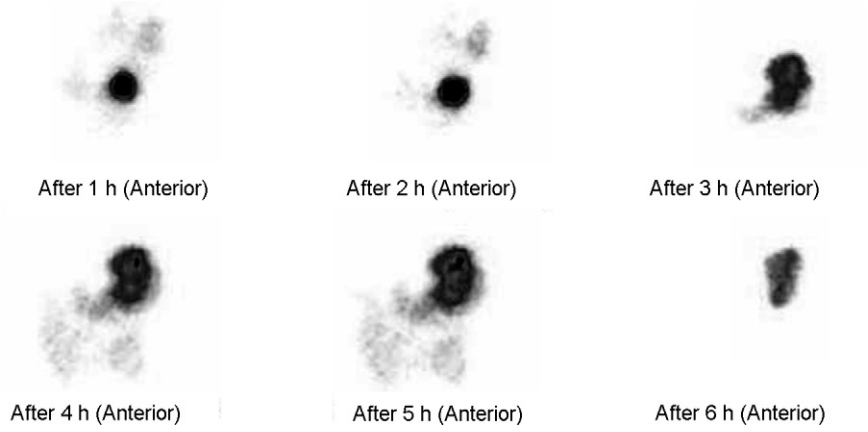

Fig. 9. Gamma scintigraphic images of Eudragit microspheres of DF in rabbit at different time intervals.

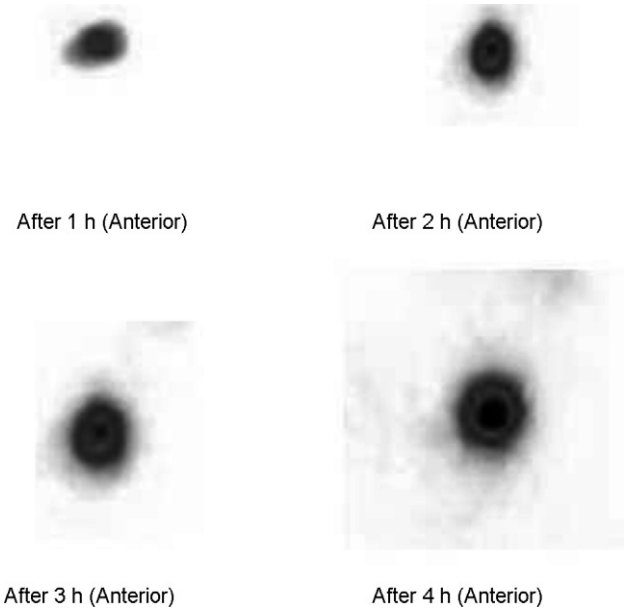

Fig. 10. Gamma scintigraphic images of Amicline (Tablet) in rabbit at different time intervals.

The scintigrams obtained in gamma scintigraphic studies of CCEM (Fig. 8) clearly showed that the capsule was completely intact in the stomach up to 2 h, the marker particles visualized as dark-colored dots allow one to recognize the shape of the capsule. The capsule started to release microspheres in small intestine in small amount after 4 h. The mean transit time from stomach to colon was found to be 5.0 ± 0.35 h. Scintigraphic images indicate that the capsule started to disintegrate in colon after 5 h and disintegrated completely within 1 h. Scintigram shows the residence of microspheres in colon up to 9 h. These results showed that formulation CCEM may be able to target Con-A conjugated microspheres of DF to the colon. The scintigram of formulation EM showed the residence of microspheres in GI tract including colon was up to 6 h (Fig. 9). The scintigraphic images of marketed formulation of DF indicate that within 1 h, Amicline started to disintegrate in stomach and completely disintegrated in 4 h study period (Fig. 10).

4. Conclusions

Optimized Eudragit microspheres of DF were successfully prepared using solvent evaporation method and Con-A was successfully attached to the Eudragit microspheres. Attachment of lectin to the Eudragit microspheres significantly increases the mucoadhesiveness and also controls the release of DF in simulated GI fluids. Gamma scintigraphy study suggested that Eudragit S100 coated gelatin capsule retarded the release of Con-A conjugated microspheres at low pH and released CCEM slowly at pH 7.4 in the colon.

Acknowledgements

One of the authors, Ms. Nalini Anande, expresses her sincere thanks to All India Council of Technical Education, New Delhi, India, for awarding the Junior Research Fellowship during her M. Pharm. program. The authors gratefully acknowledge Head, SLT Institute of Pharm. Sci., Guru Ghasidas University, Bilaspur, India, for providing the necessary facilities to carry out the research work. The authors also wish to thank Dr. Amitabh Arya, Jawaharlal Nehru Cancer Hospital & Research Centre, Bhopal, India, for allowing gamma scintigraphy study.

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