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pH RESPONSIVE CASEIN MICROPARTICLES AS A CARRIER FOR METHOTREXATE

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ABSTRACT

Casein microparticles cross linked with glutaraldehyde were prepared by steric stabilization process and the controlled release of drug from the particles were evaluated *in-vitro*. Casein is a natural biopolymer present abundantly in milk is utilized because of its biocompatibility through oral route. Methotrexate loaded Casein microparticles of size 0.5 to 5 µm were prepared. The release of methotrexate from the casein microparticles were evaluated in simulated gastric and intestinal fluids *in-vitro*. The coating of the particles with Eudragit L 100 made them as pH sensitive and therefore we found a significant release in the intestinal pH. The particles were resistant to proteolytic activity which can increase the durability of the particles in the intestine. This study demonstrates that the casein microparticles can be made pH sensitive and it can be successfully used as a targeted delivery system.

INTRODUCTION: The novel drug delivery technologies are focused to enhance the pharmacodynamic and pharmacokinetic profiles along with the patient comfort and safety. The effective delivery system should be targeted to improve the performance of the drugs. The various methods for targeting orally administered drugs to the colon are covalent linking of a drug with carrier, pH sensitive matrix, timed release systems, bioadhesive polymers etc. Protein carriers had been used as drug delivery vehicles. Casein, a naturally available protein found in milk has been used as a drug carrier.

Casein consists of a large number of proline residues and is relatively hydrophobic, soluble in alkali. Casein has been reported as a drug delivery agent for many therapeutic molecules like Doxorubicin [1], Theophylline [2,3], Mitoxantrone [4], Progesterone [5], Fluorouracil [6] and Propranolol HCL [7]. Casein, a potential matrix for controlled release of the drug has been reported [8]. The usage of polymer as steric stabilizers like poly (methyl methacrylate) for the preparation of albumin microspheres has been already established [9,10]. Similarly cellulose acetate butyrate 3% w/v has been used for the preparation of casein microparticles [11].

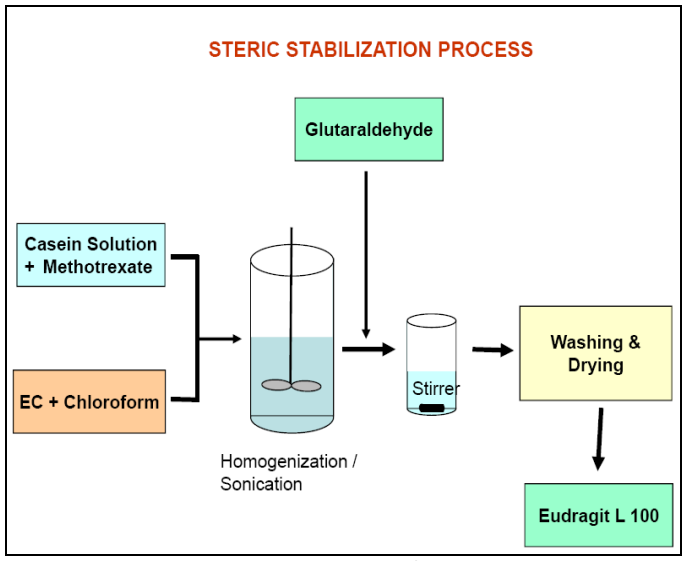
Also usage of oil in the preparation of microparticles needs excess of washing step for complete removal of oil traces, which leads loss of loaded hydrophobic drug during preparation. Hence a method which utilizes easily vaporizable solvent as organic phase and polymeric substance as steric stabilizing agent is needed.

Methotrexate (MTX), the most widely used antimetabolite, is a folic acid antagonist. It has an essential role in the treatment of acute lymphocytic leukemia, choriocarcinoma, non-Hodgkin's lymphoma, osteosarcoma and breast cancer [12]. It is a therapeutic alternative in the treatment of severe psoriasis, suppression of graft-versus-host disease after bone marrow transplantation and various rheumatic diseases. However it has a short half life time of 1.5-3.5 hours when given orally [13] and hence its tumor exposure time is considered short. In order to have an increased tumor exposure time the release of methotrexate can be prolonged. This can be achieved by means of a matrix assisted drug delivery. Eudragit exhibits favorable behavior, such as no toxicity, positive charge and controlled release profile this make them suitable for drug delivery application [14-18]. Glutaraldehyde cross linked and Eudragit L 100 casein microparticles were prepared and evaluated for their drug carrying property.

MATERIALS AND METHODS:

Preparation of casein microparticles: Steric stabilization is a mechanism that can explain the ability of certain additives to inhibit coagulation of suspensions. In the preparation of Casein microparticles ethyl cellulose was used as steric stabilizer. The microparticles were Crosslinked with glutaraldehyde and collected by centrifugation. The excess glutaraldehyde was removed by three step acetone wash.

Loading of methotrexate: For physically entrapped MTX, Casein was stirred with Methotrexate and sonicated in the presence of ethyl cellulose. The loaded microparticles were dried and then coated with Eudragit L 100 After the crosslinking time, microparticles were washed with acetone repeatedly, vacuum dried for 12 h and then coated with Eudragit L 100 (gift sample from Roehm Pharma Polymers). The preparation of Casein-MTX microparticles are shown in Scheme 1.



SCHEME 1: PREPARATION OF MTX/CS PARTICLES COATED WITH EUDRAGIT L 100

Encapsulation efficiency of casein microparticles: Encapsulation efficiency was calculated by weighing 10mg of the microparticles and dispersing them in 10 ml of phosphate buffer saline[19].The sample was ultrasonicated and left to equilibrate for 24 hours at room temperature. The suspension was centrifuged at 5500 rpm and the concentration of Methotrexate was analyzed in the supernatant at 303 nm.

$$\text{Encapsulation Efficiency (\%)} = \frac{\text{Actual Weight } (W_a)}{\text{Theroitical Weight } (W_t)} \times 100$$

Morphological characterization using SEM (Scanning Electron Microscopy): The diameter and the surface morphology of the prepared particles were analyzed using scanning electron microscopy. The particles were sprinkled on adhesive aluminium stub and then surface coating was done with gold to a thickness of ~300 Å using a sputter coater.

***In-vitro* release of Methotrexate:** Methotrexate loaded casein microparticles were subjected to *in-vitro* release in the simulated gastric fluid (as per U.S.P) and simulated intestinal fluid (Phosphate Buffer Saline) without enzymes. Known amount of methotrexate loaded casein microparticles was taken in a dialysis cassette along with 0.5 ml of simulated intestinal fluid and immersed in the 100 ml of simulated intestinal fluid. The dissolution was done at 100rpm at 37°C. Aliquots were collected at predetermined points and an equal amount of buffer was replaced to maintain the volume. The amount of methotrexate was quantified by UV-Vis Spectrophotometer (UV 1700 Schimadzu) at 303nm.The release of methotrexate in simulated gastric fluid was also analyzed.

Equilibrium swelling studies of microparticles: A preweighed amount (100 mg) of microparticles was placed in Phosphate Buffer Saline (PBS, pH 7.4) and allowed to swell up to a constant weight. The microparticles were removed and blotted with filter paper, and their changes in weight were measured. The degree of swelling (α) was then calculated from the following formula,

$$\alpha = \frac{W_f - W_i}{W_i}$$

where W_i is the initial weight of the microparticles and W_f is the weight of the microparticles at equilibrium swelling in the medium.

Fourier Transform Infra Red Spectroscopy: FTIR measurements were taken at an ambient temperature. Known amount of samples were grounded thoroughly with Potassium Bromide (KBr) and the pellets were formed under a hydraulic pressure of 600 Kg/cm^2. The characteristic adsorption bands for the polymer (casein), Ethylcellulose, Glutaraldehyde, unloaded particles and loaded particles were determined.

RESULTS AND DISCUSSION: In the present study, MTX/CS/Eudragit L 100 microparticles were prepared by steric stabilization method. Crosslinked casein microparticles are widely attracted as carrier for cytotoxic drugs. It is also

reported that removal of the free amino groups in the protein reduces its proteolytic susceptibility. This can be accomplished by crosslinking casein microparticles with glutaraldehyde; this increases stability to the microparticles in the gastrointestinal tract. Unlike synthetic polymer employed for encapsulation of drugs, biopolymers have the advantage of better biocompatibility and non-toxicity. This is particularly true with the milk protein casein as it forms an integral part of our daily diet.

The advantages of using polymeric stabilizer include improved hydrophilicity, better particle size control and the absence of adsorbed surfactant that might influence tissue reaction and drug release. However they used a 25-30% solution of polymers such as poly (methyl methacrylate) for the preparation of albumin microspheres. In this method we have used ethylcellulose, a derivative of cellulose as a steric stabilizing agent. The property of ethylcellulose as stabilizer of water-in-oil emulsion was due to its interfacial activity; Ethylcellulose can be used as a polymeric emulsifier. Its stabilizing action was identified to be in part due to the formation of a colloidal precipitate which occurs at the o/w interface. The microparticles were made enteric by coating it with Eudragit L 100 which makes the microparticles to resist the gastric pH and thereby they get solubilize above pH 6.

Methotrexate loaded CS/Eudragit L100 microparticles, observed by Scanning Electron Microscope were spherical (**Fig. 1**). SEM also reveals that the particles range from 0.5 to 5 µm in diameter. The in vitro drug release profiles of microencapsulated Methotrexate were studied using dialysis membrane. The percentage methotrexate released in the Simulated Intestinal Fluid (without enzymes) was 68.57±1.16 at the end of 12 hour and 75.64±1.36 at the end of 24 hour (Fig. 2). The percentage drug released in the Simulated Gastric Fluid (without enzymes) was 0.40±0.10 at the end of 12 hour and 0.67±0.15 at the end of 24 hour (**Fig. 2**). The decreased/no release of methotrexate in SGF reveal the enteric performance of Eudragit L100. We achieved 80.52±2.73 percentage release at the end of 24 hours in Simulated Intestinal Fluid (with Protease) (**Fig. 3**) and 0.90±0.10 percentage release at the end of 24 hours in Simulated Gastric Fluid (with Pepsin) (Fig. 3).

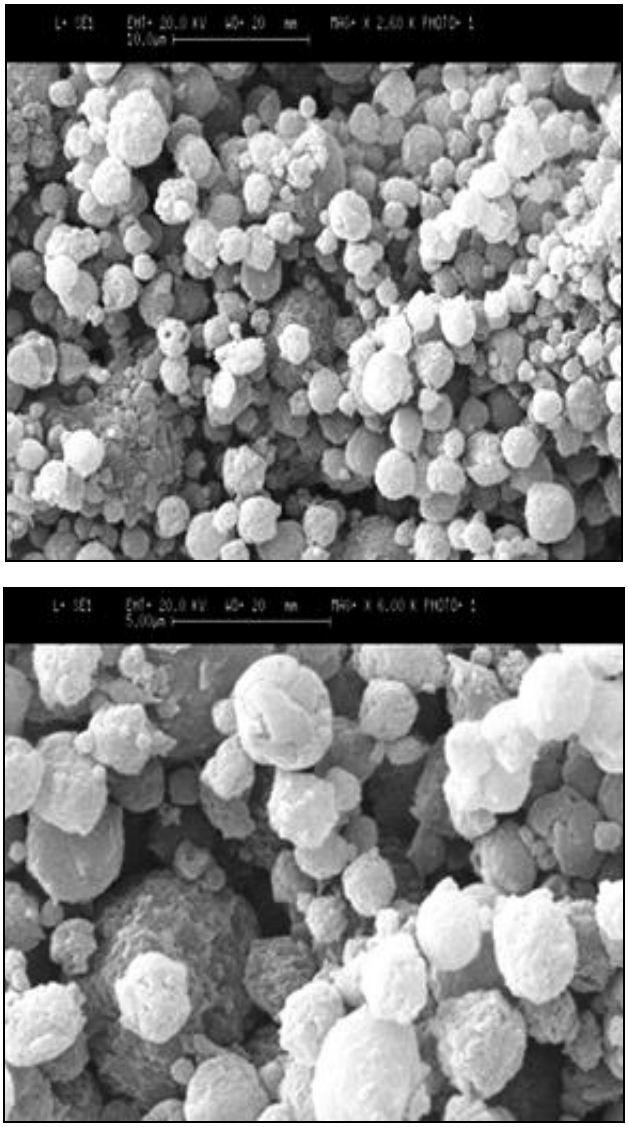


FIG. 1: SEM MICROGRAPHS OF CASIEN MICROPARTICLES LOADED WITH MTX

Release pattern of methotrexate from casein microparticles in presence of protease and pepsin doesn't deviate much from release pattern without enzymes; hence the particles were resistant to protease and pepsin activity. Methotrexate may be covalently bound to the protein mediated by glutaraldehyde.

Thus, glutaraldehyde may react in three different ways:

1. By binding to two methotrexate molecules,
2. By binding to two amino groups of the protein chain,
3. A glutaraldehyde fraction may form a bridge between the protein and methotrexate, giving origin to a protein methotrexate conjugate. The loading efficiency was found to be 78.64%.

The degree of swelling was found to be more when particles suspended in PBS than in SGF. The release from a matrix-type device, where the release is diffusion controlled is given by the Higuchi model,

$$Q = kt^{1/2}$$

Where Q is the amount released in time t and k depends on the surface area and the diffusion coefficient. Assuming that surface area and diffusion coefficient to be constant throughout the experiment, a plot of Q vs $t^{1/2}$ should be linear (**Fig. 4**). The plots gave a good fit with the correlation coefficient 0.98. Hence the microparticles follows Higuchi model in controlled drug release.

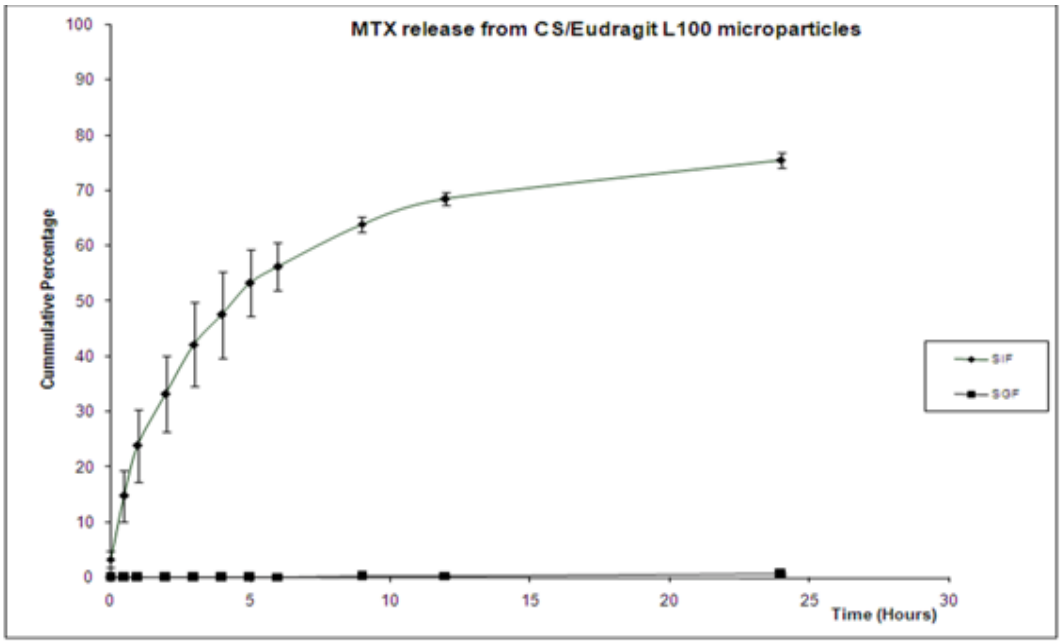


FIG. 2 : ***IN VITRO*** **RELEASE OF MTX FROM MTX/CS/EUDRAGIT L 100 MICROPARTICLES**

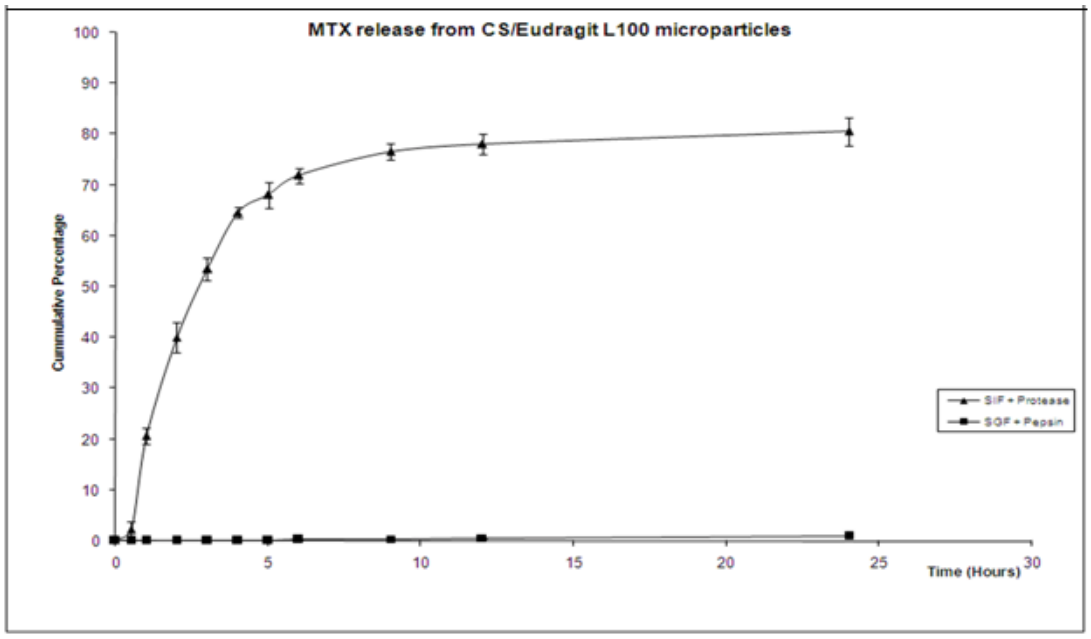


FIG. 3 : ***IN VITRO*** **RELEASE OF MTX FROM MTX/CS/EUDRAGIT L100 MICROPARTICLES IN PRESENCE OF PROTEASE AND PEPSIN**

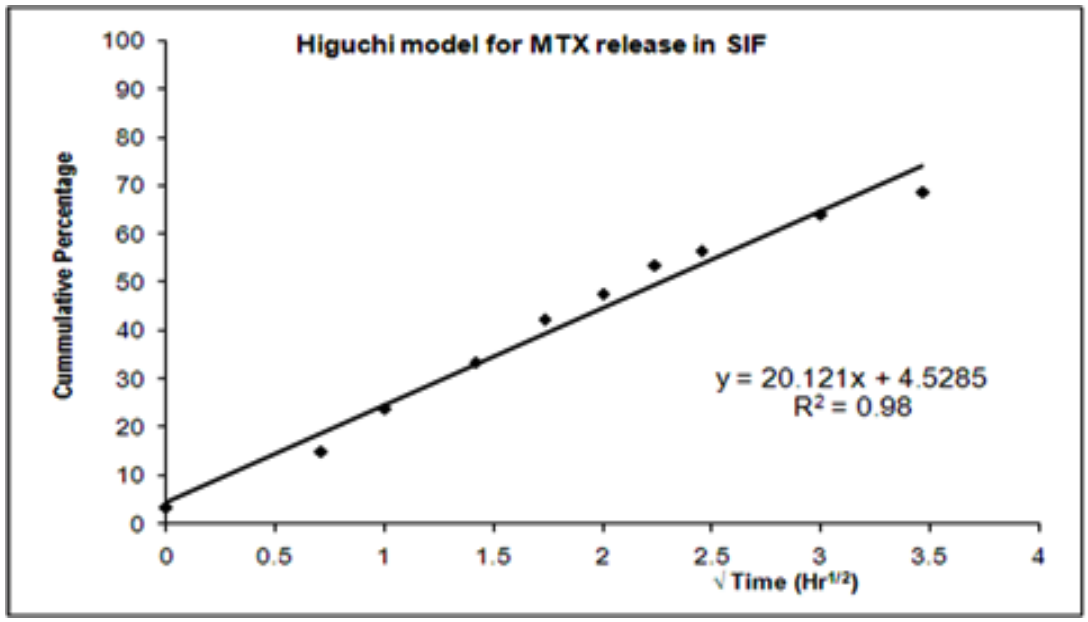


FIGURE 4: HIGUCHI MODEL FOR MTX RELEASE IN SIF

FTIR spectral data were used to confirm the chemical stability of methotrexate in casein microparticles. FTIR spectra of Casein, Loaded casein, Methotrexate, Ethylcellulose and glutaraldehyde are compared in (**Fig. 5**). In case of pure casein N-H bending vibrations is observed at 1644cm^{-1}. Due to the formation of imine group during the cross-linking reaction an extra peak appeared at 1557cm^{-1}. Methotrexate has the characteristics bands at 2.9-3.1 µm, 3.0-4.0 µm, and 6.5-6.6 µm for –NH_2, -COOH, and Amide II vibrations respectively. In drug loaded microparticles these bands were masked may be due to the identical stretching.

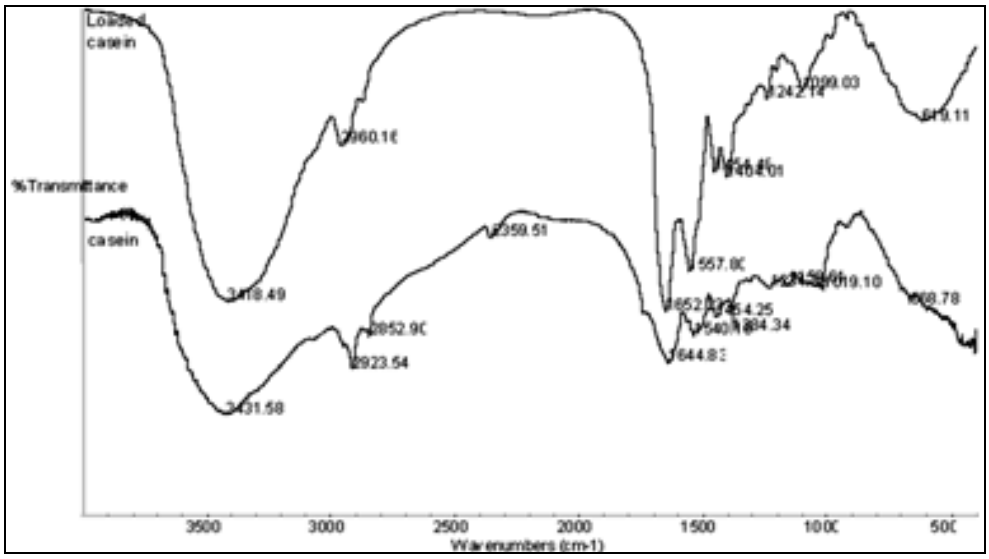


FIG. 5: FTIR ANALYSIS OF PURE CS AND MTX/CS

The appearance of band at 1099cm^{-1} suggests that excess of aldehyde groups were present in final preparation. There were also masking of bands between 1200 cm^{-1} and 1400 cm^{-1} which were characteristics of $-NH_2$ stretch in comparing casein and loaded casein, this may due to reaction between glutaraldehyde and $-NH_2$ group of casein.

CONCLUSION: The steric stabilization technique used for the production of microspheres and the Eudragit coating ensures the drug is not released in the gastric pH. The microparticles become pH sensitive due to the enteric coating and thus it enables the release of methotrexate in the intestine. Casein microparticles were resistant to the proteolytic activity of the enzymes which helps in preventing the toxic effects of methotrexate in the gastric mucosa and also it prevents the loss of methotrexate. Ultimately the release of MTX was found in a controlled fashion and the modulation of release can be achieved by changing the degree of cross linking.

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