Formulation and characterization of Methotrexate loaded sodium alginate chitosan Nanoparticles

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ABSTRACT

The aim of the present work was to formulate nanoparticles for Methotrexate drug. Methotrexate is an anticancer, disease modifying anti rheumatic drug, and BCS Class – III drug having high solubility and low permeability. Nanoparticles were prepared by ionotropic pregelation method using Box Behnen Formula. The concentration of chitosan (X1),concentration of sodium alginate (X2) and concentration of Methotrexate (X3) were chosen as independent variables while particle size, drug entrapment efficiency and percentage drug release at 36th hour, was taken as dependent variables. The dissolution profile of selected formulations was fitted to zero order, first order, Higuchi and Korsemayer Peppas models to ascertain the kinetic modeling of drug release. The prepared formulations were further evaluated for characterization like surface morphology, particle size distribution, zetapotential and drug excipient interaction study by Fourier Transformer Infra Red Spectroscopy, Differential Scanning Calorimetry and X-ray Diffraction. All independent variables were found to significantly influence the particle size, entrapment efficiency and percentage of drug release. The *in- vitro* drug release profile showed that the suitability of sodium alginate-chitosan loaded nanoparticles in controlled release of methotrexate for prolonged time.

Key words: Methotrexate, Sodium alginate, Chitosan, Ionotropic pregelation method.

INTRODUCTION

The use of natural biopolymers specifically polysaccharides in drug delivery has attracted particular interest due to their desirable biocompatible, biodegradable, hydrophilic and protective properties (Barichello JM, 1999). The interaction between biodegradable cationic and anionic biopolymers leads to the formation of polyionic hydrogels, which have demonstrated favorable characteristics for drug entrapment and delivery (Chella F, 2000). Chitosan and Alginate are two biopolymers that have received much attention and have been shown to maintain their structure and activity and protect them from enzymatic degradation (Madan T, 1997). Moreover, many of these polymers, particularly hydrogels, are naturally hydrophilic, which is advantageous since this property is thought to contribute to longer *in vivo* circulation time and allow the highest encapsulation of drug (Douglas KL,2005). Chitosan is a natural cationic polysaccharide obtained by the N deacetylation of chitin, a product found in the shells of crustaceans (Mansouri S,2004). Alginate is an anionic polysaccharide consisting of linear copolymers of a-L-guluronate and b-D-mannuronate residues. Alginates which are a group of hemocompatible polymers have not been found to accumulate in any major organs and have shown evidence of *in vivo* degradation (Mi FL, 2002). In the presence of Calcium ions, ionic interactions between the divalent Calcium ions and the guluronic acid residues cause Alginates to form gels. The properties of Calcium–Alginate gel beads make them one of the most widely used carriers for controlled release systems (Fundueanu G, 1999). Coating of these beads with other polymers including Chitosan has been shown to improve their stability during (shelf-life) storage and their half life in biological fluids.

Alginate–Chitosan polyionic complexes form through ionic gelation via interactions between the carboxyl groups of alginate and the amine groups of chitosan. The complex protects the encapsulant, has biocompatible and biodegradable characteristics, and limits the release of encapsulated materials more effectively than either Alginate or Chitosan alone (Yan XL, 2001). A further advantage of this delivery system is its non-toxicity, which permits its administration to be repeated as a therapeutic agent. Therefore, the purpose of this study was to optimize a method for the preparation of Alginate-chitosan nanoparticles by the use of Box–Behnken methodology to design the most appropriate preparation method.

Rheumatoid arthritis is an autoimmune disease in which inflammation of the cells lining the synovium produces pain, swelling, and progressive erosion of the synovial joints. Methotrexate (MTX), an antiproliferative and immunosuppressive agent, is the drug of choice in the treatment of the disease (Suarez-Almazor ME, 2000). MTX is a folic acid antagonist used alone or in association with other therapeutic agents; however, MTX has an extensive toxicity range, which is the main cause of therapy withdrawal. MTX treatment is discontinued in 8%–19% of patients due to adverse reactions that include gastrointestinal, hepatic, renal, pulmonary, and hematological disturbances, and may also affect the central nervous system (Varatharajan N, 2009).Overall, 26% of patients discontinued MTX treatment due to poor response, high toxicity, or both. Thus, in addition to the relatively high toxicity, variability, and unpredictability of the pharmacological action, there are also drawbacks to the use of MTX treatment for rheumatoid arthritis (Derviex T, 2004).

Unfortunately, progression of joint destruction cannot be inhibited completely by MTX treatment in most patients with RA. This lack of efficacy is due to the fact that large amounts of the administered MTX are rapidly eliminated by the kidneys, resulting in a short plasma half life and low drug concentration in the targeted tissue. To overcome these disadvantageous and improve the pharmacokinetic properties, recently introduced MTX-ALG-CS as a polyelectrolytic complex nanoparticle has a substantially prolonged half life in the circulation.

The nanoparticulate formulation of MTX was optimized using design of experiments by employing response surface methodology. Response surface methodology determines the optimum level of each factor by building a mathematical model. Optimization of particle size and loading efficacy as the responses were carried out by Box–Behnken response surface methodology.

MATERIALS AND METHODS

The polymer Chitosan (CS) was received as a gift sample from India Sea Foods, Cochin. Sodium alginate (ALG) was purchased from Sigma Aldrich and Pluronic F-68 from S.D fine chemicals. Methotrexate was provided as gift sample by Aptuit Pvt. Ltd., Hyderabad. Pluronic F-68 was purchased from S.D fine chemicals. All other solvents and materials used were of analytical grade.

Box- Behnken Formula: Formula combination production was beginning with determination of maximum and minimum concentration for each component that being used. Combination formula of chitosan, alginate, and MTX concentration was conducted to determine the effect of each component against nanoparticle characteristic. At early stage, each component value was entered, including chitosan 0.02-0.06% (w/v), alginate 0.05-0.10% (w/v), and MTX 0.01-0.07 (w/v) into Box-Behnken program. This whole data concentration was processed using Box-Behnken model with 3 level 3 factorial to gain representative data spread. The analysis resulted 17 formulas as recommended optimum combination, with some formula replication. The relationship between independent variables and the response was calculated by the second order polynomial Equation. (1)

$$Y = \beta_o + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 \ldots\ldots\ldots (1)$$

where Y was the predicted response; β was the model constant; x_1, x_2 and x_3 were independent variables; β_1, β_2 and β_3 are linear coefficients; β_{12}, β_{13} and β_{23} are cross-product coefficients; and β_{11}, β_{22} and β_{33} are the quadratic coefficients. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2.

Nanoparticle preparation: Nanoparticles of ALG were obtained by counter-ion induced gelification method (Rajaonarivony M,1993 , Gupta Jitendra ,2011) .Calcium chloride (0.5ml, 18mM), a cross linking agent, was added to 9.5 ml of sodium alginate solution (0.08%w/v) containing MTX under stirring condition. 2 ml of 0.5% w/v of Pluronic F-68 was added. Chitosan solution (2ml, 0.05% w/v) was added followed by sonication at 25 W for 7min and the mixture was kept at room temperature overnight. Drug loaded nanoparticles were recovered by centrifuging at 19,000 rpm for 30-45 min and washed thrice with distilled water to obtain the final nanoparticles.

Characterization of nanoparticles

Transmission Electron Microscopy: The morphological observation of drug-loaded nanoparticles was performed by transmission electron microscopy (TEM) (JEM 1200 EX, Japan), using a negative staining method. A drop of nanoparticle suspension was spread on a 200-mesh copper grid coating and the excess droplets were removed with filter paper. After 5 min, a drop of 4% (w/v) phosphotungstic acid solution was then dropped onto the grids. After being negatively stained and air-dried under room temperature, the samples were subjected to the TEM investigation.

Measurement of Particle Size: The mean particle size was obtained by Photon correlation spectroscopy (PCS) (3000SH, Malvern Instruments Ltd., UK). The MTX loaded Alg-CS nanoparticle formulations were diluted with de-mineralized filtered water to an appropriate scattering intensity. Data was analyzed by the cumulate method assuming spherical particles. Accordingly, the results are given as the effective diameter and the poly dispersity index (PDI) as a measure for the relative width of the particle size distribution.

Measurement of Zeta Potential: The zeta potential value of optimized MTX loaded Alg-CS nanoparticle formulation was measured with the Zetasizer (3000SH, Malvern Instruments Ltd., UK).To determine the zeta potential, optimized formulation was diluted with double-distilled water and placed in an electrophoretic cell.

Fourier Transform Infra-Red Spectroscopy (FTIR): MTX-ALG-CS nanoparticles separated from nanoparticulate suspensions were dried by a freeze dryer, and their FTIR transmission spectra were obtained using a FTIR-8300 spectrophotometer (Shimadzu, Japan). A total of 2% (w/w) of sample, with respect to the potassium bromide (KBr; S.D. Fine Chem Ltd., Mumbai, India) disc, was mixed with dry KBr. The mixture was ground into fine powder using an agate mortar before compressing into KBr disc under a hydraulic press at 10,000 psi. Each KBr disc was scanned at 4 mm/s at a resolution of 2 cm over a wave number region of 400–4000 cm^{-1}. The characteristic peaks were recorded for pure drug and nanoparticle samples.

Differential scanning calorimetry: Differential scanning calorimetric (DSC) analysis was used to characterize the thermal behavior of the drug methotrexate, blank and methotrexate loaded nanoparticles. DSC thermograms were obtained using an automatic thermal analyzer system (Pyris 6 DSC, Perkin-Elmer, USA). Temperature calibration was performed using Indium Calibration Reference Standard (transition point: 156.60 ˚C) as a standard. Samples were crimped in standard aluminum pans and heated from 40 to 400˚ C at a heating rate of 10˚C/min under constant nitrogen at 30 ml/min. An empty pan, sealed in the same way as the sample, was used as a reference.

X-ray Diffraction: The X-ray diffraction (XRD) patterns were determined for the drug methotrexate, blank and methotrexate loaded nanoparticles. Samples were exposed to a monochromatic nickel-filtered copper radiation (45 kV, 40 mA) in a wide-angle X-ray diffractometer (advanced diffraction system, Sci.008/ntag Inc., USA) with 2θangle.

Determination of Encapsulation Efficiency: The encapsulation efficiency of nanoparticles was determined by the separation of drug-loaded nanoparticles from the aqueous medium containing non-associated MTX by ultracentrifugation (REMI high speed, cooling centrifuge, REMI Corporation, India) at 18,000 rpm at 4 ˚C for 30 min. The amount of MTX loaded into the nanoparticles was calculated as the difference between the total amount used to prepare the nanoparticles and the amount that was found in the supernatant. The amount of free MTX in the supernatant was measured by spectrophotometer at 305 nm in suitable dilution with 0.1N HCl. The MTX encapsulation efficiency of the nanoparticles was determined in triplicate and calculated as follows:

$$\% \text{ EE } = \frac{\text{Total amount of drug} - \text{Total amount of unbound drug}}{\text{Total amount of drug}} \times 100$$

Equation—(2)

***In-vitro* release studies:** *In-vitro* release of drug from MTX nanoparticle formulation is determined by dialysis bag method in phosphate buffer saline pH 7.4. The freeze dried MTX nanoparticles (equivalent to 5.0 mg of drug) was taken in a dialysis bag (molecular cut off 12,000, pore size 0.2 µm) and placed in 100 ml of dissolution medium which was continuously stirred at 100 rpm at 37°C using shaker incubator. Definite aliquots of the dissolution medium were withdrawn at specific time intervals and the same volume of fresh dissolution medium was added to the flask to maintain a sink condition. The samples withdrawn were analyzed for drug content spectrophotometrically at 303 nm.

RESULTS AND DISCUSSION

TEM Analysis: TEM analysis confirmed that particles with target size and narrower size distributions could be prepared using a ionotropic pregelation method. Fig (1) showed that MTX-NaAlg-CS nanoparticles had spherical shape with size ranging 100 nm. This was achieved by adapting the optimized parameters for the preparation of nanoparticles.

Particle Size Determination: The particle size of optimized MTX-NaAlg-CS nanoparticle formulation was showed in Figure (2).The mean particle size of the optimised formulation was found to be 188.6 nm and the PDI was found to be 0.265.The low PDI value indicates the uniform particle size distribution which may due to the adoption of optimised formulation parameters.

Zeta potential: The zeta potentials of about -22.4 mV showed in Figure (3) indicate good stability of formulation. This might be attributed to surfactant which decreases the electrostatic repulsion between the particles and sterically stabilizes the nanoparticles by forming a coat around their surface.

FTIR Analysis: The characteristic peaks of MTX was showed (Fig.4a) at 3464 cm^{-1}(-NH stretching), 16483.4 cm^{-1} (COOH), 1653 cm^{-1} (CONH) and 853.88 (aromatic stretch). In the spectrum of MTX-NaAlg-CS nanoparticles formulation, significant peaks were obtained at 3437 cm^{-1}, 1679 cm^{-1}, 1639.1 cm^{-1} and 819.49. Because of the presence of polymer, some additional peaks were present. Fig.(4b). This indicates that there is no interaction between the drug and polymers in the formulation.

Differential scanning calorimetry: DSC studies were performed to investigate the physical state of the drug in the nanoparticles, because this aspect could influence the *in vitro* and *in vivo* release of drug from the system. DSC thermogram of MTX and MTX-NaAlg-CS nanoparticles were showed in Fig (5a, 5b) The thermogram of the drug showed a sharp melting peak at 115.82°C. MTX-NaAlg-CS nanoparticles did not show the melting peak for the MTX at 115.82°C.The endothermic peak of MTX in MTX-NaAlg-CS nanoparticles was depressed, broadened and shifted to lower temperature. These thermal characteristics of MTX corroborate with the reference value reported by (Renu Sigh, 2006). These results could indicate that MTX was not in crystalline state, but is in amorphous state after entrapment with the polymer because drug crystals completely dissolve inside the polymer matrix during the scanning of temperatures up to the melting value or because the drug remained dispersed at molecular level inside the solid dispersion after the formation of MTX-NaAlg-CS nanoparticles (Adamo, 2010).

X-ray diffraction: X-ray diffraction has been used for the study of molecular structure and polymorphism of polymeric nanoparticles (Westesen, 1993 and Bunjes, 1996). XRD pattern of pure MTX, Blank, MTX-NaAlg-CS nanoparticles formulation are illustrated in Fig 6. The XRD pattern of pure Methotrexate from 2-70° 2θ showed distinctive peaks approximately at 13.7, 14.1, 19.6, 27.8 and 29 degrees obtained were comparable with XRD pattern of crystalline MTX reported in literature (Rama, 2012). Blank nanoparticles do not show any high intensity peak revealing the amorpous nature of the polymer and stabilizer respectively. The characteristic peaks of the methotrexate was absent in MTX-NaAlg-CS nanoparticle. This indicates that MTX was molecular dispersed in to the polymeric nanoparticles and there could be less or no free drug in crystalline form on the surface of the nanoparticles. From this, it is evident that an XRD signal of encapsulated drug is very difficult to detect, which showed that the drug is dispersed at a molecular level in the polymeric matrix.(Liu,*et al*., 2006).

Drug release and Release Kinetics: The *in vitro* release pattern of MTX-NaAlg-CS nanoparticle shown the initial burst release followed by the sustained release was observed in optimized formulation (data not shown). During initial hours minimum burst release of the drug from the polymeric nanoparticles was observed followed by prolonged release (68.99%) up to 36 h. The initial burst release may be probably caused by the drug adsorbed on the surface of nanoparticles or precipitation of drug from the nanoparticles. Sustained release was obtained due to slow diffusion of the drug from the polymeric matrix.

To determine the release model that best described the drug release, the *in vitro* release data was substituted in equations of zero order, first order and Higuchi model and the results are noted. Among them the zero order model showed a high R^2 value 0.93443, indicating that the release of the drug followed zero order release kinetics.(Fig 7a) To understand the mechanism of drug release, Korsmeyer–Peppas equation was applied and it showed a good linearity. The release exponent 'n' was found to be 0.79307. (Fig 7b). According to this model, if the value of 'n' was between >0.43 and <0.85, it indicated that drug release followed anomalous transport (Non-Fickian) (Chouhan and Bajpai, 2009 b) and was controlled by more than one process (the coupling of Fickian diffusion and polymer matrix relaxation).

Optimization and validation: The experimental results were fitted into second-order response surface model. The composition of optimized formulation was achieved with 0.05%w/v chitosan, 0.08%w/v of sodium alginate and 0.04%w/v of MTX, which fulfill the requirements of optimization. The optimized formulation has particle size 183.69 nm, entrapment efficiency of about 93.59% and 68.9 % drug release, which were in good agreement with the predicted values. These figures also indicate that the developed models are adequate and predicted results are in good agreement with the measured data.

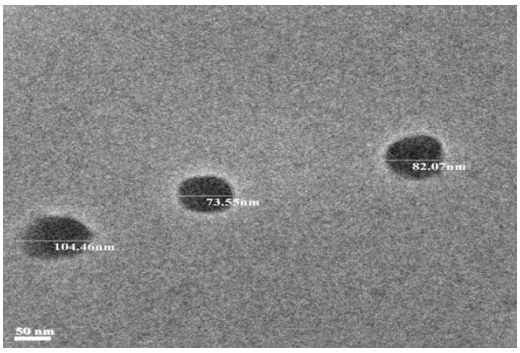

Fig 1 : TEM image of MTX-NaAlg-CS nanoparticles

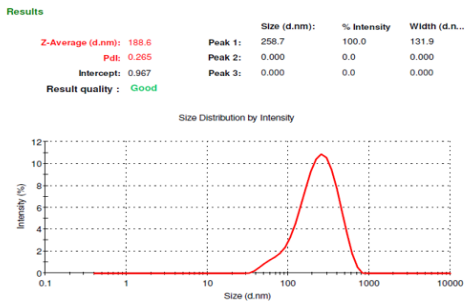

Fig 2: Particle size distribution of optimized MTX-NaAlg-CS nanoparticle

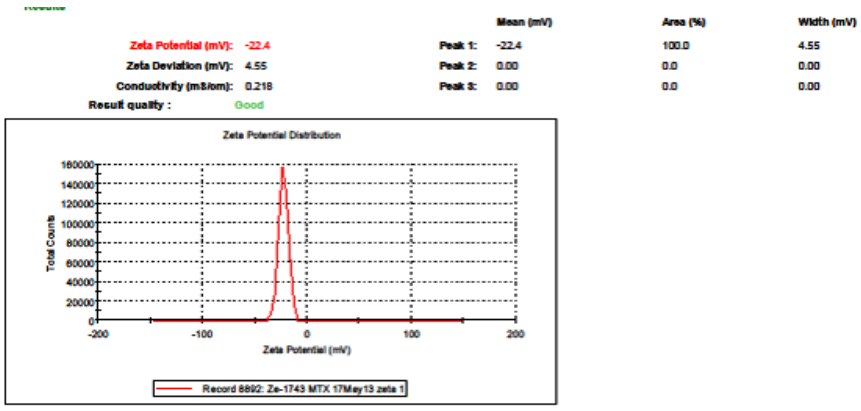

Fig 3: Zeta potential of MTX-NaAlg-CS nanoparticle

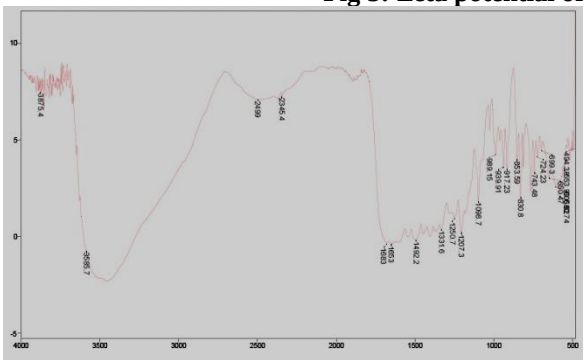

Fig 4a: FTIR spectra of pure Methotrexate

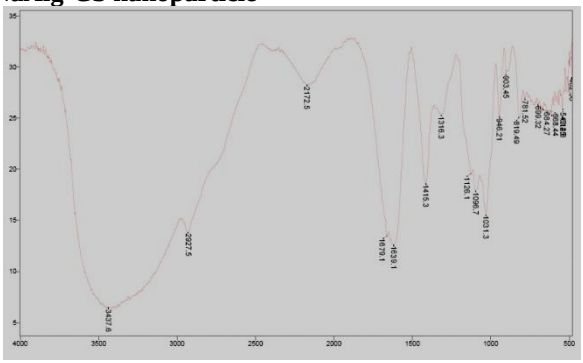

Fig 4b : FTIR spectra of MTX-NaAlg-CS nanoparticle

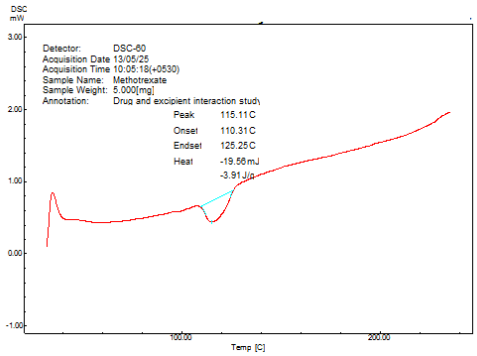

Fig 5a : DSC curve of pure methotrexate

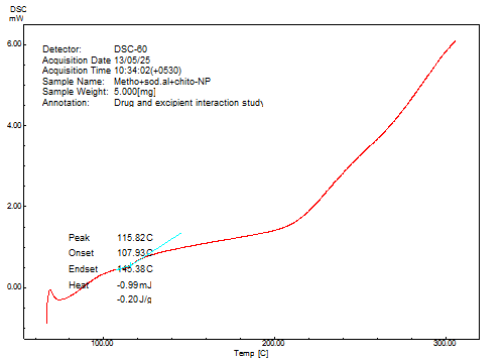

Fig 5b: DSC curve of MTX-NaAlg-CS nanoparticle

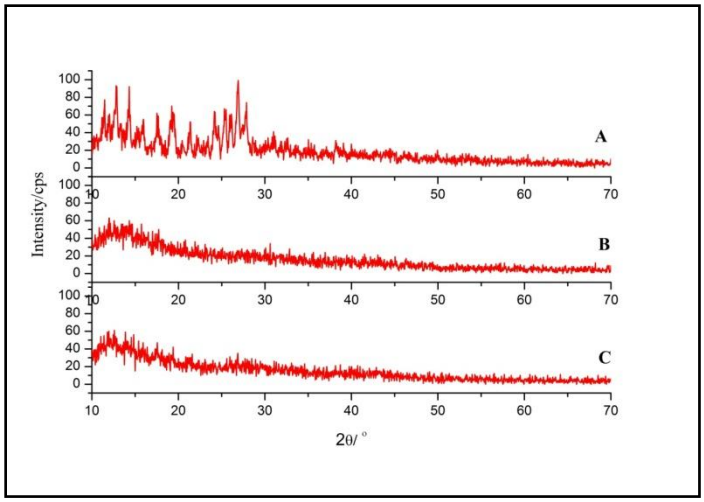

Fig 6 : X-ray diffraction pattern of A) Pure Methotrexate B) Blank nanoparticle

C) MTX-NaAlg-CS nanoparticle

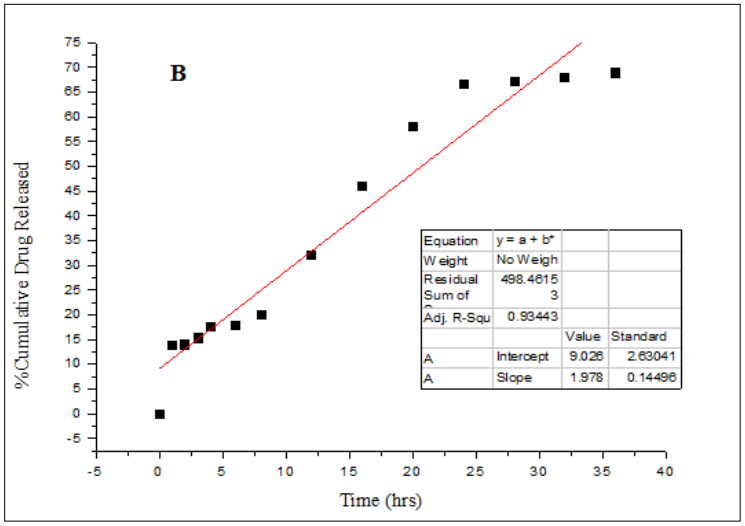

Fig 7a: Zero order release of MTX-NaAlg-CS nanoparticle

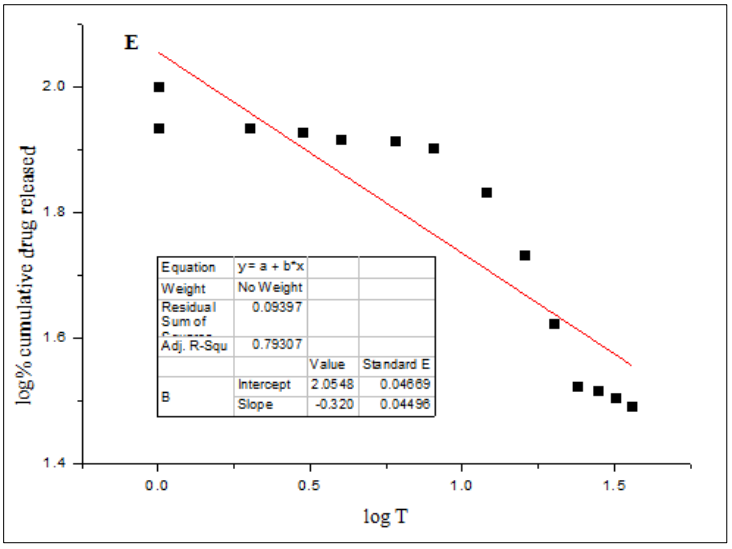

Fig 7b : Korsmeyer-Peppas drug release kinetics of MTX-NaAlg-CS nanoparticle.

CONCLUSION

Methotrexate loaded nanoparticles were prepared by the ionotropic pregelation method. The FTIR, DSC, XRD pattern study did not detect any crystalline drug material in the freshly prepared freeze dried nanoparticles. The application of factorial design gave a statistically systematic approach for the formulation of nanoparticles with desired particle size, high entrapment efficiency and % drug release. Concentration of Drug , Polymers were found to influence the particle size, Entrapment efficiency and % drug release of MTX loaded NaAlg-CS nanoparticles. The release was found to follow with non-Fickian diffusion mechanism for optimized batch. These results indicate that MTX loaded NaAlg-CS nanoparticles could be effective in controlled drug release for a prolonged period would serve the purpose for long term treatment of Rheumatoid Arthritis.

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