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R E S E A R C H A R T I C L E

On Demand Delivery of Methotrexate from Self-Assembled Nanoconstructs for Treatment of Rheumatoid Arthritis

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A B S T R A C T

The aim of the present investigation was to develop and evaluate on demand delivery of methotrexate from self-assembled nanoconstructs for treatment of rheumatoid arthritis. Methotrexate (MTX)-Dextran sulphate (DS)-Poly lactic co-glycolic acid (PLGA) nanoparticles (NPs) can target at inflammatory joints to rheumatoid arthritis if given in the form of nanoparticles as parenteral dosage form. Fourier transform infrared spectroscopy (FTIR) had employed to study drug-excipients incompatibility. Analytical method was performed using UV spectrophotometer. MTX-DS-PLGA NPs was successfully prepared using methotrexate, poly (lactide-co-glycolide) acid (PLGA), dextran sulphate and lecithin by nanoprecipitation method and evaluated for particle size, zeta potential, percent drug entrapment, percent drug loading, surface morphology (Transmission Electron Microscopy), in-vitro drug release study, sterility testing and stability study. Optimization of formulation parameter was done by Box behnken design (BBD) using Design Expert software. Drug and excipients were found to be compatible to each other which were confirmed by FTIR study. Optimization study of formulation parameter shows that batch prepared with Drug: DS (1:0.5), PLGA: (Drug: DS) (80:20), Lecithin (1.25%w/w). Particle size and zeta potential were found to be 146.40 ± 10.64 nm and -37.1 ± 6.32 respectively for optimized batch. Percent drug entrapment, Percent drug loading were found to be 98.80 ± 0.8 % and 9.6 ± 0.81% respectively. Transmission Electron Microscopy (TEM) study indicates that the particles were found to be in spherical shape and porous in nature. In-vitro drug release were found to be and 94.90 ± 0.51 % in 48 hrs. The results of sterility test which described that **MTX-DS-PLGA NPs** was successfully sterile. Stability study shows developed NPs was stable at 4-8 ± 2°C/45±5% RH (Refrigerator; RF) condition after 1 month.The present study demonstrated that nanoparticles may target at blood stream to the inflammatory joints of rheumatoid arthritis with least systemic toxicity.

Keywords: Methotrexate, Rheumatoid arthritis, Nanoparticles, Box-Behnken design

A R T I C L E I N F O

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C O N T E N T S

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease in which the white blood cells interact with antigen derived immune complexes, causing the influx and activation of lymphocytes and monocytes via increased vascularisation. The synovial membrane becomes highly vascularised, synovial fibroblasts proliferate and inflammatory cells release numerous cytokines and growth Factors into the joint[1,4,5,6].Drugs delivers to the patients in a safe, effective and compliant manner is a major challenge for the treatment of many types of disease. When local delivery of high concentration of drug while minimizing systemic toxicity, which is often, observed with oral dosing, however, local depots are typically administered less frequently and include an initial burst followed by a continuous release. To maximize efficiency of therapy, it is critical to ensure the drug is only released when needed. The ability of drugs to reach the target tissues from the point of administered. Oral administered is limited by multiple barriers including enzymatic and acidic degradation in stomach, absorption across the intestinal epithelium, hepatic clearance, and nonspecific uptake.

Methotrexate (MTX) is an anti-metabolite drug. It is considered a Disease Modifying Anti Rheumatic Drugs (DMARDs). It inhibits the activity in the joints. As a cytotoxic drug it may slow the rapid growth of cells in the synovial membrane that lines in the joints. It can reduce inflammation slow the progression of the disease. MTX is now the most commonly used slow acting antirheumatic drug for rheumatoid arthritis. MTX inhibits proliferation of the lymphocytes and other cells responsible for inflammation in the joint. From the pharmacokinetic and clinical studies it is proved that injectable MTX if preferable over oral MTX because of higher absorption and bioavailability. Oral or injectable MTX having systemic toxicities such as hepatic, renal, gastrointestinal, hematologic, immunologic, nervous system, respiratory, genitourinary, dermatologic, musculoskeletal, hypersensitivity, oncogenic, cardiovascular, endocrine, ocular and other general toxicities like malaise, fatigue, and chills which may overcome by local application of MTX[2,25].Currently marketed formulation of MTX include conventional tablet, intramuscular and subcutaneous injection. The problems with conventional formulation are its low solubility and bioavailability and its effect on gastric environment and hepatic metabolism while in injection, systemic toxicity is increased.

We have focused our attention on the development of Methotrexate loaded Dextran sulfate- poly lactic co-glycolic acid (MTX-DS-PLGA) self-assembled nanoparticles (NPs) that can easily administer via intravenous route. Self-assembled MTX-DS-PLGA NPs have amphiphilic block copolymer which composed of hydrophilic DS as the targeting ligand and PLGA as the hydrophobic segment. PLGA NPs encapsulate the lipophilic drugs such like MTX. MTX is a folate analogue to inhibit dihydrfolate reductase. MTX combined with Dextran sulphate used as targeting ligand. Dextran sulphate is a representative ligand for macrophage scavenger receptor class A (SR-A).[13,14]PLGA polymer can encapsulate MTX and increase encapsulation efficiency. In that MTX-DS-PLGA NPs have potential to effectively reach the inflammatory joint region of RA via both active and passive mechanism.[3]

The aim of present investigation was to develop and evaluate Self assembled Methotrexate loaded Dextran sulphate-Poly lactic co-glycolic acid nanoparticles[24,28] for the treatment of rheumatoid arthritis.

2. Materials and Methods

Table 1: List of Materials

Materials	Use	Suppliers
Methotrexate[15,16]	Anti rheumatoid agent- API	Gift sample from Intas Biopharmaceuticals Pvt. Ltd., Ahmadabad, India
Poly (D, L – lactide -co-glycolide) acid[17,18,30]	Hydrophobic polymer	Strides Arco Lab., Bangalore, India
Dextran sulphate[19]	Hydrophilic polymer	Aatur chemicals, Vadodara
Lecithin[20]	Phospholipid as a surfactant	Aatur Chemicals, Vadodara
Acetonitrile, Ethanol	As a solvent	Sisco Research Lab. Ltd. Mumbai, India

Table 2: List of Equipments/ Machine

Equipments	Company and Model no.	Purpose
UV Spectrophotometer	Shimadzu1800 240 V Japan	For Drug Analysis
Fourier transform infrared spectroscopy (FTIR)	Bruker, Mumbai, India	For identification &compatibility study
Malvern Zetasizer Nano ZS	Malvern instruments ltd., UK	For particle size &zeta potential measurement
Magnetic stirrer	System Anatech, Mumbai, India	For preparation of nanoparticles
Transmission Electron	JEOL JEM-2100 HR	For morphological

Microscopy (TEM)		characteristics

Preformulation Study

Preformulation study is desired to ensure the development of a stable as well as the therapeutically effective and safe dosage form. Preformulation testing is designed to assess the influence of physicochemical properties of drug substances and excipients on the formulation properties of the dosage form, method of manufacture and pharmacokinetic-biopharmaceutical properties of the resulting product. A thorough understanding of physicochemical properties may ultimately confirm that no significant barriers are present for the formulation development.

Fourier transforms infrared spectroscopy (FTIR):

FTIR spectra for drug alone and with excipients mixture were recorded using a FTIR spectrophotometer with KBR pellets to study drug-excipients interaction and compatibility. The FTIR studies were carried out by the pressed pellet technique using a KB rpressin which the KBr was taken and Kept in a hotairoven for two hours for the removal any moisture. The above dried KBr was taken for the preparation of pellets of drug, and the selected formulation excipients. The prepared pellet was placed in the sample holder and kept in the instrument to record the IR peaks. The results of the infrared studies for the drug and with powder mixture werere corded. FTIR spectra of Methotrexate and physical mixture of drug and excipients are shown in figure 5.1 and 5.2 respectively. The spectral elucidations for drug alone and powder mixture are shown in table 5.1.

Analytical Method: Analytical method was performed by UV Spectrophotometer[26].

Calibration curve of Methotrexate in Phosphate Buffer saline pH 7.4

Preparation of stock solution:

Accurately weighted 5mg of methotrexate was transferred to 50 ml volumetric flask and add 10 ml of phosphate buffer saline pH 7.4 to dissolve methotrexate. Final volume was made up to 50 ml with phosphate buffer saline pH 7.4.

Preparation of calibration curve:

The above stock solution was scanned for the maximum absorbance using UV Visible double beam spectrophotometer. The λ_{max} of Methotrexate in phosphate buffer saline pH 7.4 was found to be 302 nm. The above stock solution (100 µg/ml) was further diluted to get concentration of in the range of 4-16 µg/ml. Absorbance of each solution was measured using UV-Vis double beam spectrophotometer by putting reference standard of respective medium. The standard curve was generated for entire range of concentrations and the experiment was performed in triplicate. The standard calibration curve data and graph are shown in table 5.2 and figure 5.3 respectively.

Preparation of Methotrexate loaded Dextran sulphate-Poly lactic co- glycolic acid nanoparticles:[7,11,12]

Nanoprecipitation method was used for preparation for NPs. It is a straightforward technique, rapid and easy to perform. The NPs formation is instantaneous and the entire procedure is carried out in only one step. Briefly, it requires two solvents that are miscible. Both the polymer and the drug must dissolve in the first one (the solvent), but not in the second system (the non-solvent). Nanoprecipitation occurs by a rapid desolvation of the polymer when the polymer solution is added to the non-solvent. Indeed, as soon as the polymer-containing solvent has diffused into the dispersing medium, the polymer precipitates, involving immediate drug entrapment.[11]

DS-PLGA NPs was prepared by a combined self-assembly technique and nanoprecipitation method as previously described with some modifications. In brief, PLGA polymer (with different ratio) was dissolved in acetonitrile. Lecithin as a surfactant (different Percentage) and Dextran sulphate was dissolved in 4% w/v of ethanol aqueous solution. Simultaneously the MTX was dissolved in PBS pH 7.4. Then MTX solution was mixed with Lecithin-DS solution under magnetic stirring. After 5 min of gentle stirring of the combined solution, the PLGA solution was injected drop by drop into the Lecithin-DS-MTX solution. The mixed solution was stirred on magnetic stirrer at room temperature. Removal of organic solvent leads to formation of DS-PLGA NPs. To confirm the effect of DS on the enhancement of encapsulation efficiency, the DS-PLGA NPs without DS the PLGA NPs was prepared as the same procedure by replacing DS solution with distilled water. Different surfactants such as hydrogenated soya phosphotidyl choline (HSPC) and pluronic F127 effect was studied to optimize MTX-DS-PLGA NPs.

Formulation Development

Optimization of Formulation Variables:

There are various formulation parameters which may have impact on the various characteristics of product. Thus, first attempt were made to evaluate the effect of these parameters on formulation of nanoparticles.

Drug: Dextran sulphate (DS) (molar ratio), PLGA: Drug: DS (molar ratio) and Lecithin (%) were taken as formulation variables have major impact on the nanoparticles suspension. Quantitative aspects of the effects and relationships among various formulation parameters affecting spray drying of drug were in vestigatedusingresponsesurfacemethodology (RSM). To study this, weper formed, “Box Behnken Design” (BBD)on three critical process parameter known to affect the ir results. The BBD is a popular template for RSM because it requires only three-levels of eachprocess factor and only a fraction of all the possible combinations. In this design, the experimental region is assumed to be a cube, and experiments are performed at points corresponding to midpoint of each edge and replicated experiments at the center of this multi dimensional cube. This design is suitable for exploring gquadratic response surfaces and constructing second-order polynomial models. Thecompletedesignconsistedof17experimentalpointsthatincluded17 factor points and three replications at the centre point. The non-linear quadraticmodel generated by the design is as follows:

$Y = \beta0 + \beta1\ A + \beta2\ B + \beta3\ C + \beta12\ AB + \beta13\ AC + \beta23\ BC + \beta11\ A^2 + \beta22B^2 + \beta33\ C^2$ ---------- Equation 1

Where; Y is the measured response (dependant variable)

associated with each factor- level combination; expressed in terms of particle size, zeta potential, Percent drug entrapment of the formulation. A,B and Carethe (formulation variables studied) respectively. The Design Expert (Version 9, State Ease Inc.,USA) program was used for design of experiment and analysis of this second-ordermodel and for drawing of three- dimensional response surface and contourplots. Dependent and Independent Variables in BBD are shown in table 4.3 and Matrix ofBox Behnken design foroptimizing formulation variables are shown in table 4.Results for optimization of formulation variables are shown in the table 5.

Lyophilization of optimized batch: B_{13}

In aqueous suspensions, the chemical and physical stability of nanoparticles has been reported to be poor. Freeze-drying has been the most utilized drying method of nanoparticles suspensions. Because the freeze-drying process is highly stressful for nanoparticles, addition of cryoprotectants becomes essential. For nanoparticles carbohydrates have been perceived to be suitable freeze-drying protectants. There are considerable differences in the cryoprotective abilities of different carbohydrates. The optimized batch of nanoparticles was lyophilized using mannitol (at 1:1, 1:2 and 1:3 NPs: cryoprotectant) to select suitable cryoprotectant and its concentration. The redispersibility of the freeze-dried formulations and particle size of the NPs before and after freeze-drying were evaluated. Results are shown in table 5.5.

Statistical Analysis: [42]

For optimization Box-Behnken design was employed to study the effect of independent variables (A) Drug: Dextran sulphate (molar ratio) (B) PLGA: (Drug: DS) (molar ratio) and (C) Lecithin (%) on dependent variable (Y1) particle size, (Y2) percent drug entrapment. All the batches are prepared according to the design and analyzed using the design expert 9.0software.

The non-linear quadratic model generated by the design is as follows:

$$Y = \beta 0 + \beta 1\, A + \beta 2\, B + \beta 3\, C + \beta 12\, AB + \beta 13\, AC + \beta 23\, BC + \beta 11\, A^2 + \beta 22 B^2 + \beta 33\, C^2$$

Where; Y is the measured response (dependant variable) associated with each factor - level combination; expressed in terms of particle size (µm) (Y1), percent yield (Y2) percent drug entrapment A, B and C are the (Independent variables) Drug: Dextran sulphate (molar ratio)PLGA: (Drug: DS) (molar ratio) and Lecithin (%) respectively.

Equation expresses the quantitative effect of the process variables (A, B, and C) and combination thereof on the responses (Y1 and Y2) in terms of interaction coefficients. The values of the coefficients A to C are related to the effect of these variables on the response (Y). Coefficients with more than one factor term and those with higher order terms represent interaction terms and quadratic relationships respectively. A positive and negative signs suggest a positive and negative effect on response respectively. The results of analysis of variance ANOVA with the values of R^2, adjusted R^2, predicted R^2, Degree of freedom (Df) and Mean square (MS) are given in table 5.6 and 5.7 along with the response surface & contour plots generated for each response, which are shown in figure 5.4 to 5.9. Also the validation was done by preparing check point batch. The t_{cal} value was calculated and compare with t_{tab} value to check the similarity between the predicted responses and actual responses.Figure 5.10 shows overlay plot showing combined effect of Particle size, Percent drug entrapment and table 5.8 shows results of Check Point Batch 13.

Characterization of Methotrexate loaded Dextran sulphate-Poly lactic co- glycolic acid nanoparticles[7]:

Particle size measurement and zeta potential:

Particle size and zeta potential of prepared nanoparticles was measured by dynamic light scattering using Malvern Zetasizer.Results of particle size are shown in table 5.3 and Results of optimized batch shown in table 5.9 and figure 5.11. Results of zeta potential shown in table 5.

Percent drug entrapment and percent drug loading:

The percentage of drug encapsulation was determined by UV visible spectrophotometric method. Nanoparticles suspension was centrifuge at 18000 rpm and at 4°C for 30min for removal of free drug. Sediment was collected and dissolved in acetonitrile and estimated spectrophotometrically using UV visible spectrophotometer at 302 nm for entrapped drug. Percent drug entrapment and Percent drug loading was calculated from Eq 1 and Eq 2.

$$\text{Percent drug entrapment (PDE)} = \frac{\text{Amount of drug present in Nanoparticles}}{\text{Theoretical drug loading}} \times 100 \quad (1)$$

$$\text{Percent drug Loading (PDL)} = \frac{\text{Amount of drug present in Nanoparticles}}{\text{Weight of Nanoparticles sample analyzed}} \times 100 \quad (2)$$

Results of percent drug entrapment and percent drug loading are shown in table 5.9.

Transmission electron microscopy (TEM):

The morphology of NPs was examined using transmission electron microscopy JEOL JEM-2100 HR. A drop of NPs suspension was visualized after staining with phosphotungstic acid on a copper grid under TEM. Photograph of TEM images of optimized batch B_{13} are shown in figure 5.13.

***In-vitro* release studies:**

The drug release from NPs was determined using dialysis bag technique in phosphate buffer saline (pH 7.4). An aliquot of 1 ml of NPs suspension in water with MTX concentration of 0.5 mg/ml was seal in a dialysis bag (molecular weight cut off 12,000 Da) and immersed in 50 ml of preheated release medium continuously stirred at 50 rpm and maintained at 37 °C on magnetic stirrer. Asampleof5mlwastakenfromthereceiversolutionat predeterminedtime(hr)intervalsandreplacedwithanequal amountof PBS pH 7.4. Samples were analyzed at 302 nm using UV spectrophotometer after suitable dilutions, if required. Result of % cumulative drug release and graph of release profile for nanoparticles are shown in the table 5.10 and figure 5.14respectively.

Sterility testing: [43]

Sterilization of optimized batch was performed by dry heat sterilization method using hot air oven. Sample was placed at 170 ° C for 2 hrs. After sterilization, sterility testing was performed using fluid thioglycollate medium as per USP standards. Three test tubes were taken for negative control,

positive control and sample testing. In first test tube, sterilized fluid thioglycollate medium was taken as a negative control. In second test tube, Staphylococcus aureus (S.aureus) was incubated in fluid thioglycollate medium for positive control and in third test tube, sample was placed for sterility testing. All the test tubes are incubated for 14 days and checked for turbidity on everyday which was the measure for microbial growth. Results of sterility testing are shown in table 5.11 and figure 5.15 and 5.16 respectively.

Stability studies:

Comparative stability study was performed on the formulation at two different temperature conditions, i.e., 4-8 ± 2°C/45±5% RH (Refrigerator; RF) and 25 ± 2°C/ 65 ± 5% RH (Room temperature; RT) as per ICH guidelines. The physical characterization of nanoparticles was observed periodically by evaluating percent drug entrapment, percent drug loading and particle size. Results of stability studies are shown in table 5.12.

3. Results and Discussion

Preformulation Study

Fourier transforms infrared spectroscopy (FTIR):

FTIR spectra for drug alone and with excipients were taken using a FTIR spectrophotometer with KBr pellets for the identification of the drug and excipients and to study drug - excipients compatibility. FTIR spectra of Methotrexate and physical mixture of drug and excipients are shown in figure 5.1 and 5.2 respectively.The spectral elucidations for drug alone and powder mixture are shown in table 5.1.

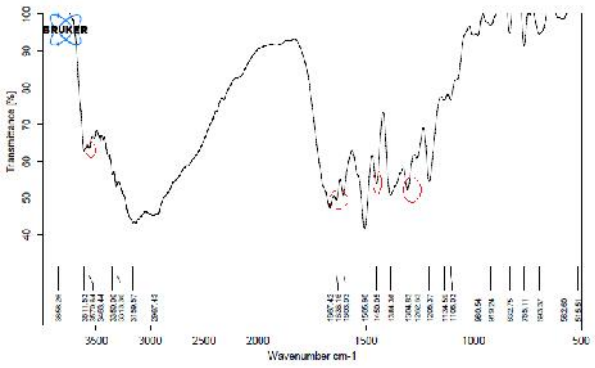

Fig 1:FTIR Spectra of Methotrexate

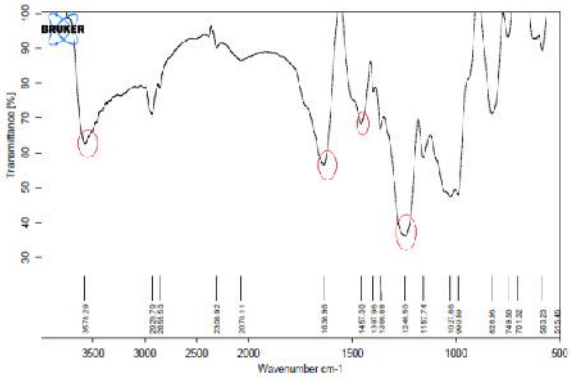

Fig 2: FTIR Spectra of Physical mixture of methotrexate and excipients

Discussion:

Frequencies of principle peaks in FTIR spectra of physical mixture of methotrexate and excipients were nearly similar to the frequency of principle peaks present in FTIR spectra of methotrexate. So, these results revealed that the drug was compatible with excipients and neither drug decomposition nor drug-excipients and excipient- excipient interactions occurred in the formulation.

Analytical Method:

Standard curves of Methotrexate in Phosphate buffer saline pH 7.4 (PBS pH 7.4) were analyzed in the range of 4-16µg/ml. The selected range of MTX was found to be linear. Regression coefficients at 302 nm were found to be 0.996. The standard calibration curve data and graph are shown in table 5.2 and figure 5.3 respectively.

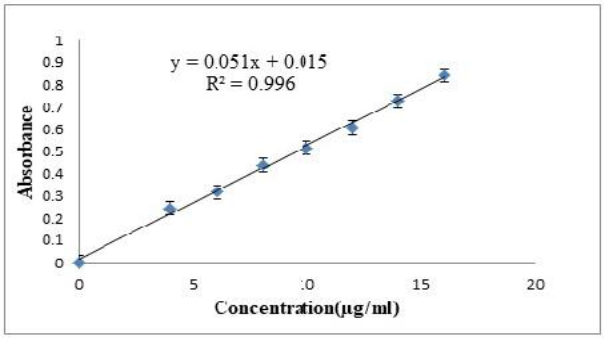

Fig 3: Calibration curve of Methotrexate in PBS pH 7.4

Discussion:

Regression co-efficient (R^2) for the drug in distilled water and PBS pH 7.4 was found to be (0.996) near to one and in the linearity range (4-16µg/ml) which showed linear relationship between absorbance and concentration. This standard concentration method obeys Beers law and found to be suitable for the determination of percent drug entrapment and *in vitro* drug release study.

Preparation of Methotrexate loaded Dextran sulphate-Poly lactic co- glycolic acid nanoparticles:

Nanoparticles were prepared by nanoprecipitation method. Various batches for the optimization of Drug: DS molar ratio, PLGA: Drug: DS molar ratio and concentration of lecithin in percentage were prepared by nanoprecipitation method and characterized for percent drug entrapment, percent drug loading, particle size and zeta potential.

Discussion:

Various batches were prepared for optimization of three formulation variables i.e. Drug: Ds (molar ratio), PLGA: Drug: DS (molar ratio), and Lecithin (%). Drug: DS ratio was varied by increasing the DS molar ratio from 0 to 1. PLGA: Drug: DS molar ratio was taken by increasing PLGA molar ratio from 70 to 90 and decreasing (Drug: DS) ratio from 30 to 10. Effect of lecithin was also measured by taking three different concentration 0.5%, 1.25%, 2%. All the batches were characterized for particle size, percent drug entrapment, percent drug loading.

Optimization of Formulation Variables:

For selection of optimum drug to polymer ratio, concentration of lecithin and PLGA and DS ratio,

nanoparticles were formulated with different molar ratio of drug to dextran sulphate to drug ratio (1: 0, 1: 0.5 and 1:1) PLGA to Drug and Dextran sulphate ratio with different molar ratio (70:30, 80:20, 90:10) using different concentration of lecithin surfactant (0.5,1.25,2 % w/w). All batches were analyzed for particle size, percent drug entrapment, percent drug loading. Results for optimization of formulation variables are shown in the table 5.3

Discussion:

Above results shown in table 5.4 shows results of evaluation parameter of optimized batches which shown when increases surfactant and polymer concentration increases drug entrapment and decreases particle size but when decreases polymer concentration and surfactant increases particle size decreased but entrapment also decreased so here concluded that optimized batch 13 which has ratio of PLGA: DS: Drug (80:20) Drug: DS ratio (1:0.5) and Lecithin concentration (1.25%) shows that particle size of 146.40±0.05 nm, Percent drug entrapment 98.80±0.051 % and Percent drug loading 9.70±0.25 % was selected to optimized different surfactants. Table 5.4 shows the results of different surfactants such as hydrogenated soya phosphotidyl choline (HSPC) and pluronic F127 effects on particle size and percent drug entrapment.Table 5.4 shows effect of different surfactant on particle size and percent drug entrapment and percent drug loading. The results indicates that the pluronic F 127 and HSPC (Hydrogenated soya Phosphotidyl choline) shows comparative larger particle size and lower percent drug entrapment and percent drug loading than the lecithin. So the lecithin was selected as a surfactant for the preparation of nanoparticles. Based on above study batch 13 was selected as optimized batch and taken for further evaluation.

Lyophilization of optimized batch:

Optimized batch B_{13} was lyophilized using mannitol in a different ratio. Redispersibility of the freeze-dried formulations and particle size of the NPs before and after freeze-drying were evaluated.

Discussion:

Optimized batch B8 was lyophilized using mannitol as a cryoprotectant. Different ratios of NPs: Mannitol were studied at 1:1, 1:2 and 1:3. Results revealed that among these three, product of NPs: Mannitol (at 1:3) ratio shows easy redispersibility after lyophilization. Particle size was also observed. Particle size was increased from 145.2 ± 10.9nm to 147.4 ± 17.2 nm which is not significant.

Statistical Analysis:

All the batches are prepared according to the design. All the batches were analyzed using the design expert 9 software. The software itself suggests Quadratic Model and also gave model equation for dependent variables.

Response 1 – Particle Size:

Particle Size from the B_1 to B_{17} batches of nanoparticles varied from 89.62±0.061 to 290.6±0.045 nm.

Polynomial Equation for Particle Size:

Particle size= +142.50-6.87 *A -36.19*B +24.71*C +5.48*AB +17.23*AC -37.10*BC -25.15* A^2+12.83* B^2+27.47*C^2.

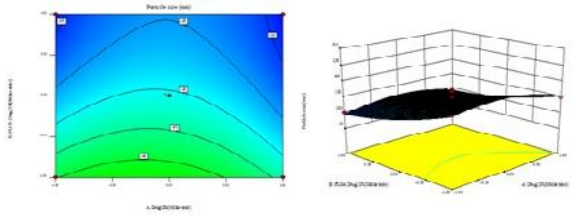

Fig 4: Response surface plot and Contour plot of effect of (a) Drug: DS (A) and (b) PLGA: Drug: DS (B) on Particle Size

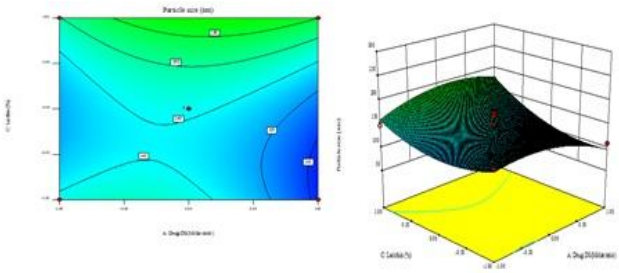

Fig 5: Response surface plot and Contour plot of effect of (a) Drug: DS (A) and (b) Lecithin (C) on Particle Size

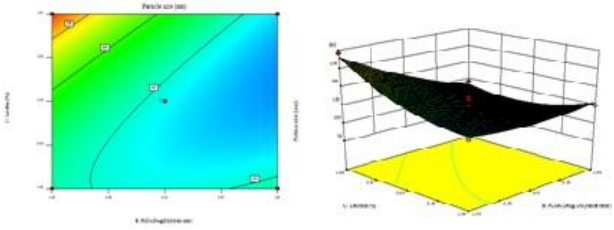

Fig 6: Response surface plot and Contour plot of effect of (a) PLGA: Drug: DS (B) and (b) Lecithin (C) on Particle Size

Discussion:

From the ANOVA results the F value of the model was found to be 5.29 which implies the model was significant. The predicted R^2 value 0.5621 was found to be close to the adjusted R^2 value 0.6477, which indicate that there was no need of model reduction.

Response 2 – Percent Drug entrapment:

Percent Drug entrapment from the B_1 to B_{17} batches of nanoparticles varied from 40.2±0.015% to 98.8±0.051%.

Polynomial Equation for Percent drug entrapment:

Percent drug Entrapment= +58.22-3.82 *A -8.89*B +23.01*C +7.65*AB -7.94*AC +3.93*BC +2.28* A^2+3.24* B^2+3.41*C^2.

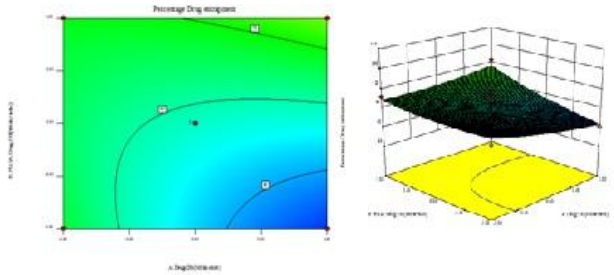

Fig 7: Response surface plot and Contour plot of effect of (a) Drug: DS (A) and (b) PLGA: Drug: DS (B) on Percent drug entrapment

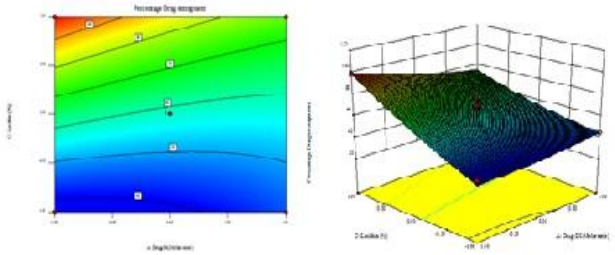

Fig 8: Response surface plot and Contour plot of effect of (a) Drug: DS (A) and (b) Lecithin (C) on Percent drug entrapment

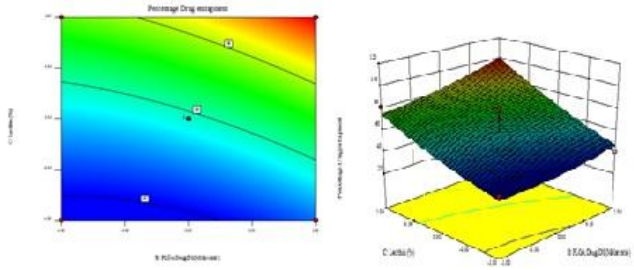

Fig 8: Response surface plot and Contour plot of effect of (a) PLGA: Drug: DS (B) and (b) Lecithin (C) on Percent drug entrapment

Discussion:

From the ANOVA results the F value of the model was found to be 10.80 which implies the model was significant. The predicted R^2 value 0.6677 was found to be close to the adjusted R^2 value 0.7072, which indicate that there was no need of model reduction.

Validation of optimized batch:

Numerical and graphical optimization was carried out Design Expert software for optimization of final batch of nanoparticles which should have following criteria. Selected criteria for independent and variable for optimized formulation.

Independent variables:

A.Drug:DS-1:0.5

B.PLGA:Drug:DS-80:20

C.Lecithin-2% w/w

Dependent variables:

Y1 Particle size- Less than 200nm

Y2 Percent drug entrapment- Maximum (100%)

Overlay plot for combined efffect of PLGA:Drug:DS and Drug:DS and lecithin are shown in figure 5.10

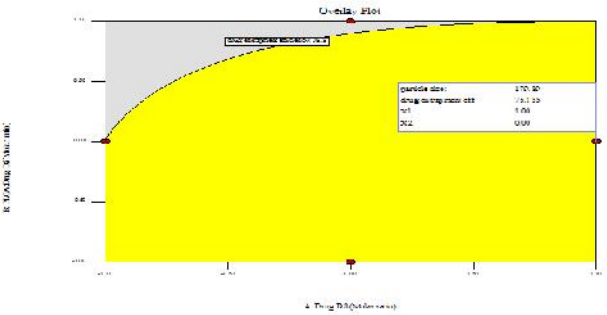

Fig 9: Overlay plot showing combined effect of Particle size, Percent drug entrapment

Discussion:

The overlay plot reflected that "yellow region" of the area shown in the figure is the area of interest (experimental region). Formulation having particle size 134.228 nm and percent drug entrapment 94.32% was found in experimental region of the overlay plot and having higher desirability than other check point batches. So, it was selected as optimized batch.

Validity of regression equations of box behnken design:

Comparative table of the observed responses with that of the predicted responses along with t- values are listed in table 5.8.

Discussion: From the results it had been found that t_{cal} and t_{tab} value were found to be 1.392 and 6.314 respectively. Here, t_{cal} value was less than t_{tab} value for all responses at all the levels, which suggest that there were no significant difference between two results. So equation obtained for selected responses are validated in selected ranges of variables. The close resemblance between the observed and predicted response value assessed the robustness of predictions. These values indicate the validity of generated model.

Characterization of Methotrexate loaded Dextran sulphate-Poly lactic co- glycolic acid nanoparticles:

Particle size measurement:

Particle size was measured by Malvern zetasizer. Results of particle size of optimized batch B_{13} are shown in table 5.9 and figure 5.11.

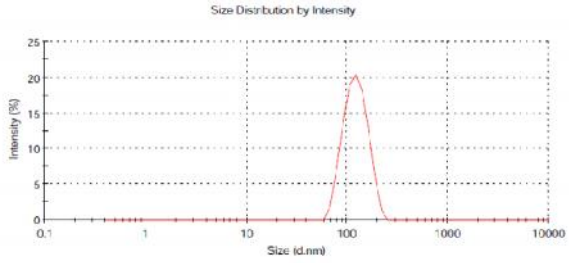

Fig 10: Particle size of optimized batch B13

Discussion:

Particle size of the prepared nanoparticles was measured by zetasizer. Particle size for optimized batch was found to be 146.40 ± 10.64nm. The obtained particle size is in required range and is not susceptible to be taken up by RES.

Zeta potential measurement:

Surface charge measurement was done using Malvern zetasizer. The zeta potential of a particle is the overall charge that the particle acquires in a particular medium. It is a physical property which is exhibited by any particle in suspension. The zeta potential of optimized batch was found to be -37.1±6.32 mV.

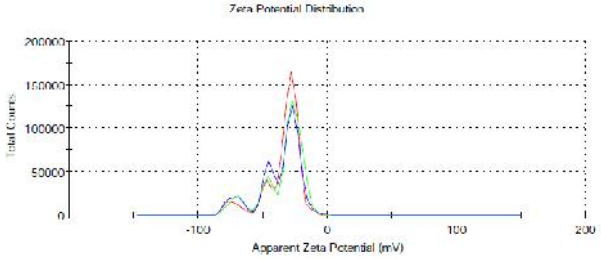

Fig 11: Zeta potential of optimized batch B13

Discussion: Surface charge measurement was done using zetasizer. The zeta potential of a particle is the overall charge that the particle acquires in a particular medium. It is a physical property which is exhibited by any particle in suspension. The zeta potential of optimized batch was found to be -37.1±6.32 mV. PDI is a measure of distribution of the particles in the sample. It was found to be 0.342ï0.002 which is acceptable.

Percent drug entrapment (PDE) and percent drug loading (PDL):

Results of percent drug entrapment and percent drug loading are shown in table 5.9.

Discussion: The results of above batches shows that the batch B_{13} having a good particle size (146.4±10.64m) and highest percent drug entrapment (98.8±0.8%) and percent drug loading was (9.6±0.81%). Particle size of PLGA-DS-NPs was found to be below 200nm. This batch reduces opsonization and minimizes the clearance by the RES, leading to longer blood circulation and improved pharmacokinetic properties.

Transmission electron microscopy (TEM):

The morphology of the particles was studied using a Transmission electron microscope (TEM). It was performed for batch B-2 (with 1 % phototungusting acid). TEM images of batches B_{13} are shown in figure 5.13.

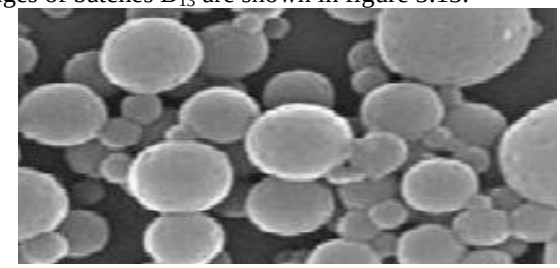

Fig 12: Surface morphology of MTX-DS-PLGA nanoparticles using Transmission electron microcopy

Discussion: TEM study shown that particle size for batch B_{13} was found to be in the range of 10-1000nm. Particles of optimized batch B_{13} were found to be spherical in shape. So, optimum size range and morphology may be suitable for targeting the nanoparticles to the blood stream.

***In-vitro* Drug Release Study:** *In-vitro* release study was performed using dialysis bag with a receiver compartment volume of about 20 ml with continuous stirred on magnetic stirrer which was kept at a temperature of 37 ± 0.5 ℃. Result of % cumulative drug release and graph of release profile for nanoparticles and marketed preparation are shown in the table 5.10 and figure 5.14 respectively.

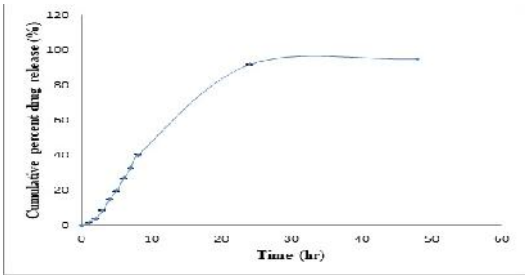

Fig 13: Cumulative percentage drug release of MTX-DS-PLGA NPs

Discussion:

The *in vitro* drug release was found to be 94.90± 0.518% for nanoparticles after 48 hrs, which shows slow and sustain release of drug from the dosage form. Drug release from the formulation was found to be slow and sustained over 48 hrs. So it can be conclude that the release rate of the drug from the nanoparticles was found to decrease drastically if we incorporate hydrophobic polymer in the formulation.

Sterility testing:

Sterilization study was carried out by dry heat sterilization. The optimized batch B_{13} was sterilized by dry heat sterilization method using hot air oven. Sample was placed at 170° C for 2 hrs. After performing sterilization, sterility test was done using fluid thioglycolate medium as per USP method. Results are shown below.

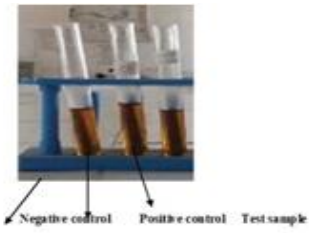

Fig 14: Sterility test of nanoparticles using fluid thioglycollate medium for optimized Batch B13

Fig 15: Results of sterility test after incubation of 14 days for optimized Batch B13

Discussions:

Sterility study was performed as per USP method using fluid thioglycollate medium and staphylococcus aureus bacteria. Figure 5.15 shown three test tubes which have negative control containing fluid thioglycollate medium, positive control containing fluid thioglycollate medium and staphylococcus aureus bacteria and test sample containing MTX-DS-PLGA NPs and fluid thioglycollate medium. Figure 5.16 shown results after incubation in which negative control has no growth of microorganism, positive control has growth of microorganism which describes as fluid thioglycollate medium provide nutrition to the bacteria and test sample has no growth of micro-organism which described that sample was sterilized. Table 5.11 shown results of sterility test which described that MTX-DS-PLGA NPs was successfully sterile.

Stability study:

The stability study was carried out for optimized formulation B_{13}4-8 ± 2°C/45±5% RH (Refrigerator; RF) and 25 ± 2°C/ 65 ± 5% RH (Room temperature; RT) for 1 month as per ICH guidelines. The formulation shown good stability with no remarkable change in appearance, particle size, percent drug entrapment and *in vitro* drug release profile up to 1 month. Results of stability data are shown in table 5.12 for the optimized batch B_{13}.

Discussion: Stability study was performed for final optimized B_{13} batch. From the stability studies of the optimized NPs formulation for one month, it was found that there was not significant change in appearance, particle size, percent drug entrapment, percent drug loading. No significant change in any of above parameter during storage at 4-8 ± 2°C/45±5% RH (Refrigerator; RF). indicated that the developed NPs formulation of MTX was stable after 1 month.Significant change in particle size, percent drug entrapment and percent drug loading was observed at 25 ± 2°C/ 65 ± 5% RH (Room temperature; RT) indicated that the developed NPs formulation of MTX was not stable after 1 month.

Table 3: Dependent and Independent Variables in BBD

Independent variable	**Variable level**		
	Low(-1)	Medium(0)	High(+1)
Drug: Dextran sulphate (DS) (molar ratio)	1: 0	1: 0.5	1:1
PLGA: (Drug: DS) (molar ratio)	70:30	80:20	90:10
Lecithin (%)	0.5	1.25	2
Dependent variables	1. Particle size (nm) (Y1) 2. Percent drug entrapment (PDE) (Y2)		

Table 4: Matrix of Box Behnken design for optimizing formulation variables

Batch No.	**Drug: DS(molar ratio)**	**PLGA:(Drug: DS)(molar ratio)**	**Lecithin (%)**
B_1	1:1	70:30	1.25
B_2	1:1	80:20	2.0
B_3	1:1	80:20	2.0
B_4	1:1	80:20	2.0
B_5	1:0.5	90:10	0.50
B_6	1:0.5	70:30	0.50
B_7	1:0	90:10	1.25
B_8	1:1	80:20	2.0
B_9	1:0.5	70:30	2.0
B_{10}	1:0	80:20	2.0
B_{11}	1:1	80:20	2.0
B_{12}	1:1	90:10	1.25
B_{13}	1:0.5	80:20	1.25
B_{14}	1:0	70:30	1.25
B_{15}	1:1	80:20	0.50
B_{16}	1:0	80:20	0.50
B_{17}	1:0.5	90:10	2.0

Table 5: Effect of different surfactants such as hydrogenated soya phosphotidyl choline (HSPC) and pluronic F127 on particle size and percent drug entrapment

Batch No	**Surfactant**	**Drug: DS (molar ratio)**	**PLGA:(Drug: DS) (molar ratio)**	**Lecithin (%)**
B_{18}	**Pluronic F 127[21]**	1:0.5	80:20	1.25
B_{19}	**HSPC[22]**	1:0.5	80:20	1.25

Table 6:FTIR spectra Interpretation

Functional group	**Principle Peaks(cm^{-1})**			
	N-H Stretching	N-H deforming	C=C stretch aromatic ring	C-O stretching
MTX	3570cm^{-1}	1636cm^{-1}	1450cm^{-1}	1262cm^{-1}
MTX with powder mixture	3578cm^{-1}	1636cm^{-1}	1457cm^{-1}	1246cm^{-1}

Table 7: Calibration curve of Methotrexate in PBS pH 7.4

SR No.	**Concentration (µg/ml)**	**Mean Absorbance(±) SD**
1	4	0.245 ±0.04
2	6	0.318 ±0.02
3	8	0.437 ±0.06

4	10	0.514 ±0.02
5	12	0.605 ±0.02
6	14	0.727 ±0.06
7	16	0.842 ±0.08

(Where n=3, Mean± SD)

Table 8: Results of Optimization of Formulation Variables

Batch No.	Drug: DS(molar ratio)	PLGA: (Drug: DS) (molar ratio)	Lecithin (%)	Particle size(nm)		Percent drug entrapment (PDE)		Percent drug loading (PDL) Mean ï SD
				Actual value Mean ï SD	Predicted value	Actual value Mean ï SD	Predicted value	
B1	1:1	70:30	1.25	181.70±5.95	178.72	59.60±0.70	66.33	8.82±0.30
B2	1:1	80:20	2.0	149.20±6.65	142.50	40.20±1.00	58.22	7.22±0.12
B3	1:1	80:20	2.0	149.20±6.65	144.21	40.20±1.00	36.78	7.22±0.12
B4	1:1	80:20	2.0	149.20±6.65	157.17	40.20±1.00	36.90	7.22±0.12
B5	1:0.5	90:10	0.50	290.60±7.51	280.80	81.68±1.01	75.07	5.37±0.13
B6	1:0.5	70:30	0.50	173.10±9.55	142.50	69.90±2.25	58.22	13.80±0.35
B7	1:0	90:10	1.25	110.20±10.66	142.50	49.40±6.22	58.22	4.87±0.21
B8	1:1	80:20	2.0	149.20±6.65	159.00	40.20±1.10	46.81	7.22±0.37
B9	1:0.5	70:30	2.0	113.20±13.06	142.50	51.20±4.2	58.22	10.03±0.51
B10	1:0	80:20	2.0	142.20±9.30	134.23	97.40±1.05	100.70	19.09±0.50
B11	1:1	80:20	2.0	149.20±6.65	154.01	40.20±1.05	43.39	7.22±0.54
B12	1:1	90:10	1.25	100.20±14.44	95.39	72.00±1.10	68.81	3.55±0.06
B13	**1:0.5**	**80:20**	**1.25**	**146.40ï10.64**	**159.19**	**98.80ï0.8**	**98.69**	**9.70ï0.81**
B14	1:0	70:30	1.25	174.90±7.82	179.89	71.73±2.19	75.16	21.2±1.15
B15	1:1	80:20	0.50	166.80±12.59	142.50	80.40±1.4	58.22	8.0±0.16
B16	1:0	80:20	0.50	108.80±8.07	96.02	44.90±1.27	45.01	8.93±0.25
B17	1:0.5	90:10	2.0	89.62±4.94	92.60	83.18±1.49	76.45	5.43±0.44

Table 9: Effect of different surfactants such as hydrogenated soya phosphotidyl choline (HSPC) and pluronic F127 on particle size and percent drug entrapment

Batch No.	Surfactant	Drug: DS (molar ratio)	PLGA: (Drug: DS) (molar ratio)	Lecithin (%)	Particle size(nm) Mean ï SD	PDE (%) Mean ï SD	PDL (%) Mean ï SD
B_{18}	**Pluronic F 127**	1:0.5	80:20	1.25	331±2.23	43.8±0.12	4.29±0.015
B_{19}	**HSPC**	1:0.5	80:20	1.25	463.4±4.41	52.8±0.52	5.17±0.022

Table 10: Results of redispersibility and particle size of lyophilized batch

Sr.No.	NPs : Mannitol	Redispersibility	Particle size (nm) (n = 3, Mean ï SD)	
			Before	After
1	1:1	Not easily dispersible	154.5 ± 21.5	163.8 ± 26.1
2	1:2	Not easily dispersible	160.3 ± 18.6	171.4 ± 23.4
3	1:3	Easily dispersible	145.2 ± 10.9	147.4 ± 17.2

Table 11: Result of ANOVA for Particle Size

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob> F	
Model	28811.65	9	3201.29	5.29	0.0195	significant
Residual	4233.64	7	604.81	-	-	-
Lack of Fit	760.12	3	253.37	0.29	0.8303	not significant
R-squared	0.7137	-	-	-	-	-
Adj R-Squared	0.6477	-	-	-	-	-

Pred R-Squared	0.5621	-	-	-	-	-
Adeq Precision	10.605	-	-	-	-	-

Table 12: Result of ANOVA for Percent drug entrapment

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F	
Model	4985.93	3	1661.98	10.80	0.0008	significant
Residual	1999.74	7	153.83	-	-	-
Lack of Fit	919.57	9	102.17	0.38	0.8960	not significant
R-Squared	0.8719	-	-	-	-	-
Adj R-Squared	0.7072	-	-	-	-	-
Pred R-Squared	0.6677	-	-	-	-	-
Adeq Precision	9.978	-	-	-	-	-

Table 13: Results of Check Point Batch 13

Batch Code	Parameter	Predicted Values	Experimental Values	t_{cal}	t_{tab}
B_{13}	Particle size (nm)	**159.19**	**146.40ï10.64**	**1.392**	**6.314**
	percent drug entrapment (%)	**98.69**	**98.80ï0.8**		

Table 14: Results of evaluation of optimized batch

Batch	Particle size (nm)	PDI Meanï SD	Zeta potential (mV)	PDE	PDL
B_{13}	146.40±10.64	0.342ï0.002	-37.1±6.32	98.8±0.8%	9.6±0.81%.

Table 15: In-vitro Release Study of MTX-DS-PLGA NPs

Time (hr)	%CDR of MTX-DS-PLGA NPs
1	1.45 ± 0.2326
2	3.62 ± 0.1620
3	8.25 ± 0.1353
4	14.80 ± 0.4038
5	19.67 ± 0.2193
6	26.81 ± 0.5631
7	32.56 ± 0.2610
8	39.82 ± 0.1343
24	91.66 ± 0.4400
48	94.90 ± 0.5181

Table 16: Results of sterility study data for Optimized Batch B13

Days	Negative control	Positive control	Test sample
1	-	+	-
2	-	+	-
3	-	+	-
4	-	+	-
5	-	+	-
6	-	+	-
7	-	+	-
8	-	+	-
9	-	+	-
10	-	+	-
11	-	+	-
12	-	+	-
13	-	+	-
14	-	+	-

Table 17: Stability Study Data for Optimized Batch B13

Sr. No.	Parameters	StoragePeriods(Days)					
		4-8 ï 2îC/45ï5% RH (Refrigerator; RF)			25 ï 2îC/ 65 ï 5% RH (Room temperature; RT)		
		Before Storage	After 15days	After 30days	Before storage	After 15days	After 30 days
1	Particle Size (µm)	146.40±10.64	145.3±9.84	149.50 ±8.9	146.40±10.64	170.3±7.84	188.50 ±9.8
2	Percent Drug Entrapment (%)	98.80 ±0.8	97.60±0.65	96.50±0.42	98.80 ±0.8	85.60±0.65	78.50±0.82
3	Percent Drug loading (%)	9.70±0.81	9.65±0.65	9.45±0.71	9.70±0.81	5.5±0.55	4.85±0.61

Table 18: Optimized formulation

Sr. No.	Materials	Amount
1	Methotrexate	5 mg
2	Poly (D, L – lactide -co glycolide) acid	40 mg
3	Dextran sulphate	5 mg
4	Lecithin	1 mg

Table 19: Result Summary for Optimized Batch B13

SR. No.	Parameters	Result
1	Particle size	146.40±10.64nm
2	Zeta potential	-37.1±6.32 mV.
3	Percent drug entrapment	98.80 ± 0.8%
4	Percent drug loading	9.6 ± 0.81 %
5	Transmission electron microscopy study	Spherical shape
6	In vitro drug release study	91.66 ± 0.44 %after 24 hrs
7	Stability	Stable at 4-8 ± 2°C/45±5% RH (Refrigerator; RF) condition up to 1 month
8	Sterility test	Pass in sterility test

4. Conclusion

FTIR study shows that neither drug decomposition nor drug-excipients and excipient- excipient interactions occurred in the formulation.Analytical method was performed by UV Spectrophotometer. Regression co-efficient (R^2) was found to be near to one and which showed linear relationship between absorbance and concentration.Optimizations of process parameters were done by RSM using BBD. Experimental design was developed using software Design Expert 9 for optimization of process variables. Nanoparticles of MTX developed by nano precipitation method. Optimization study of formulation parameter shows that batch prepared with Drug: DS (1:0.5), PLGA: (Drug: DS) (80:20), Lecithin (1.25%w/w). Particle size and zeta potential were found to be 146.40 ± 10.64 nm and -37.1 ± 6.32 respectively for optimized batch. Percent drug entrapment, Percent drug loading were found to be 98.80 ± 0.8 % and 9.6 ± 0.81% respectively. Transmission Electron Microscopy (TEM) study indicates that the particles were found to be in spherical shape and porous in nature. I*n-vitro* drug release were found to be and 94.90 ± 0.51 % in 48 hrs. The results of sterility test which described that MTX-DS-PLGA NPswas successfully sterile. Stability study shows developed NPs was stable at 4-8 ± 2°C/45±5% RH (Refrigerator; RF) condition after 1 month. The present study demonstrated that nanoparticles may target at blood stream to the inflammatory joints of rheumatoid arthritis with least systemic toxicity.

Conflict of Interest: We declare no conflict of interest.

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