**ABSTRACT**

Objective: Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are essential part of the administration of Rheumatoid Arthritis (RA). Methotrexate (MTX) is effective for tumor necrosis factor alpha (TNF-α) biologic agents, indicated only in minority of patients suffering from severe RA. MTX remains the "anchor drug" in the treatment of RA. For delivery improvement, novel pharmaceutical drug delivery system i.e. MTX-Cubosomes were developed.

Methods: Poloxamer 407 and Glycerol monooleate (Monoelin, MO) used and the formulation were characterized as a sustained release drug delivery system for Methotrexate. Different ratios of Monolein, Poloxamer 407 and water were used to develop the different cubosomes using homogenization and emulsification method. Characterization of formulations for morphology was performed and also particle size distribution by Transmission Electron Microscopy (TEM).

Results: Formulation showed the internal cubic structures of the vesicles. The particle size of the formulations was found to be ranging from 53.21 to 185.32 nm, zeta potential of the formulations varied from 18-20-36.10 mV. The cubosomal formulation exhibited good entrapment efficiency along with high drug loading. Compatibility with the excipients was also established. An in vitro release study was done using Franz Diffusion cell indicated sustained release of the formulation at a rate of 1.25 %/h. Cubosomes proved to be reliable system for sustained transdermal drug delivery system.

Conclusion: Methotrexate cubosomes is a novel medication delivery framework and in this examination it has been developed and characterized. The formulations were found to be promising in terms of its characterization parameters like particle size, zeta potential, entrapment efficiency, loading capacity, release kinetics, and stability, suitable for topical delivery.

Keywords: Rheumatoid arthritis, NSAIDs, Cubosomes, Methotrexate

INTRODUCTION

RA is an autoimmune system sickness related with cutting edge incapacity, foundational inconveniences, early passing, and financial costs [1]. RA is an ailment depicted by methods for intense and constant fundamental disease that incorporates the joints, and affects tissues and organs that comprise of veins, heart, skin, lungs, and muscles. The beginning and seriousness of infection are alterable and trickly. RA at first bears with weakness, musculoskeletal agony, and firmness and after certain weeks to months it develops to include joints. The little joints are influenced first, the little bones of the fingers than the bigger joints are influenced, winds up swollen, warm, and painful [2]. Morning firmness or stiffness is additionally the signs and manifestations of RA and proposes enthusiastic illness. The influenced individual for the most part characterizes gradualness or challenge in development [3].

It creates a fiery reaction of the synovium (synovitis) of synovial cells and the pannus improvement in the synovium. The pathology of the confusion once in a while prompts the decimation of articular ligament and ankylosis of the joints (wrists, shoulders, knees, lower legs and feet). The synovium is emphatically bioadhesive with great compatibility with topical and mucosal deposition and conveys dynamic fixings due to its skin penetration enhancing capability [32]. Hence, the objective behind this study was to formulate topical cubosomes, novel delivery system that could help in Rheumatoid Arthritis improving absorption to the skin and simultaneously reduce the systemic diffusion of the skin-absorbed drug.

**MATERIALS AND METHODS**

**Materials**

Methotrexate was a kind gift from IPC, India. Poloxamer 407, MO was obtained from Gattefosse, France. Dialysis membrane (molecular...
weight cut-off 12,000 g/mole) was purchased from Sigma Chemical Company (Sigma-Aldrich Corp., St. Louis, MO, USA). Polyvinyl alcohol, Potassium dihydrogen phosphate and disodium hydrogen phosphate was purchased from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA). All other chemicals used were of analytical grade.

Methods

Preparation of methotrexate-loaded cubosomes nanoparticles

Cubosomes Dispersions were made by emulsification of the cube-like lipid part comprising of MO and Poloxamer 407 in water containing PVA 20 as appeared in table 1. The cubosomes were shaped by softening MO and Poloxamer 407 with the guide of hot plate kept up at 60 °C (MLH). At that point, the medication was dissolved down inside the melted blend. Distilled water containing 2.5 %w/w PVA (low viscous cube-like part is accomplished by utilizing natural solvents, preheated at indistinguishable temperature) was added to the melted blend underneath mechanical mixing at 500 rpm. Dispersions were kept up under blending at temperature for 2 h to harden the lipid. The Dispersions were exposed to mixing at 15 000 cycles for each min at 60 °C for 1 min (Heidolph Homogenizers, Silent gadget M, and Germany). When the formulations were cooled, they were kept up at room temperature in glass vials.

Characterization of methotrexate-loaded cubosomes

Determination of pH

Digital pH meter (Metttler Toledo, Langacher, Switzerland) was used for measuring the pH of the formulated Cubosomes. For this, each formulation was taken sufficiently to dip the electrode accurately and it was kept for 5 min to stabilize (n=6).

Rheological behavior

The rheological conduct of MTX-loaded cubosomes was discovered utilizing cone and plate rheometer (Brookfield DV3THB cone/plate rheometer) kept up at 25±2 °C and revolution were extended 90 ° fixed angle. Before the measurement, one drop of the sample (n=6) was taken from each selected formulation and diluted and 90 ° fixed angle. Before the measurement, one drop of the sample (n=6) was taken from each selected formulation and diluted

Drug excipients compatibility studies

The drug compatibility with polymer was determined with the help of Fourier transform infrared (FTIR) spectra of Methotrexate and Methotrexate-loaded cubosomes were determined by using Perkin Elmer RX1 model. The pellets were prepared by gently mixing freeze dried formulation (1 mg) with 200 mg of potassium bromide and compacting at high pressure. The pellets thus prepared were scanned at a resolution of 4 cm⁻¹ from 400 to 4000 cm⁻¹

Shape and surface morphology

The MTX cubosomes was observed under TEM to determine the surface morphology and size of the formulation. The formulation was diluted 50 times with double distilled water and stained negatively by phosphotungstic acid and then dried on the carbon coated grid. Excess phosphotungstic was removed using filter paper. Finally it was observed using Morgagni 268D (magnification x 2 50 000, Fei Electron Optics, Netherlands) transmission electron microscope

Particle size, particle size distribution, polydispersity index (PDI) and zeta potential

Cubosomes formulations were measured for the Particle size and its distribution, PDI and zeta potential by Dynamic Light Scattering using Zetasizer (NanoZS90, Malvern Instruments Ltd. Worcestershire, UK). It was determined at a temperature of 25 °C and 90 ° fixed angle. Before the measurement, one drop of the sample (n=6) was taken from each selected formulation and diluted in 10 ml of the dispersion medium (distilled water) [21].

Entrapment efficiency and drug loading

Cubosomes entrapment efficiency and drug loading were determined using high-speed centrifugation (Sigma 3K30, Osteorode am Harz, Germany) for 15 min at 15 000 rpm. Supernatant was separated once centrifugation is done and filtered using Millipore VR membrane (0.2 µm) [33]. Required dilution was done and it was analyzed (n=6) by HPLC [23] to examine MTX content at 303 nm. The entrapment efficiency (%) and drug loading was calculated by using the following equation

\[
\text{Entrapment Efficiency} (\%w/w) = \frac{\text{Amount of Encapsulated Drug}}{\text{Total amount of the drug}} \times 100
\]

\[
\text{Drug Loading} (\%w/w) = \frac{\text{Amount of Encapsulated Drug}}{\text{Total amount of cubosomes}} \times 100
\]

In vitro drug release studies

The MTX releases from cubosomes across dialysis cellulose membrane (molecular weight cutoff 12 000 g/mole) were performed by mounting it on vertical Hansson Franz diffusion cell system. The donor medium consisted of 1 g of vehicle containing Methotrexate. The diffusion area available between the cells was 1.785 cm2. The receptor medium consisted of 1 g of vehicle containing Methotrexate. The samples were analyzed using HPLC [34].

Release kinetics

The drug release kinetics of Methotrexate Cubosomes was studied; the in vitro drug release study data was fitted to various kinetic models like, Higuchi matrix model, Hixon Crowell model, Korsmeyer-Peppas model and zero-order, first-order model. For each model, the correlation coefficient (R2) was calculated. The best fit model for the drug release was that giving R2 never to 1 was selected [35, 36].

Stability studies

The stability studies of the finalized formulations were done on the basis by ICH Q1A (R2) guidelines [45]. The samples of Methotrexate loaded cubosomes were stored under the following temperatures:

a. 25±2 °C/65±5 %RH  

b. 40±2 °C/75±5 %RH  

Table 1: Formulation table

| S. No. | Formulation code | PLX: MO ratio | PLX (%w/w) | MO (%w/w) | Plx and MO: water |
|-------|------------------|---------------|------------|-----------|------------------|
| 1)    | F1               | -             | 0.00       | 4.76      | 1:20             |
| 2)    | F2               | 1:6           | 0.68       | 4.08      | 1:20             |
| 3)    | F3               | 1:4           | 0.96       | 3.80      | 1:20             |
| 4)    | F4               | 1:2           | 1.58       | 3.18      | 1:20             |
| 5)    | F5               | -             | 0.00       | 9.10      | 1:10             |
| 6)    | F6               | 1:6           | 1.30       | 7.80      | 1:10             |
| 7)    | F7               | 1:4           | 1.82       | 7.28      | 1:10             |
| 8)    | F8               | 1:2           | 3.04       | 6.06      | 1:10             |
| 9)    | F9               | 1:10          | 0.00       | 16.66     | 1:5              |
| 10)   | F10              | 1:6           | 2.38       | 14.28     | 1:5              |
| 11)   | F11              | 1:4           | 3.34       | 13.32     | 1:5              |
| 12)   | F12              | 1:2           | 5.56       | 11.10     | 1:5              |
in stability chambers (Thermolab, Mumbai, India) for 6 mo. Withdrawn samples at different time intervals i.e. 0, 1, 2, 3 and 6 mo were analyzed for particle size distribution, PDI, Entrapment efficiency, drug loading and phase separation [37].

RESULTS AND DISCUSSION

Preparation of methotrexate cubosomes

The MTX cubosomes were prepared according to the plan referenced in table 1. Surfactants, that were used in the plan of cubosomes, are poloxamer 407 of every an exceedingly focus differ somewhere in the range of 0 and 20 % w/w with respect to the scatter part. The grouping of the monoglyceride/surfactant blends somewhere in the range of 0 and 20 % w/w with respect to the cubosomes, are poloxamer 407 of every an exceedingl y focus referenced in table 1. Surfactants, that were used in the plan of

Table 2: Characterization parameters of MTX cubosomes (mean±SD; n=6)

| S. No. | Formula- tion code | pH   | Particle size (µm) | Zeta potential (mV) | Entrapment efficiency % | Loading capacity (%) | Viscosity (cp) | PDI          |
|--------|--------------------|------|-------------------|---------------------|-------------------------|---------------------|---------------|--------------|
| 1      | F1                 | 6.62±0.01 | 185.3±12.10       | -18.20±1.27         | 64.39±0.94              | 11.22±1.34          | 2.93±0.21    | 0.75±0.03    |
| 2      | F2                 | 6.41±0.05 | 95.9±6.30         | -33.95±2.19         | 79.44±0.81              | 5.81±0.13           | 2.99±0.13    | 0.39±0.02    |
| 3      | F3                 | 6.88±1.10 | 68.3±2.32         | -31.75±0.92         | 80.31±0.72              | 6.41±0.28           | 3.14±0.17    | 0.42±0.03    |
| 4      | F4                 | 6.71±1.40 | 23.2±1.89         | -31.85±0.78         | 88.95±0.61              | 13.31±2.13          | 3.36±0.24    | 0.12±0.03    |
| 5      | F5                 | 6.42±2.60 | 154.1±11.94       | -32.70±1.14         | 60.39±0.24              | 12.01±0.36          | 3.41±0.13    | 0.65±0.05    |
| 6      | F6                 | 6.32±1.90 | 168.7±5.32        | -32.35±0.49         | 74.89±0.90              | 6.54±0.32           | 3.37±0.31    | 0.33±0.03    |
| 7      | F7                 | 6.68±2.40 | 143.5±6.10        | -31.00±0.71         | 77.62±0.53              | 6.46±0.29           | 4.13±0.18    | 0.21±0.06    |
| 8      | F8                 | 6.94±1.30 | 130.7±1.35        | -32.95±0.49         | 78.49±0.42              | 8.04±4.23           | 4.09±0.41    | 0.39±0.02    |
| 9      | F9                 | 6.65±1.9  | 121.3±2.97        | -28.20±0.14         | 60.94±0.94              | 10.92±2.19          | 6.63±0.48    | 0.73±0.03    |
| 10     | F10                | 6.39±2.4  | 78.2±5.47         | -33.60±0.28         | 69.37±0.72              | 7.10±0.31           | 7.39±1.43    | 0.31±0.01    |
| 11     | F11                | 6.92±0.8  | 82.5±6.11         | -36.10±2.97         | 70.63±0.44              | 7.52±3.72           | 7.97±4.9     | 0.28±0.03    |
| 12     | F12                | 6.77±1.4  | 83.3±6.28         | -32.70±0.99         | 76.12±0.52              | 7.91±2.51           | 8.43±5.3     | 0.32±0.03    |

Rheological behavior

The rheological behavior of the formulations was assessed to study the spreadability of the formulation and its residence for longer time. Increased concentration of Monolein and Poloxamer 407 showed better like consistency that increases the formulation viscosity (table 2). All the formulations have viscosities in the range that will provide better penetration.

Drug excipients compatibility studies

FTIR Spectroscopy of Methotrexate and Methotrexate loaded Cubosomes is presented in fig. 1. Strong and characteristic absorbance can be seen with Standard Methotrexate showing IR peaks at 1644 cm⁻¹ and 1603 cm⁻¹. Absorption peaks between 2500 cm⁻¹ and 3000 cm⁻¹indicative of unchanged excipients property. Spectra of cubosomes altered with Methotrexate show expanded absorbance at 1600 cm⁻¹ and the presence of another top at 1490 cm⁻¹ demonstrates the nearness of Methotrexate on the cubosomes. The intensity and the number of the peaks were found to be reduced suggesting possible interactions of the excipients with Methotrexate thereby reducing the crystalline nature and making the drug amorphous helpful in better absorption.

Shape and surface morphology

The surface morphology and mean distance across of the Methotrexate stacked cubosomes was imaged utilizing TEM. TEM pictures demonstrated nearly cube shape like surface of the detailing with uniform distribution for example<200 nm (table 2) and it supported the molecule size estimation by the zetasizer. The outcomes demonstrate the particles were consistently appropriated and isolated from one another. Decreased molecule size of the definition F4 was because of the nearness of higher grouping of Poloxamer 407 which was adsorbed on surface going about as a covering layer that balances out the little cubosomes nanoparticles surface [21]. Decreased Poloxamer concentration brought about the expansion of molecule size which was because of the increment in interfacial pressure consequently bringing about aggregation expanding the molecule site. All the cubosomes at the magnification as high as 250000x were found not circular or round, they showed a cube like structure. TEM photomicrograph of the MTX loaded cubosomes is given in fig. 2.
Particle size and polydispersity index

The particle size of the formulations was found to be ranging between 53.21±0.8 nm to 185.32±12.1 nm (fig. 3) and PDI was found ranging from 0.214±0.03 to 0.754±0.03 (table 2). Reduction in the surface tension between the lipid phase and the aqueous phase due to increase in the surfactant concentration might have lead to reduction of particle size. High surfactant concentration has also caused reduction in the steric hinderances [36, 37].

Zeta Potential of the formulations were found ranging from -18.20±1.27 to -33.95±2.19 (n=6) (table 2). Zeta potential indicates the stability of the dispersed particles in dispersion medium. High zeta potential shows high repulsion between the particles and particle aggregation is less likely to occur. A negative value of the zeta potential shows that formulations have good stability and dispersion quality.

Entrapment efficiency and drug loading

Different formulations were differentiated by determining the amount of drug incorporated in the formulation. The encapsulation efficiency of the formulations was found between 60.39±0.24 and 88.95±0.61 % (table 2). As the drug is lipophilic in nature it is found that the encapsulation efficiency was high. The drug loading capacity was found to be varying from 5.81±0.13 to 12.01±0.36 %. Binary lipid matrices showed high loading and entrapment efficiency of the cubosomes [24].

In vitro drug release

In vitro drug release study profile of the cubosomes in the initial stage showed (fig. 4) controlled release upon contact with bulk release media. No burst drug release indicated the entrapment of the drug in the lipid layer and MTX slow diffusion. The hydrophobic drug in the external layer may partition through the polymer phase to the cubic phase. The drug release rate of all the formulation was observed varying from 1.55-1.89 %/h showing a greater potential for the sustained release pattern of the cubosomes nano-formulation whereas gel showed a release of 23 %/h. Cubosomes showed a
controlled drug release with $t_{50}$ approximately 16 h. The cumulative release percent almost achieved 100 % after 48 h, showing a complete release ability of formulation. Formulations F3 and F4 showed better release, droplet size and other characteristic properties. This pattern of slow release of the drug can be related to the slow expulsion due entrapment of the drug [38].

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**Fig. 4:** *In vitro* drug release profile of MTX cubosomes and MTX Gel (mean±SD; n=6)

**Fig. 5:** Drug release kinetics of various models (mean±SD; n=6)
Release kinetics

The acquired in vitro release information was joined into different release kinetic models. The Highest estimation of the correlation coefficient was watched for the Higuchi matrix model \((R^2=0.9959)\) and it pursues non-fickian discharge. Higuchi depicts the drug release as a diffusion mechanism dependent on the Fick’s law, contingent upon the square foundation of time. [39] This connection can be utilized to portray the drug dissolution from different sorts of altered or controlled release pharmaceutical dosage forms (fig. 5) [38].

Stability studies

The developed Methotrexate cubosomes were observed for particle size distribution, phase separation, Precipitation, % Entrapment efficiency and % Drug loading after keeping the product in specified conditions (table 3).

| Condition | Time | Phase separation | Precipitate | Particle size±SD | PDI ±SD | % Entrapment | % Drug loading |
|-----------|------|------------------|-------------|------------------|---------|--------------|---------------|
| Initial   | 0    | No               | No          | 78.45±0.42      | -30.42±0.11 | 87.28±0.51 | 14.40±4.21    |
| 25=±65±5%RH | 3M   | No               | No          | 69.32±5.31      | -31.26±0.48 | 85.72±4.36 | 13.37±3.91    |
| 40=75%±5%RH | 3M   | No               | No          | 102.48±4.71     | -29.46±0.11 | 80.50±3.17 | 12.94±4.20    |
| 6M        | No   | No               | No          | 95.18±1.42      | -29.42±5.15 | 76.20±4.19 | 12.90±3.21    |

It was found that the formulation remained stable during 6 mo of stability studies. According to the results received, the kinetic analysis showed that the degradation of Methotrexate was found to follow zero-order reaction for the cubosomes formulation F4, based on the values of the correlation coefficient \((r)\).

CONCLUSION

MTX is the best helpful specialist accessible to treat an assortment of malignancies, for example, non-Hodgkin’s lymphoma, osteosarcoma, choriocarcinoma, lymphocytic leukemia, and head, neck, and bosom diseases. Utilized against non-neoplastic conditions, for example, serious psoriasis and immune system illnesses, for example, RA. Methotrexate cubosomes are additionally a novel medication conveyance framework and in this examination, it has been created and contemplated. The created conveyance framework is viewed as a means of fruitful treatment of rheumatoid as it gives controlled conveyance of the medication in human by means of the noninvasive skin course with additionally continuing, less successive dosing.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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