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Research Article

Development and *In Vitro* Characterization of Solid Lipid Nanoparticles (SLN) Containing Methotrexate And Doxycycline

Vijaya R*, Ram Kishan K R

*Department of Pharmaceutical Technology, University College of Engineering, Anna University, BIT Campus, Trichy, Tamilnadu, India

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ABSTRACT: Solid lipid nanoparticles (SLN) containing Disease Modifying Antirheumatic drugs (DMARDs) Methotrexate (MTX) and Doxycycline (DOX) was developed using a triglyceride (tristearin) and a polaxamer (pluronic F68). Hot homogenization of melted lipid and aqueous phase at temperature above the melting point of lipid had produced SLN dispersion. Optimization of process and formulation variables have yielded SLN having an entrapment efficiency of 65.07%±1.23% and 79.56%±0.92% for MTX and DOX respectively. Particle size and zeta potential measured using Malvern Zetasizer showed 157.2nm and -9.6mv respectively for the optimized SLN formulation. The compatibility between the drug and the formulation excipients was tested by Fourier Transform Infrared Spectroscopy (FTIR) and found to be compatible. Powder X-ray diffraction (PXRD) study revealed that the drugs and lipid were dispersed in crystalline state in SLN. The *in vitro* drug release studies performed in phosphate buffer of pH 7.4 using dialysis bag showed a sustained release of both the drugs (>75%±1.4%) up to a period of two days. From the *in vitro* results, it can be concluded that SLN was found to be a suitable nano carrier for the incorporation of DMARDS: MTX and DOX without any significant interaction. The developed system produced sustained release of both the drugs (based on their concentration) for longer duration and thus suitable for the chronic inflammatory conditions of RA.

Keywords: Solid Lipid Nanoparticle; Methotrexate; Doxycycline; DMARDs; Rheumatoid Arthritis; Characterization; Drug release.

INTRODUCTION

Rheumatoid arthritis is a chronic inflammatory disease affecting the quality of life of many people in their old age. Recent treatment modality includes combinational DMARD's are in the management of disease condition in the assumption that that the two drugs acts in two different pathways so as to reduce (or) delay the progression of disease. It is very useful in cases of not knowing the actual status of disease in early rheumatoid arthritis [1]. Among the combinations MTX (methotrexate)+DOX(doxycycline) proposed in the literature has been selected for the present study. As these small molecules of matrix metaalo proteinase inhibitors (MMPI's) (eg. DOX) have been reported to replace biological DMARD to certain extent in the combination therapy [2, 3]. MTX which is used as a parent drug in combination DMARD therapy is an antineoplastic antimetabolite with immunosuppressant properties whereas DOX is a tetracycline, in addition to its antibacterial effect, inhibit matrix metalloproteinases (MMPs) and is an immunomodulatory. The conventional combination drug deliveries of DMARD's are not effective in reducing the side effects of combined drugs. However, the use of Nano carriers for the delivery of dual drugs tends to improve the efficacy of both drugs by sustaining the drug action for long term with possible reduction on side effects has been found to be favorable for treating the chronic inflammatory conditions of RA [4]. Out of the several Nano carriers, the formulation of nano sized solid lipids is a very attractive idea to achieve controlled drug release in different routes, because drug mobility in a solid lipid should be considerably low [5]. Increased drug stability, high drug payload, incorporation of lipophilic and hydrophilic drugs is some of the advantages of solid lipid nanoparticles (SLNs [6]. The SLNs have been proved for efficient single or combined delivery of numerous therapeutic agents by various administration routes [7, 8]. Sustained efficacy without any increase in drug toxicity is the major requisite in the long term treatment of RA. Therefore, the kind of dual drug delivery of MTX and DOX using SLN should further reduce the toxicity of these drugs with the possibility for improving their therapeutic efficacy. Moreover, DOX is unstable in aqueous solution and irritates the mucous membrane. These problems can be rectified by its incorporation in the lipid matrix. With MTX, the poor pharmacokinetic and the narrow safety margin limits the therapeutic outcomes of conventional MTX delivery systems. The use of SLN as a carrier for MTX would

*Author for Correspondence: vrssvrs@gmail.com

improve its pharmacokinetic and safety margin. Hence, an attempt has been made to develop SLN for the delivery of MTX and DOX and to analyze for size, stability and drug release characteristics.

MATERIALS AND METHODS

Materials

Methotrexate was purchased from Madras Pharmaceuticals.Pvt.Ltd, (Chennai, India). Doxycycline was purchased from Micro Labs Pvt. Ltd, (Hosur, India). Tristearin was purchased from Tokyo Chemicals Industries (Japan). Stearic acid, Pluronic F68, Dialysis Membrane and Membrane Filter were purchased from Himedia Laboratories Pvt.Ltd, (Mumbai, India). Hydrochloric acid, Sodium hydroxide and Methanol were purchased from SD fine-chem. Limited, (Mumbai, India).

Determination of lipid solubility of drugs

Solubility of drugs (MTX and DOX) in different solid lipids was determined by adding a known quantity of drug (5mg MTX and 50mg DOX) in 100mg of lipid melts, namely, tristearin and stearic acid maintained at a temperature above the melting point of lipids. The amount of lipid melt was increased by 100mg each time until the drugs were completely solubilized. A formation of clear transparent solution was considered as the end point of the solubility study.

Compatibility study

The compatibility between the formulation ingredients was tested by FTIR (Fourier Transform Infrared Spectroscopy) (Perkin Elmer, India) by analyzing the interactions. A pinch of sample was added to potassium bromide (KBr) and pellets were formed by hydraulic press method. A graph showing the functional groups present in the sample was obtained. Samples of MTX, DOX, were examined individually and Physical mixtures of MTX-DOX and MTX-DOX-Stearic acid-Tristearin were analysed by FTIR spectra.

Development of MTX and DOX Solid Lipid Nanoparticles

SLNs were prepared by high shear hot homogenization method [10-12]. In this method Methotrexate and Doxycycline, were added to the lipid melts of stearic acid, tristearin by maintaining a temperature above the melting point (about 80°C) of these lipids. This hot lipid phase was dispersed in a 50ml of hot surfactant solution subsequently (Polaxamer 1.5% w/w), at 15000rpm, 80°C for 3min, using a high-speed stirrer (Ultra Turrax, IKA homogenizer). The pre-emulsion was then diluted up to 200ml with cold de-ionized water and stirred at 10,000 rpm for 2min. The obtained nanoemulsion (O/W) was cooled down in an ice bath and lyophilized to get solid lipid nanoparticles. Lyophilization removes excess water and enhances the storage stability of SLN. Three formulation batches of SLNs loaded with methotrexate and doxycycline were prepared as given in table 1.

Measurement of Particle size distribution and zeta potential

Particle size and size distribution are the essential characteristics of nanoparticle systems. The particle size and size distribution analysis of SLNs was performed by Dynamic Light Scattering (DLS) technique. All the samples were diluted with distilled water to a suitable concentration and the obtained o/w nano emulsion were made to pass through 0.22µ membrane filter before analysed using Malvern Zetasizer (Nanoseries, United Kingdom). All measurements were performed in triplicate. The surface charge of SLN was determined by measuring of the zeta potential of the lipid nanoparticles calculated according to electrophoretic mobility. The field strength was 20 V/cm on a large bore measuring cell (4mm).

Estimation of incorporated drug & entrapment efficiency

Of drugs MTX/DOX, a calibration curve was constructed using the standard solutions of MTX, DOX separately with the final concentrations of 10, 20, 30, 40, 50µg/mL. The amount of drugs present in the SLN formulation was quantified by an assay using a simple and sensitive spectrophotometric method. For the estimation of entrapped drug, previously lyophilized samples were dissolved in suitable solvents (0.1N NaOH for MTX; 99 volumes of 1M HCl and 1 volume of methanol for DOX). To obtain a clear solution, the turbid solutions were filtered through 0.22µ membrane filter. The absorbance was noted at a wavelength of 303nm for MTX and 349nm for DOX. For the UV/Vis spectrophotometric determination the amount of MTX and DOX present (entrapped) in the formulation was determined using an indirect method; i.e., the amount of drug that was present outside the SLNs was determined by subtracting the amount of MTX /DOX that remained in the aqueous phase after centrifugation of the sample at high speed, to total amount of MTX and DOX added in the preparation of SLNs. i.e. Entrapment efficiency = (Total amount of MTX/DOX − Amount of MTX/DOX in the supernatant)/ (Total amount of MTX/DOX)

Storage stability of SLN

To assess the storage stability of SLN, the size of the SLNs were recorded after 30days of storage at 2 to 8°C. The storage at high temperature conditions was limited as this may trigger the thermodynamic instability as well as partial melting of surface solid lipids leading to aggregation.

Degree of crystallinity

The degree of crystallinity of entrapped drugs MTX/DOX was measured by powder X-ray diffraction analysis (Panalytical XPERT PRO powder diffractometer (Cu κα radiation) operating at a voltage of 40V.XRD).The geometric scattering of radiation from crystal planes within a solid allow the presence or absence of the former to be determined thus permitting the degree of crystallinity to be assessed.

RESULTS AND DISCUSSION

Compatibility study

The functional group peaks of MTX & DOX was found to be present in the FITR of their physical mixture. In the case of MTX, the peaks were observed at wavenumbers 3335.2 cm^{-1}(hydroxyl & amino groups), 1644cm^{-1}, 1606cm^{-1}, 1551cm^{-1} (aromatic ring), 830&764 cm^{-1} (skeletal C-C

Table 1 Formulation Composition of MTX and DOX SLNs.

S.no	Formulation	Methotrexate (mg)	Doxycycline (mg)	Stearic acid (mg)	Tristearin (mg)
1	F 1	5	50	250	250
2	F 2	5	50	500	-
3	F 3	5	50	-	500

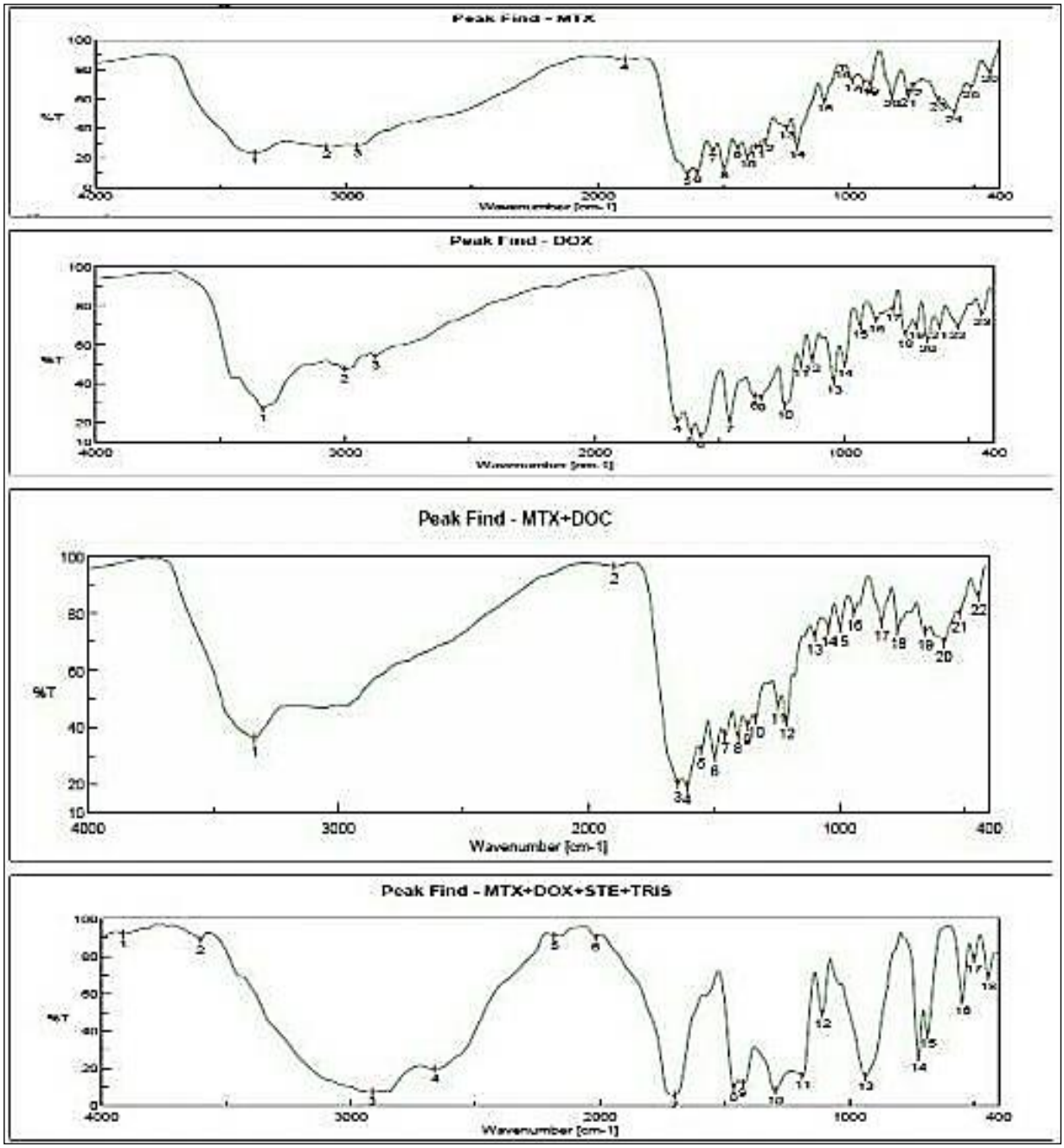

Fig. 1. FTIR of MTX, DOX, MTX+DOX, MTX+DOX+Stearic acid+Tristearin.

vibrations). In case of DOX wavenumbers of 3335.2cm^{-1} (N-H Stretching) 1606cm^{-1} to 1403cm^{-1} (aromatic C=C), 1242cm^{-1} (C=O bending) were observed. Hence, the physical mixture of MTX&DOX showed compatibility between them. With respect to the FITR of SLN containing MTX & DOX, elicited the functional groups peaks of lipid tristearin such as 2914cm^{-1} (methylene C-H Stretching), 1701cdg.m^{-1} (simple ester group), 1701cm^{-1} & 722cm^{-1} are indicative of

Table 2 Particle size of Solid Lipid Nanoparticles formulations F1, F2 and F3 containing MTX and DOX.

S.no	Formulation	Size (nm)
1	F 1	320.6
2	F 2	442.8
3	F 3	157.2

Table 3 Particle size of formulation F3 upon storage at refrigerated temperature.

Formulation	Day 0	Day 15	Day 30
F 3	157.2nm	157.4nm	157.6nm

Table 4 Release kinetics Methotrexate and Doxycycline from SLN (F3).

Kinetics model	R^2(MTX)	R^2(DOX)
Zero order	0.9413	0.9765
Higuchi	0.9865	0.9731
First order	0.9895	0.9578
Korsmeyer-peppas	0.9224	0.8733
Hixson crowell	0.9780	0.9073

long linear aliphatic chain. . The presence of peaks at 3604cm^{-1} (O-H stretching), (C-H stretching) & 1108cm^{-1} (C-O group) were due to polaxamer associated with lipid. Moreover, the presence of MTX & DOC aromatic peaks at 1615cm^{-1}-1495cm^{-1} and 1108cm^{-1}, 985cm^{-1}, 722cm^{-1}, 685cm^{-1} (due to C-H bending of aromatics) in the spectra suggests that the drugs were embedded in the lipid nanoparticles stabilized by polaxamer. The FTIR of samples are shown in figure.1

Particle size analysis

The particle size of the formulations F1, F2, F3 obtained is shown in table 2 and fig 2. The formulation F3 had the particle size less than 200nm (157.2nm, PDI value of 0.390) hence, it has been lyophilized and used for further characterization. The obtained zeta potential of F3 was found to be -9.6mV which is enough to ensure good physical stability. As the PDI was greater than 0.1, filtration was carried out using a membrane filter. Particle size has influences the drug loading, stability and its release characteristics. They also decide the in vivo distribution, biological fate and the targeting capability of nanoparticle drug delivery systems [13, 14]. Particles less than 200nm are said to be invisible to RES and prolong their circulation in vivo. The size also depends on the crystallization of lipid. i.e formation of small particles with high crystallization of low melting point lipids. Also the added polaxamer having high HLB and high molecular weight might have resulted in the formation of nano particles of lesser size [15].

Determination of drug content & entrapment efficiency

The percentage purity of the MTX was found to be 65.07% and 79.56% for DOX. The entrapped quantity of drugs was found to be 3.2535mg of MTX and 39.78mg of DOX respectively. It has been reported that the particle size, drug loading capacity of SLNs is found to vary with lipid (triglycerides, fatty acids, steroids, waxes etc), emulsifier (anionic, cationic, non - ionic) and the method of preparation etc. especially the loading capacity depends upon the solubility, miscibility of drug in the melted lipid, chemical and physical structure of solid lipid matrix & the polymorphic state of lipid material. The pre – requisite to obtain a sufficient loading capacity is a sufficiently high solubility of the drug in the lipid melt. Typically the solubility should be higher than required because, it decreases when cooling down the melt and might be even lower in the solid lipid. To enhance the solubility in the lipid melt one can add solubilizers [16]. In addition, the presence of mono and diglycerides in the lipid matrix material promotes drug solubilization. The chemical nature of the lipid is also important because lipids which form highly crystalline particles with a perfect lattice lead to drug expulsion. Further the solubility of most of the therapeutic agents has been reported to be more in polaxamer than tweens and spans [15].

Stability Studies

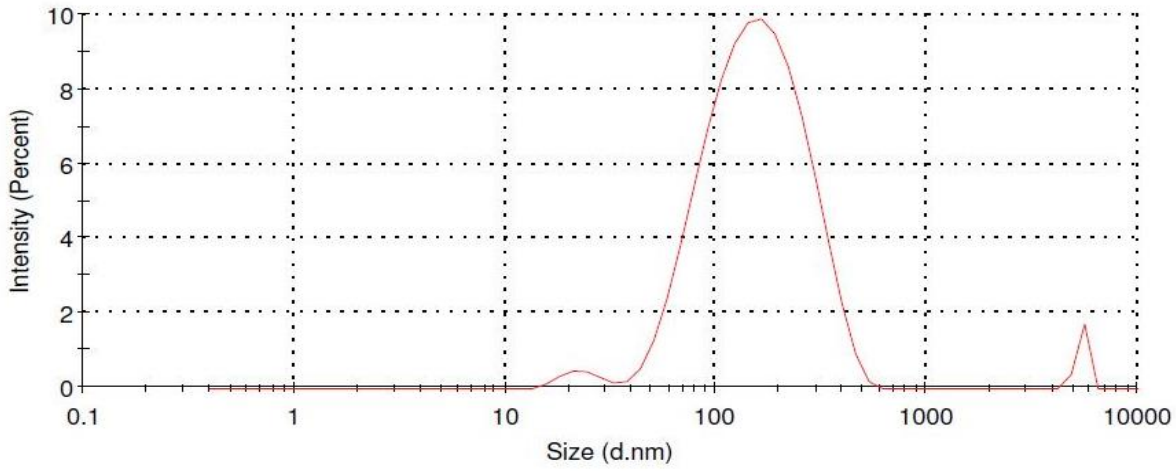

Fig. 2. Zeta size of F3 (SLN).

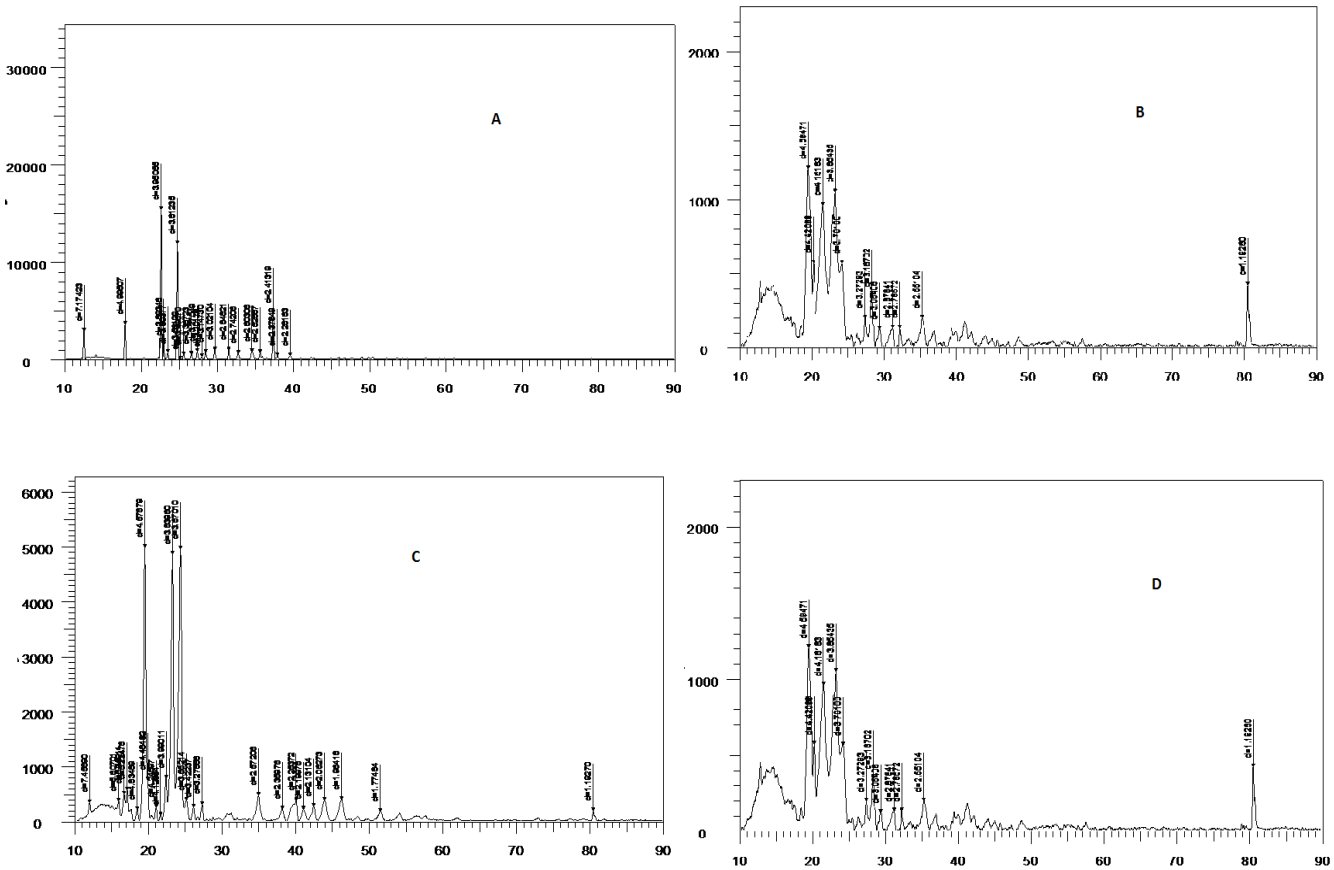

Fig 3.The PXRD patterns of (A) MTX, (B) DOX, (C) Tristearin and (D) Formulation F3 (SLN).

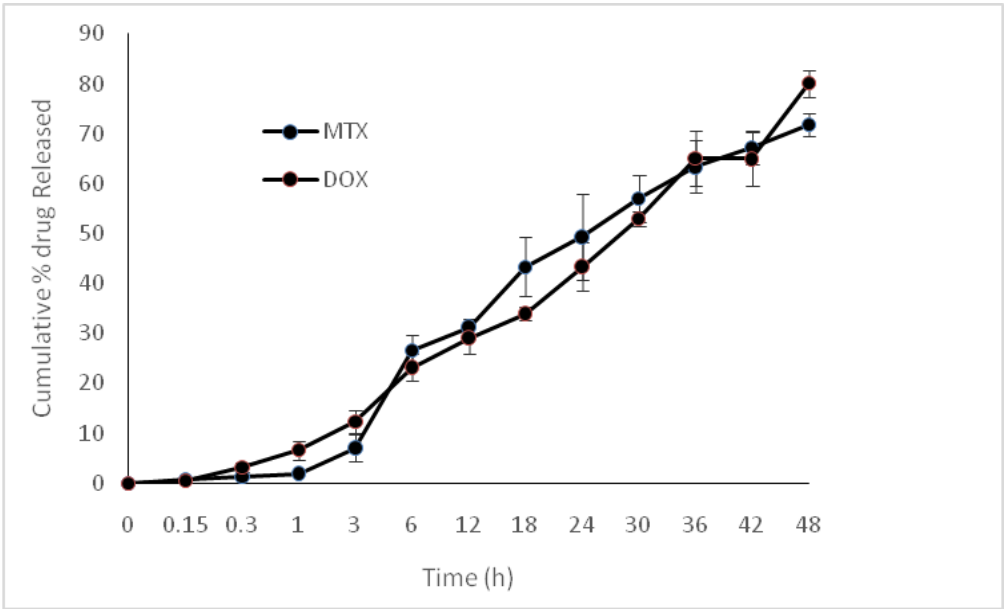

Fig 4. *In-vitro* release of Methotrexate and Doxycycline from F3 (SLN).

The SLNs was found to retain physicochemical characteristics at refrigeration upon 30days storage.
Therefore, MTX-DOX-SLNs are recommended to be stored at refrigerated temperature. The physical properties of SLNs during prolonged storage were determined by monitoring changes in particle size as the function of time. The particle size analysis at different time points is given in table 3. No much deviation in particle size on day 30 compared with day

0 reflects their physical stability at the conditions tested.

Degree of crystallinity

The recorded PXRD patterns of MTX, DOX, Tristearin and their SLNs (F3) are shown in figure 3. Generally XRD dictates the polymorphic transition of lipid nanoparticles which have impact on loading, release and long term stability. PXRD pattern of MTX and DOX exhibiting sharp peaks at 2θ scattered angles 10.5, 17.4, 19.4, 19.7 and 23.7 indicates their crystalline nature. In formulation F3 simultaneous peak intensities were observed showing that degree of crystallinity of MTX – DOX SLNs remains the same suggesting that F3 was in crystalline form and thus more stable.

In-vitro release of DOX and MTX from SLN

The results of the in vitro release studies are shown in table 4 & figure 4. Both MTX & DOX were found to follow higuchi first order & higuchi zero order release kinetics respectively. Initially (upto 3h) the release was very less as it takes time for the drugs to diffuse out of the lipid indicating a strong embodiment of drugs in the lipid matrix & surrounding polaxamer coating & its thickness (which is based on concentration)[17]. As less amount of MTX has been loaded in the SLN, the release has become concentration dependant. For DOX, the loaded amount was high & it followed a constant rate of release i.e. independent of DOX concentration. It could be stated that the results of the release kinetics is suited for their pharmacokinetics behavior in vivo. The sustained release up to 48h of study, made this SLN formulation suitable for sustained effect of drugs MTX & DOX in all routes of administration which may helps in the reduction of drug toxicities in vivo.

CONCLUSION

Hot homogenization method was found to be suitable to produce SLNs with a nanosize of 157.2nm and to combine a lipophilic and a hydrophilic drug successfully with a triglyceride (Tristearin) and a surfactant (Polaxamer). Sterilization of SLNs as has not been done since the preparation was accompanied by heat. High speed stirring induced optimum zeta potential & size for MTX-DOX-SLN. PXRD analysis showed a stable crystalline state of MTX and DOX in SLN. Positively charged MTX-DOX-SLN has elicited sustained and independent release of MTX and DOX from the SLNs for longer duration. Thus, the initial results are promising for the delivery of dual drugs MTX and DOX from the developed SLNs without any incompatibility. However, in vivo studies relevant to RA are needed to be conducted to confirm their in vivo efficacy.

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