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Original Article

FORMULATION AND EVALUATION OF PARENTERAL METHOTREXATE NANOLIPOSOMES

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ABSTRACT

Objective: The objective of the present study was to encapsulate Methotrexate in liposomal formulation for treatment of cancer. Conventional compositions of Methotrexate are available but in high doses showvariation in bioavailability and they are associated with a number of toxicities when administered orally. To overcome these problems, in the present study, inclusion of Methotrexate in parenteral liposomal formulation was approached with the aim of increasing retention time at the site of action which leads to improvement in bioavailabilityand better tumor targeting.

Methods: In this study, PEGylated Methotrexate liposomes containing Hydrogenated Soy Phosphatidyl Choline and Cholesterol were prepared by thin film hydration method. The main advantage of PEGylated lipid vesicles lies in the possibility of active-targeted delivery of drugs to the tissues or organs that need those most. Attempts were made to enhance the encapsulation by use of non-ionic surfactants such as Tween-80, Tween-20 and solubilityenhancers such as β-cyclodextrin. The characterization of formulated liposomes was carried out by vesicle size, zeta potential, %free drug and in-vitro dissolution.

Results: Formulation containing 10mg/ml of Tween-20 and 20 mg/ml of β-cyclodextrin showed highest encapsulation efficiency. The optimized formulation has exhibited more than 90% release of the drug within a period of 4 days. The accelerated stability studies (40±2°C/ 75±5% RH) of the Methotrexate liposome were conducted for a period of three months and the formulation was found to be stable.

Conclusion: These results suggest that the liposome encapsulated MTX may serve as a useful targeted drug delivery system for effective management of neoplastic diseases.

Keywords: Methotrexate, Liposomes, HSPC, Cholesterol, mPEG-DSPE.

INTRODUCTION

Phospholipid vesicles (liposomes) were originally developed as a model membrane system. However, it was soon realized that their capacity to entrap ions and small and large molecules along with their low permeability presented considerable advantages as a drug delivery system. The idea was that drugs entrapped in the aqueous space(s) inside liposomes could be delivered to the site of action with greater selectivity and/or less degradation than for the free drug. Their ability to potentiate the pharmacological efficacy of various drugs *In vitro* against mammalian cultured cells enhanced their prospects as a drug delivery system. The goal of any drug delivery system is to provide a therapeutic amount of the drug to the proper site in the body, to achieve prompt effect and then maintain the desired drug concentration. Liposomes are microscopic, fluid-filled pouches, whose walls are made up of layers of phospholipids identical to the phospholipids that make up cell membrane [1].

The potential of liposomes for delivering anticancer drugs has been investigated extensively in different cancer types. Especially the chemotherapeutic drug doxorubicin has received much attention, since it is easy to load high amount of this drug into liposomes [2]. In fact, commercially available untargeted liposomes loaded with doxorubicin, marketed under the name Caelyx®, have already been approved for clinical use in several cancers [3] and have also been investigated in clinical trials for glioblastoma multiforme [4]. Untargeted liposomal formulations of doxorubicin benefits especially from the improved toxicity profile and have also demonstrated some improvements in therapeutic efficacy compared to the free drug [5]. So far, targeted liposomes have only been investigated in animal models of human cancers, where targeting of liposomal drugs to the cancer cells or the tumor vasculature have demonstrated a more pronounced inhibition of tumor growth than administration of free drug or untargeted liposomes [6]. Some recent publications employ a combination of several liposome types or conjugate a number of different targeting molecules to the surface of a single liposome, in order to achieve a more efficient tumor targeting than with only one targeting agent. Several of these studies indeed demonstrated a synergistic effect compared to only using one targeting agent [7, 8].

Methotrexate (MTX) is widely used in the treatment of neoplastic disorders. Methotrexate inhibits dihydrofolic acid reductase. Dihydrofolates must be reduced to tetrahyrofolates by this enzyme before they be utilized as carriers of one-carbon groups in the synthesis of purine nucleotides and thymidylate. Therefore, methotrexate interferes with DNA synthesis, repair, and cellular replication. The drug side-effect profile includes mouth sores, stomach upset, and low white blood counts. MTX can cause severe toxicity of the liver, kidneys, and bone marrow, which requires regular monitoring with blood tests. It can cause headache and drowsiness, itching, skin rash, dizziness, and hair loss. MTX when given orally has a short elimination half-life. In leukemic pediatric patients, oral absorption of methotrexate appears to be dose dependent and has been reported to vary widely (23% to 95%). The absorption of doses greater than 40 mg/m^2 has been reported to be significantly less than that of lower doses. Food has been shown to delay absorption and reduce peak concentration. This can be overcome by encapsulating MTX in lipid vesicles with the aim of increasing the retention time at the site of action. PEGylated vesicles of MTX can be prepared which are "stealth" lipid vesicles that evade detection and destruction by phagocytes by virtue of the presence of hydrated PEG (polyethylene glycol) molecules. The other main advantage of PEGylated lipid vesicles lies in the possibility of active-targeted delivery of drugs to the tissues or organs that need them most. Not only does this maximize delivery efficiency for the agent in question, but it also minimizes the chances of toxicity to other organs [9].

MATERIALS AND METHODS

Materials

Methotrexate was obtained from Fermion-Finland; Hydrogenated Soy Phosphatidyl Choline (HSPC), mPEG2000-Distearyl Phosphatidyl Ethanolamine and Cholesterol were obtained from Lipoid AG, Germany. β-cyclodextrin was obtained from Cydex pharmaceuticals.

Tween-20 and Tween-80 were obtained from Croda Inc. New Jersey. All other chemicals used were of analytical grade.

Construction of calibration curve by UV

A stock solution of (100mg/ml) standard drug was prepared, required dilutions were made with phosphate buffer pH 7.4. To a series of 10 mL volumetric flasks aliquots standard solutions were added and the volume was made up using phosphate buffer pH 7.4. The absorbance of these solutions was measured at 303 nm using 1 cm quartz cuvette in UV-Visible double beam spectrophotometer.

Preparation of liposomes

Lipid nano vesicles were prepared by the thin-film hydration method using passive-loadingtechnique [10]. The molar ratios of lipids (phospholipids-mPEG-DSPE/HSPC and cholesterol) were accurately weighed and dissolved in a minimal quantity of a mixture of chloroform: methanol (2:1) in a 250 mL round bottom flask with a ground glass neck to obtain a clear solution. The solvent removal was achieved by using Buchi Rota evaporator rotated at 60 rpm in order to completely evaporate the solvent and to obtain a thin dry lipid film. Hydration of the dry lipid film was achieved by adding the MTX in PBS pH 7.4 buffer at a concentration of 1.5 mg/mL and the temperature of the hydrating medium was maintained above the gel-liquid crystal transition temperature (Tm) of the phospholipids with the highest Tm, before adding to the dry lipid. Once stable multilamellar vesicles (MLV) suspension was produced, it was subjected to sonication for a period of 20 minutes by transferring the suspension into a glass vial.

In-process Checks

RPM: 60rpm (film formation), 100rpm (Hydration).

Temperature: 45°C (film formation), 60°C(Hydration).

Physical characterization of liposomes

Particle size and Zeta potential determination

Determination of particle size (in nanometers) and size distribution (as the PDI) of the liposomal suspension was measured by using Horiba, Nanoparticle SZ-100 series. Zeta potential measurement of the optimized liposomal suspension was done by using the Malvern nano zeta sizer instrument.

% Drug entrapment efficiency (EE)

Encapsulation efficiency was determined as the percentage of methotrexate encapsulated in liposome to the original amount of Methotrexate added. To determine drug loading efficiency of liposome, lipid vesicles were lysed using methanol: ethanol (2:1). Entrapped %, free drug % were calculated by using the equations…

Entrapment % = (Total Drug-Free Drug)/Total Drug*100

= (A2-A1)/A2*100

Free Drug % = (Total Drug-Entrapment %)

***In vitro* drug release**

The *In vitro* release of drug from the liposomal formulation was carried out by using membrane diffusion technique using the dialysis membrane. 5 ml of liposomal suspension containing known amount of drug was placed in a dialysis membrane previously soaked overnight. A glass cylinder open at both ends was placed in 200 ml of PBS (pH 7.4), maintained at 37^{0}C and stirred with the help of a magnetic stirrer. Aliquots (5 ml) of release medium were withdrawn at different time intervals and the sample was replaced with fresh PBS (pH 7.4) to maintain constant volume. The samples were filtered through 0.45µm membrane filter and were analyzed by UV spectrophotometry at a λ max of 303 nm.

Release kinetics

To analyze the *In vitro* release data various kinetic models were used to describe the release kinetics. The zero order rate equation (At = A0 – K0t) describes the systems where the drug release rate is independent of its concentration. The first order equation (Log C = log C0 – Kt / 2.303) describes the release from system where release rate is concentration dependent. Higuchi described the release of drugs from insoluble matrix as a square root of time dependent process based on Fickian diffusion. Mechanism of drug release from the liposomal solution is described by Korsmeyer equation / Peppas model [11].

Accelerated Stability Studies

The accelerated stability study was conducted to monitor physical and chemical stability of the liquid form of parenteral Methotrexate liposomal formulation for up to three months. The stability parameters, such as description, pH and assay were determined as function of the storage time.

RESULTS AND DISCUSSIONS

Calibration curve of Methotrexate in UV spectrophotometer

The UV absorbance of Methotrexate standard solution in the range of 2-20 µg/ ml of drug in buffer, pH 7.4 showed linearity at λ max 303 nm. The linearity was plotted for absorbance against concentration with the slope equation y = 0.055x - 0.014. The standard curve is shown in Fig 1.

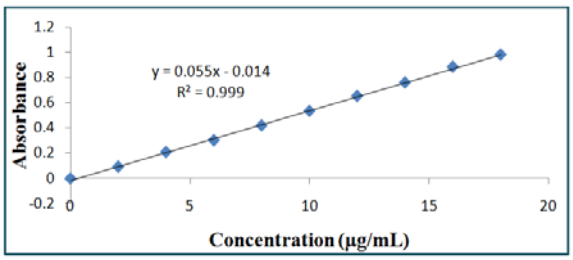

Fig. 1: Standard graph of Methotrexate in phosphate buffer of pH 7.4Fourier Transformer Infra Red (FT – IR) Spectroscopy

The compatibility between the drug and the selected lipid and other excipients was evaluated using FT-IR peak matching method. There was no appearance or disappearance of peaks in the drug-lipid mixture, which confirmed the absence of any chemical interaction between the drug, lipid and other chemicals as shown in Fig 2 and 3.

Preparation of Methotrexate liposomal formulation

The Liposomes were prepared by dried thin film hydration technique using rotary evaporator. The parenteral liposomal formulation containing Methotrexate was prepared by using lipids, cholesterol and non-ionic surfactants- Tween-20 and Tween-80 and β-cyclodextrin was used as a solubility enhancer and all other parameters like temperature and RPM were kept constant. Drug to lipid ratio was varied and for optimized formulation it was found to be 1:20. The composition of the formulations is shown in table 1.

Among compositions four formulations, namely F7, F9, F10 & F12 were selected as optimized batches for further evaluation as shown in table 2.

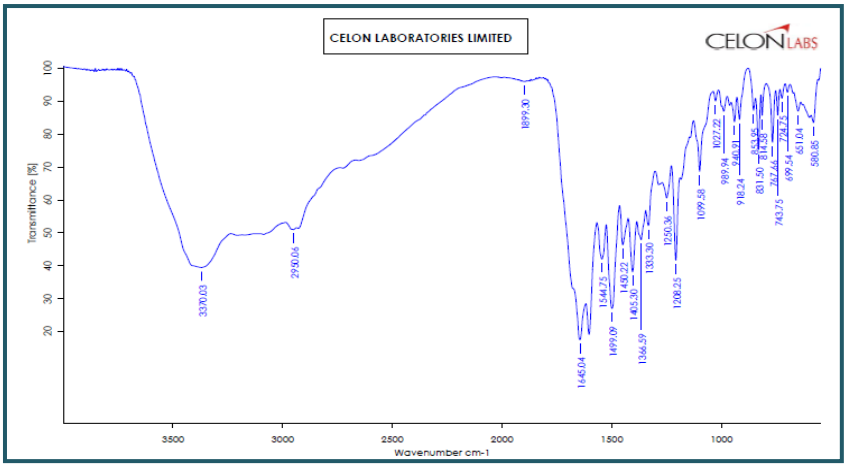

Fig. 2: FTIR of Methotrexate (Pure API)

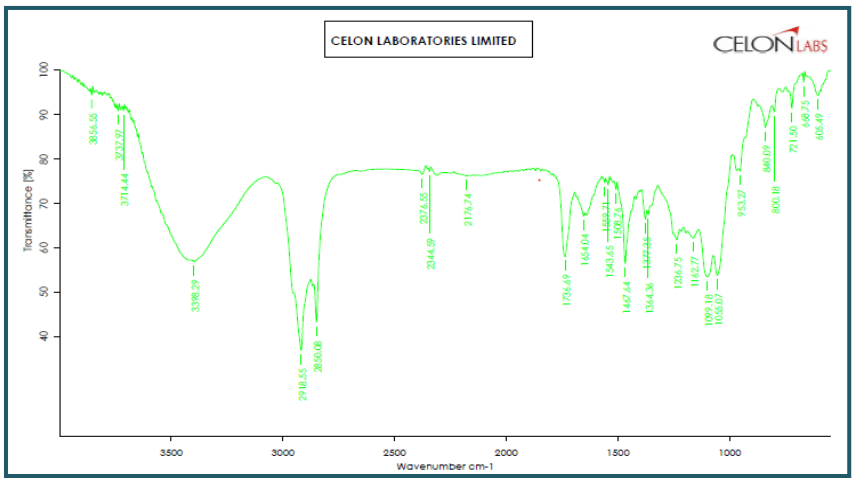

Fig. 3: FTIR of Methotrexate liposomal formulation

Table 1: Formulation studies of Methotrexate liposomes

S. No.	Ingredient	Qty/ml												
		F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13
1.	Methotrexate (mg)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
2.	Hydrogenated Soy Phosphatidyl Choline (mg)	30	30	40	50	50	50	50	50	50	50	50	50	50
3.	mPEG2000-DSPE (mg)	8	8	8	8	8	8	8	8	8	8	8	8	8
4.	Cholesterol (mg)	8	18	18	18	24	36	50	50	50	50	50	50	50
5.	Tween-80 (mg)	-	-	-	-	-	-	-	5	10	-	-	-	-
6.	Tween-20 (mg)	-	-	-	-	-	-	-	-	-	10	10	10	10
7.	β-Cyclodextrin (mg)	-	-	-	-	-	-	-	-	-	-	10	20	30
8.	Phosphate buffer (7.4)	q. s	q. s	q. s	q. s	q. s	q. s	q. s	q. s	q. s	q. s	q. s	q. s	q. s

Table 2: Optimized Formulations of Methotrexate liposomes

S. No.	Ingredient	Qty/ml			
		F7	F9	F10	F12
1	Methotrexate(mg)	1.5	1.5	1.5	1.5
2	HSPC(mg)	50	50	50	50
3	mPEG2000-DSPE(mg)	8	8	8	8
5	Cholesterol(mg)	50	50	50	50
5	Tween-80(mg)	-	10	-	-
6	Tween-20(mg)	-	-	10	10
7	β-Cyclodextrin(mg)	-	-	-	20
8	Phosphate buffer (7.4)	q. s	q. s	q. s	q. s
9	Molar ratio of Drug/Lipid	1:20	1:20	1:20	1:20
10	Molar ratio of Lipid/Cholesterol	1:1	1:1	1:1	1:1

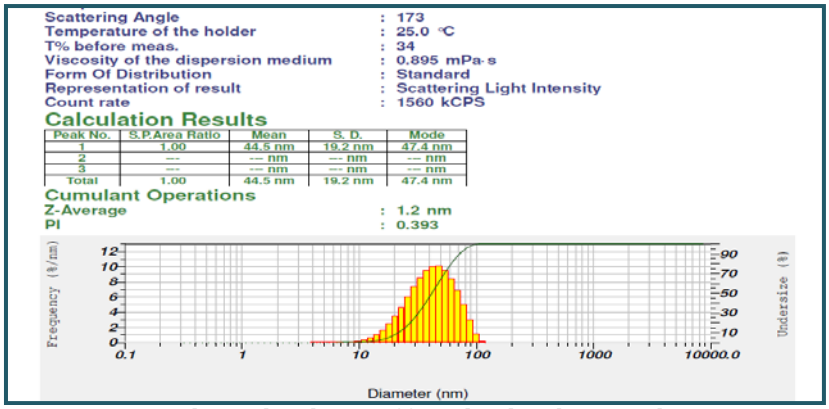

Fig. 4: Particle size distribution of formulated Methotrexate liposomes

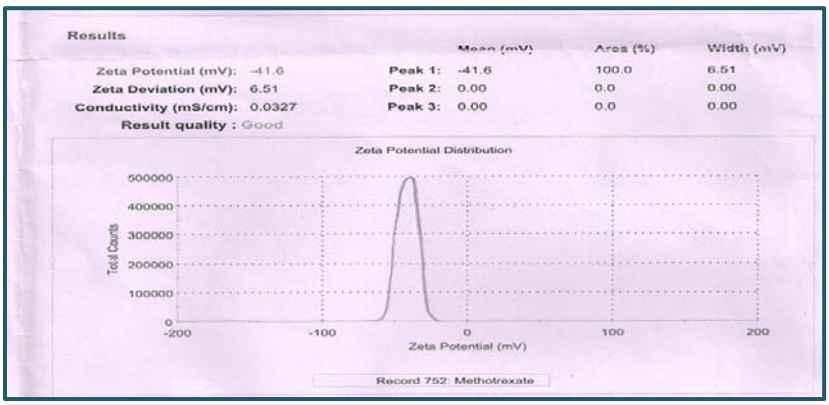

Fig. 5: Zeta Potential of Formulated Methotrexate liposomesEntrapment efficiency of Methotrexate in liposomes

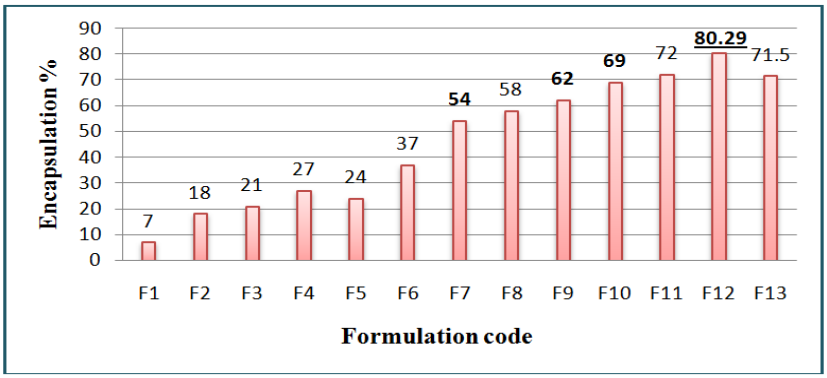

Fig. 6: Graphical representation of Encapsulation efficiency of formulated Methotrexate liposomes

Characterization particle size analysis

Particle size analysis of the sonicated lipid vesicles was determined using Horiba, Nanoparticle SZ-100 series. The results are in Fig. 4. It was observed that the average particle size was found to be 44.5 nm for optimized formulation and PDI (poly dispersity index) was found to be 0.393 indicating good dispersion of uniformly sized lipid vesicles.

Zeta potential analysis

The zeta potential value of liposomal solution F12 was found to be -41.6which lie near to the arbitrary value indicating good stability and the negative value indicates no agglomeration of particles. The results are represented in Fig. 5.

The drug entrapment efficiency studies were conducted for various formulations. The entrapment efficiency of all formulations is graphically represented in Fig. 6. The formulation F12 showed maximum entrapment efficiency.

***In vitro* dissolution data**

The *In vitro* dissolution profile of optimized formulations was determined by membrane diffusion method. The dissolution was carried out for a period of 96 hrs in 7.4 pH phosphate buffer. The cumulative percent release of optimized formulations at various time intervals was calculated. In optimized formulations sustained release was observed up to 96 hrs. These results indicate slow release and more encapsulation of the drug.

Release Kinetics

The release kinetics of F7, F9, F10 and F12 formulations was studied. It was observed that the total drug present in the

formulated liposomes was released in 96 hrs. The regression co-efficient and diffusion co-efficient values for four optimized formulations are tabulated in table 3. Plots of zero order, first order, Higuchi and Peppasare depicted in Fig. 7-10. These values are compared with each other for model and drug equation. Based on highest regression [r^2] values, the best-fit model for optimized formulations was found to be zero order release in case of F12and the formulations F7, F9, and F10 followed higuchi diffusion model. All the formulations were then fitted into korsmeyer-peppas model and n values are reported in table 3. For all the optimized formulations, the 'n' value was in the range of 0.85-0.9 indicating case-II transport.

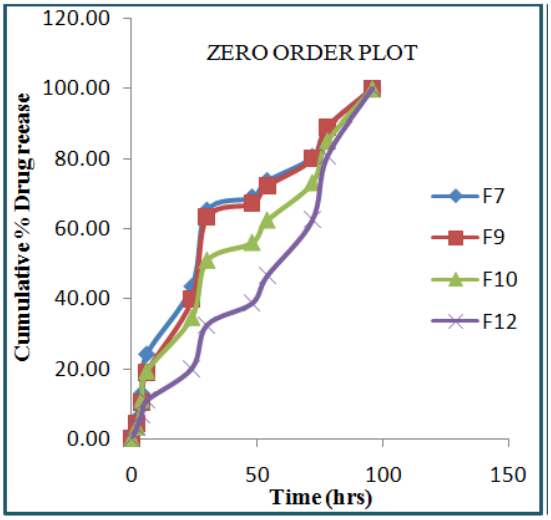

Fig. 7: Zero order release graph for optimized formulations F7, F9, F10 & F12

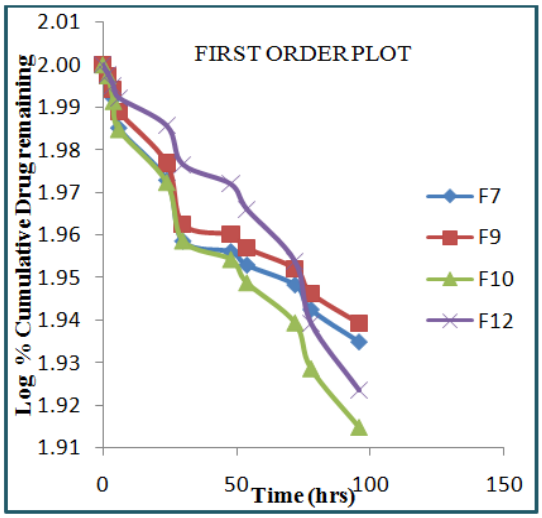

Fig. 8: First order release graph for optimized formulations F7, F9, F10 & F12

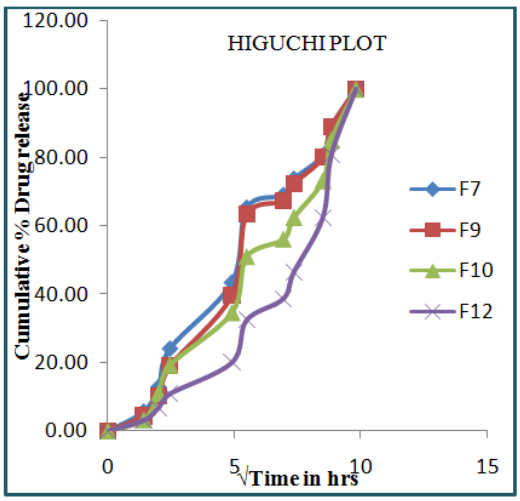

Fig. 9: Higuchi release graph for optimized formulations F7, F9, F10 & F12optimized

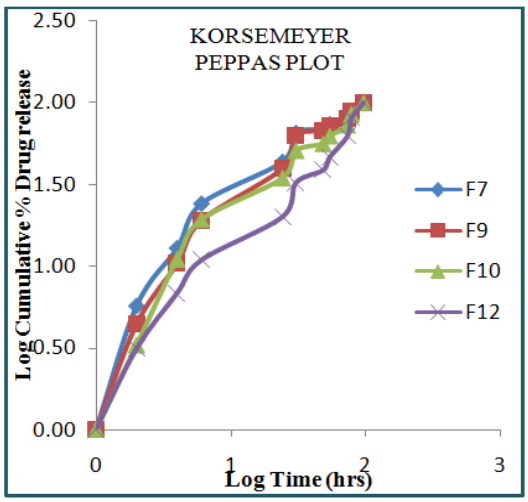

Fig. 10: Korsemeyer Peppas release graph for formulations F7, F9, F10 & F12

Table 3: Regression coefficient and diffusion coefficient values

Formulation	Zero Order		First order		Higuchi		Korsemeyer	
	R^2	K_0	R^2	K_1	R^2	K_H	R^2	n
F7	0.917	0.997	0.925	0.0007	0.981	10.52	0.910	0.856
F9	0.930	1.023	0.937	0.0007	0.979	10.70	0.941	0.895
F10	0.968	0.980	0.972	0.0006	0.973	10.02	0.940	0.889
F12	0.974	0.961	0.968	0.0007	0.889	9.359	0.973	0.889

Table 4: Results of Stability testing of Methotrexate liposomal formulation

Time point	F12			
	Initial	1 month	2 months	3 months
Description	Yellow coloredcake	Yellow colored cake	Yellow colored cake	Yellow colored cake
Ph	6.91	7.02	7.02	7.11
Assay	80.22%	78.53 %	77.12%	77.12%

Stability studies

The stability of Methotrexate liposomes was evaluated for optimized formulation F12 after storage at accelerated condition at $40^0C \pm 20^0C/75\% \pm 5\%$ RH for 3 months. The description, pH, and assay of the sample was determined as a function of the storage time. The liposomes stored at accelerated condition were found to be stable for duration of 3 months. The results are shown in table 4.

CONCLUSION

The parenteral liposomal formulations containing Methotrexate can be successfully formulated by thin film hydration method using passive loading technique. Improvement in encapsulation efficiency and sustained release was achieved by increasing the concentration of lipids, non-ionic surfactants Tween-80, Tween-20 and solubility enhancing agents – β-cyclodextrin. From the above study it is concluded that parenteral liposomal formulations of Methotrexate can be explored as a potential candidate for treatment of various neoplasticdiseases.

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CONFLICT OF INTERESTS

Declared None

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