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Optimized Moxifloxacin Nanoliposomes with Enhanced Stability

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ABSTRACT

Nanoliposomes (NLs) prepared by three different methods were compared in terms of their ability to encapsulate moxifloxacin hydrochloride (MXF). Formulation parameters namely, phospholipid to cholesterol ratio, drug to lipid ratio, and pH, were optimized. NLs with size 277.07±2.18nm and entrapment efficiency (EE) of 66.25±1.89% were obtained. The stability of MXF-NLs was enhanced by lyophilization using different ratios of trehalose. Lyophilization with lipid/trehalose ratio 1:6 was the best ratio in terms of size and EE obtained after rehydration of NLs.

INTRODUCTION

Liposomes are well recognized controlled drug release vesicular carriers composed of one or more concentric phospholipid bilayers surrounding an aqueous compartment allowing encapsulation of both hydrophilic and hydrophobic bioactive. They, selectively, deliver the encapsulated drug to the site of action, prolonging duration of exposure, improving its therapeutic index and potentially overcoming development of resistance [1]. In spite of their biocompatibility, biodegradability and non-immunogenicity, their physical and chemical instabilities while in aqueous dispersions limit their potential application in drug delivery. The fatty acid chains components of the phospholipids tend to oxidize or hydrolyze increasingly with unsaturated components leading to a rise in bilayer permeability. Fusion, drug leakage or precipitation of ingredients during storage are some of the commonly encountered aspects of physical instability.

Freeze drying had been suggested to limit chemical and physical instabilities manifestations with special attention to the membrane integrity with the possibility of drug leakage **[2].** Selection of a suitable cryoprotectant, liposome bilayer composition and freeze drying protocol can protect the lipid bilayer from damage via ice crystal formed during freezing, inhibit vesicles fusion or aggregation after dehydration and prevent large decline in drug entrapment efficiency **[3].**

Moxifloxacin HCl is a broad spectrum fourth generation fluoroquinolone. It is highly effective against several gram–positive and gram-negative microorganisms compared to second and third generation fluoroquinolones (i.e ofloxacin, ciprofloxacin and levofloxacin) **[4].** Encapsulation of moxifloxacin HCl in liposome may prolong its duration of action via a controlled release of it so reducing the dosing frequency and minimizing systemic side effects. In this study, a special emphasis was given to the choice of fabrication and formulation parameters leading to enhanced stability following freeze drying.

MATERIALS AND METHODS

1.1. Materials

Moxifloxacin hydrochloride (MXF) was kindly provided by Medical Union Pharmaceuticals Company, Cairo, Egypt. L-α-phosphatidylcholine, type X-E (PC), from dried egg yolk and

cholesterol were purchased from Sigma Chemical Co. Chloroform, methanol and ethanol, absolute, analytical grade reagent were purchased from Fisher Scientific UK, Bishop Meadow Road, Loughborough. Diethyl ether: Tedia Company, Inc, 1000 Tedia Way, Fairfield, OH45014, USA. Spectra/Por dialysis membrane (12,000-14,000 molecular weight cut off): Spectrum Laboratories Inc, Rancho Dominguez, CA. Trehalose dihydrate from Fisher Scientific, Fairlawn, New Jersey.

1.2. Experimental design:

The effect of some fabrication and formulation parameters namely: method of preparation, phosphatidylcholine to cholesterol ratio (PC: CH), MXF concentration, pH of buffer of aqueous core, was evaluated by varying one factor while keeping all other factors constant during preparation of NLs. Table (1) shows the variables, levels and formulae prepared.

Table (1): Fabrication and formulation parameters evaluated in the preparation of MXF loaded conventional liposomes.

Variable	Level	Formula code
Method of preparation	Reverse phase evaporation (REV)	F1
	Thin film hydration (TF)	F2
	Ethanol injection (EI)	F3
PC: CH	10:0	F4
	9:1	F5
	8:2	F1
	7:3	F6
	6:4	F7
	5:5	F8
MXF: total lipids molar ratio	0.05:1	F9
	0.1:1	F6
	0.15:1	F10
	0.2:1	F11
	0.3:1	F12
pH of buffer	7.4	F10
	5.5	F13

1. 2.1 Preparation of conventional MXF loaded liposomes using various methods

Three methods were used for the preparation of MXF loaded liposomes at constant PC/CH ratio of 8:2 and drug concentration (0.1:1 molar ratio to total lipids used, namely PC and CH). Formulae F1, F2 and F3 were prepared by reverse phase evaporation, thin film hydration and ethanol injection method respectively.

1. 2.1.1. Reverse phase evaporation method (REV)

MXF liposomes were prepared by reverse phase evaporation method (REV) previously described in literature **[5, 6].** Briefly, 200mg of lipid components including PC and CH in a molar ratio of 8:2 were accurately weighed, transferred to a long-neck quick fit round bottom flask and then adequately dissolved in 10mL of a 2:1 v/v mixture of chloroform/methanol mixture. The organic solvent was removed by rotary evaporator (Janke and Kunkel, IKA Laboratories, Staufen, Germany) and the obtained lipid film was dissolved in 12mL diethyl ether. The drug, dissolved in 4mL phosphate buffer solution (PBS) of pH 7.4 was added to the lipid mixture and was sonicated for 5min in a bath sonicator (Crest ultrasonics ETL testing laboratories, Cortland, NY) forming a homogeneous water-in-oil (w/o) emulsion. The organic solvent was then evaporated under reduced pressure at 40°C using a rotary evaporator, rotating at approximately 200rpm until a gel was formed. Upon further vigorous rotary evaporation (300rpm), the resultant gel was broken to give the liposomes. The remaining aqueous phase (16mL PBS, pH 7.4) was then added portion wise with gentle vortex mixing. Then the flask was stirred up at ambient temperature on rotary evaporator at 200rpm for 45min. The liposomes were passed through a stack of polycarbonate membrane with defined pore size (400nm) using an extruder (Lipex extruder, Avestin Inc) to obtain NLs. The liposome suspension was left to mature overnight at 4^{o}C to ensure hydration of the lipid.

1. 2.1.2. Thin film hydration method (TF)

Thin film hydration method (TF) was used as described in literature to prepare F2 **[7, 8].** Lipids, PC and CH at a ratio of 8:2 were dissolved in 10mL chloroform/methanol (2:1 v/v) to obtain a limpid solution. Then the solvent was evaporated from the limpid solution in a rotary evaporator at controlled reduced pressure until a fine film was formed on the walls of the receptacle. The aqueous phase consisting of MXF in 20mL PBS, pH 7.4 was introduced into the aforementioned

receptacle portion wise with gentle mixing and was stirred up vigorously at ambient temperature on rotary evaporator at 200rpm. The agitation continued until there was no lipid deposited on the wall. Prepared liposomes were then extruded and the procedure was completed as before.

1.2.1.3. Ethanol injection method (EI)

According to **[7]**, formula F3 was prepared as follows: The aqueous phase consisting of MXF dissolved in 20mL PBS , pH 7.4, was heated up to 60^0C, stirred well on a magnetic stirrer (Yellow Line MAG HS7, IKA, Germany). The lipid mixture of PC and CH, at a mole ratio of 8:2, was dissolved in absolute ethanol heated at about 60^0C. The ethanolic lipid solution was then quickly injected using a syringe into the above well-stirred aqueous phase, keeping for 30min on the magnetic stirrer. The aqueous phase immediately became milky as a result of liposome formation. The liposomal system was transferred to a round-bottom flask (250mL volume). The flask was attached to a rotary evaporator, lowered into a 40^0C water bath and ethanol was removed under reduced pressure during the hydration reaction. The obtained liposomes were then extruded and treated as explained in section 1.2.1.1.

1.2.2. Optimization of formulation variables

Table (2) shows the composition of the formulae prepared for optimizing the formulation variables used in preparing conventional MXF loaded liposomes.

1.2.2.1. Effect of PC to CH molar ratio

Using the REV method, various PC to CH molar ratios namely 10:0, 9:1, 8:2, 7:3, 6:4 and 5:5 were used for the preparation of liposomes. A constant MXF to total lipid molar ratio of 0.1:1 was maintained and the method was applied as previously explained in section 1.1.1.

1.2.2.2. Effect of varying MXF concentration

MXF liposomes were prepared by REV method as described above in section 1.1.1, keeping PC to CH molar ratio constant at its optimum level, while varying MXF to total lipid molar ratio. The following ratios were tested: 0.05:1, 0.1:1, 0.15:1, 0.2:1 and 0.3:1 mole/mole of MXF: total lipid.

1.2.2.3. Effect of buffer pH

The effect of varying the pH of the buffer of the aqueous core from 7.4 to 5.5 was investigated by preparing liposomes showing optimum PC: CH and MXF molar ratio using PBS of pH 5.5 by REV as previously explained.

2. NLs Characterization

2.1. Determination of drug entrapment efficiency

The entrapment efficiency (EE) of MXF in NLs was determined indirectly by measuring the concentration of the free drug in the aqueous phase of NLs. Centrifugation was carried out using Nanosep® centrifuge tube fitted with an ultrafilter (MWCO 100KD; Pall Life Sciences, Port Washington, NY). About 100µl of NLs dispersion sample was placed in the upper chamber of the Nanosep® and was diluted to 500µL with PBS. The unit was centrifuged at 9000rpm for 90min at 4^0C. The NLs along with the encapsulated drug remained in the upper chamber while the aqueous phase moved into the sample recovery chamber through the filter membrane. The amount of free MXF in the aqueous phase was estimated spectrophotometrically using UV-visible spectrophotometer (Model UV-1601PC; Shimadzu, Kyoto, Japan) at the predetermined λ_{max} (289nm). The individual value for three replicate determinations and their mean values were reported. EE% was calculated according to the following equation:

$$EE\% = \{(W_{\text{initial drug}} - W_{\text{free drug}})/W_{\text{initial drug}}\} \qquad \textbf{(Equation 1)}$$

Where "$W_{\text{initial drug}}$" is the total amount of the drug used and "$W_{\text{free drug}}$" is the amount of free drug detected in supernatant after centrifugation of the aqueous dispersion.

2.2. Particle size analysis

The particle size (PS) analysis was performed by dynamic light scattering (DLS) using Malvern Zetasizer 4 (Malvern Instrument, UK). The DLS yielded the hydrodynamic average diameter of the main population and the polydispersity index (PDI) which is the measure for the width of the PS distribution. All samples were diluted with PBS until the particles count reached 200-300 kilo count per second (KCPs) before analysis. Ressults were performed in triplicates of different batches.

2.3. Zeta potential determination

The surface charge of NLs was determined by the measurement of the zeta potential (ζ) of the freshly prepared NLs dispersion using Malvern Zetasizer 4 (Malvern Instrument, UK). ζ was calculated according to Helmholtz Smoluchowsk from their electrophoretic mobility. All samples were diluted with PBS to the same concentration used in PS analysis before determination. Results were performed in triplicates of different batches.

2.4. Morphological examination by transmission electron microscope (TEM)

Transmission electron microscopic (TEM) analysis was used to examine the vesicle ultrastructure **[9]** after applying a negative stain. A drop of NLs suspension was applied on carbon-coated copper grid to allow its absorption in the carbon film and after 2–3 min the excess was drawn off with filter paper. Subsequently, a drop of 2% (w/v) phosphotungstic acid was placed on the grid. Negatively stained NLs were examined by TEM (Hitachi-H-600, Joel, Japan), at a power of 120 kV.

2.5 *In vitro* release study

The dialysis membrane diffusion technique was adopted to study the *in vitro* release of MXF from selected formulae **[10, 11].** Briefly, an accurately measured amount of NLs dispersion containing about 1mg of MXF was placed in the dialysis bag. Both ends of the bag were clamped and each bag was individually immersed in vial containing 3mL PBS (0.05M, pH 7.4) as a release medium. All vials were placed in water bath maintained at 37 ± 0.5^0C and shaking was kept constant at 50rpm throughout the release study. At predetermined time intervals, (0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 24 and 48 h); samples were withdrawn, replaced with fresh buffer and assayed spectrophotometrically at λ_{max} (289nm) for MXF content. The release experiments were carried out in triplicate and the results were expressed as mean ± standard deviation.

2. 6. Stability studies

2. 6.1. Determination of the physical stability of MXF liposomal dispersions

Selected MXF loaded NLs were stored at refrigeration temperatures (4^0C) for three months. Samples from each batch were withdrawn at specified time intervals to determine the residual

amount of the drug in the vesicles as described previously in section 2.1.3 and the average PS and ζ of the selected formulations were also determined, results can be taken as an index of stability **[12, 13].**

2.6.2. Freeze drying

2.6.2.1. Nanoliposomes freeze drying

Lyophilization of liposomal dispersions was performed using trehalose as cryoprotectant. The liposome dispersions with different mass ratios of lipid to cryoprotectant (1:1 to 1:8) were first frozen at - 20˚C. Then the frozen liposomal dispersions were placed in the freeze dryer (Christ alpha 1-2 LD plus, Christ, Germany) for 48 h to yield the lyophilized liposomal powders. The temperature of the freeze dryer was set at -50^{0}C and the vacuum was below 0.07 mBar.

2.6.2.2. Estimation of recovery of NLs from freeze dried powder:

The ability of FDNLs to disintegrate into a homogenous suspension of NLs in aqueous media was evaluated. An amount of 10mg of the dry powder was dispersed in 3ml of PBS and vortexed for 1-2 min. The size, EE and zeta potential of the dispersed particles were determined compared to their values for the NLs before freeze drying.

2.6.2.3. Differential scanning calorimetry (DSC)

Samples of MXF, PC, CH, plain MXF loaded NLs colloidal dispersion and FDNLs were subjected to DSC analysis (DSC-60, Shimadzu, Tokyo, Japan). The analysis was carried out on 40µl of NLs or 1mg of individual component samples sealed in standard aluminum pans. Thermograms were obtained at a scanning rate 10^{0}C/min to temperature 300^{0}C using dry nitrogen at a flow rate of 30mL/min.

3. Statistical analysis

All formulations were prepared and reported in triplicates. Blank formulae were prepared along with MXF loaded NLs formulae. Results are expressed as mean ± SD (standard deviation). The statistical significance of difference between groups were evaluated by one-way ANOVA and Tukey's post hoc test with a significance level of $p < 0.05$.

4. RESULTS AND DISCUSSION

The objective of this study was to prepare NLs with high drug EE, uniform size suitable as a controlled release delivery system of MXF antibiotic. For this purpose, the work focused on preparation and characterization of conventional liposomes including selection of the optimum preparation method and formulation parameters.

4.1 Preparation and optimization of MXF loaded NLs

4.1.1. Effect of preparation method of EE% MXF in liposomes

As showed in Figure (1), liposomes prepared by REV method had an EE% of 50% exceeding those prepared by TF and EI methods with respective EE% of 23.5 and 19.1%. During preparation of liposomes via REV method, following evaporation of the organic solvents (methanol and chloroform), the formulation contained 200mg total lipids equivalent to ~ 266µmol lipid per 4mL (~ 66µmol/mL of the aqueous phase) mixed with a total volume of 12mL diethyl ether to form w/o emulsion **[6]**. Diethyl ether was then evaporated and phase inversion occurred resulting in formation of LUV **[8, 14].** The high lipid concentration at this step (~66µmol/mL of aqueous phase) resulted probably in the entrapment of a large amount of the hydrophilic drug MXF. All water soluble drug molecules were contained in the small water droplets of the w/o emulsion which may coalesce into liposomes. Addition of the remaining aqueous phase did not extract the drug contained within the small droplets of the aqueous internal phase **[7].**

In TF hydration method, it was necessary to ascertain that no residue of the organic solvent remained, so the lipid film was placed under a high vacuum for at least 4 h to remove any residual solvent. Since, the aqueous phase (20mL of PBS, pH 7.4) containing the whole drug amount was added to hydrate the lipid film and left agitated on rotary evaporator till no lipid was seen attached to the wall of the rotary flask. Similarly, in EI method, the solution of lipid mixture was mixed with the aqueous solution of MXF so the lipid concentration during the processing was lower than that in REV.

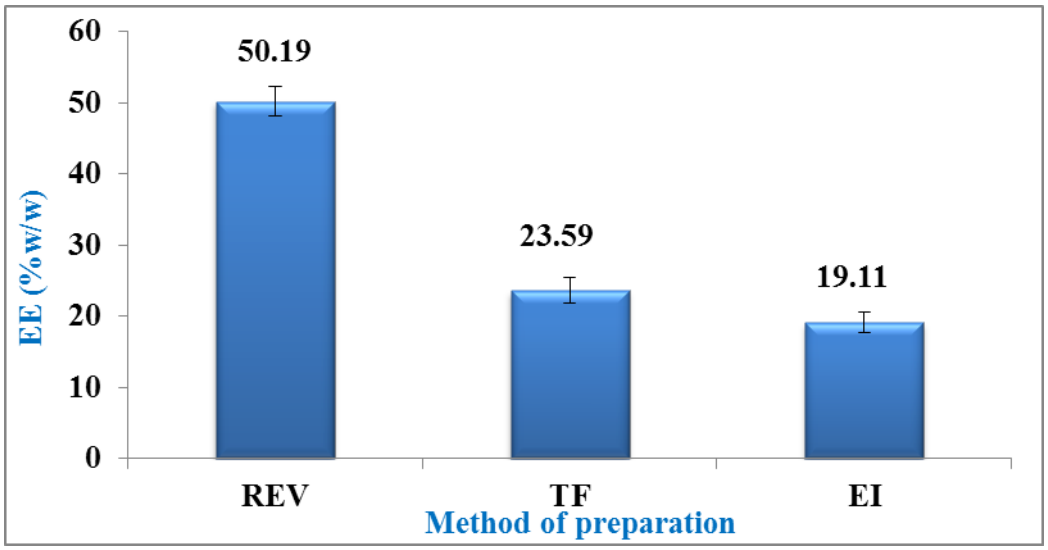

Figure (1): Entrapment efficiency of MXF in liposomes obtained by various methods of preparation.

4.1.2. Effect of PC to CH molar ratio:

Using the REV method and at constant drug loading (0.1:1 MXF/lipid molar ratio), as can be seen from Table (2), F4 lacking cholesterol and F8 containing the highest CH content (PC/CH ratio 1:1) exhibited the largest PS (371.98±6.98 and 379.23±12.25 respectively) and highest PDI (0.645±0.039 and 0.625±0.046 respectively). Compared to F4, addition of CH in a PC/CH ratio of 9:1 decreased the PS and PDI significantly to 243.96±1.78 and 0.557±0.093 respectively as can be seen in F5. Table (2) and Figure (2) reveal that further increase in CH amount, caused significant increase in PS and decrease in PDI as can be noticed in F1 and F6 prepared with PC/CH ratios of 8:2 and 7:3 respectively. With lower PC/CH ratios or higher CH amounts considerable increase in PS and PDI was then seen as could be seen by comparing F7 and F8 to F6. An optimum CH level should, therefore, be maintained in the liposomal formulation.

The absence of CH in the formulation probably favored vesicles aggregation **[15].** Incorporation of CH in the formulations led to the formation of liposomes with homogenous distribution as evidenced by the small PDI **[2]**. This can be explained on the basis of the increased stability of the liposomal membrane during hydration due to the increased rigidity **[16, 17]** and microviscosity of the lipid bilayer as well as reduced permeability **[18]** imparted by CH. Moreover, CH has always been considered as a crystal-breaker of the gel phase and inducer of

chain ordering in the fluid phase without rigidification of the overall phase **[18, 19]**. The increase in PS of liposomes of F6 and F1 compared to F5 can also be explained based on the increased EE%. However, further increase in CH content beyond a certain level (PC/CH ratio of 7:3) in the formulations started to disrupt the regular bilayer structure by interfering with the close packing of phospholipids in the vesicles leading to increasing in PDI. This can also be evidenced by the lower EE% seen with F7 and F8.

EE% of MXF, a water-soluble compound was greatly affected by varying CH content in the NLs as demonstrated in Figure (3a). It is increased with increasing CH amount as could be seen by comparing F5, F1 and F6 to F4 devoid of CH and then decreased with further increase in CH amount and decrease in PC (formulae F7 and F8). Increasing CH level from (PC/CH 10:0 to 7:3, molar ratio) led to significant increase in EE that can be ascribed to the ability of CH to cement the leaking spaces in the phospholipid bilayers. Furthermore, it has been postulated that the EE of hydrophilic compounds in lipid vesicles greatly depend on the inner volume of the vesicle **[18, 19]**. On the other hand, using higher level of CH (PC/CH 6:4 in F7, and 5:5 in F8) with a lower PC level starts disrupting the regular bilayer structure leading to leaching of drug and lowering of drug entrapment **[20, 21].** In accordance with our finding, Chen and co-workers reported decrease in PS, PDI and EE% of paclitaxel with decrease in PC:CH ratio from 2:1 to 1:2 and attributed this observation to the destruction of liposome membrane at high CH concentration **[22]**.

Based on the results presented in Table (2) and Figures (2 and 3a), there was no significant difference between the PS of F1 and F6 but F6 showed more homogeneous PS distribution. Hence, a molar ratio of (7:3) of PC/CH was selected for further work. At this ratio, the highest EE% and lowest PDI with a small PS were noted.

Table (2): Characteristics of conventional MXF loaded liposomes prepared by REV method.

Formula Code	PC:CH (molar ratio)	MFX Mole fraction**	PS (nm)	PDI	EE (%w/w)	ζ (mV)
F4	10:0:0	0.1	371.98 (0.98)	0.645 (0.039)	27.45 (3.57)	-7.85 (0.78)
F5	9:0:1	0.1	243.69 (1.78)	0.557 (0.093)	34.39 (2.19)	-9.84 (0.55)
F1	8:0:2	0.1	265.39 (2.55)	0.434 (0.005)	50.19 (2.04)	-12.45 (0.74)
F6	7:0:3	0.1	272.23 (1.63)	0.286 (0.008)	53.94 (2.53)	-12.08 (0.97)
F7	6:0:4	0.1	323.23 (9.95)	0.389 (0.015)	40.36 (2.02)	11.12 (0.45)
F8	5:0:5	0.1	379.97 (12.25)	0.625 (0.046)	35.28 (2.85)	-10.21 (0.81)
F9	7:0:3	0.05	265.57 (5.79)	0.377 (0.038)	42.07 (5.32)	-11.93 (0.55)
F10	7:0:3	0.1	277.07 (2.18)	0.303 (0.024)	66.25 (1.89)	-12.31 (0.76)
F11	7:0:3	0.15	280.87 (1.34)	0.291 (0.009)	50.96 (1.57)	-11.52 (0.66)
F12	7:0:3	0.2	284.77 (2.63)	0.318 (0.031)	33.53 (2.33)	-11.42 (0.51)
F13	7:0:3	0.3	272.62 (5.42)	0.318 (0.013)	59.42 (1.31)	-9.84 (0.96)

Results are mean of three determinations (SD).*All formulae were prepared using PBS, pH 7.4 except F13 where PBS of pH 5.5 was used. **Calculated as mole/1mole of total lipids. PC: egg phosphatidylcholine, CH: Cholesterol and MXF: moxifloxacin hydrochloride.

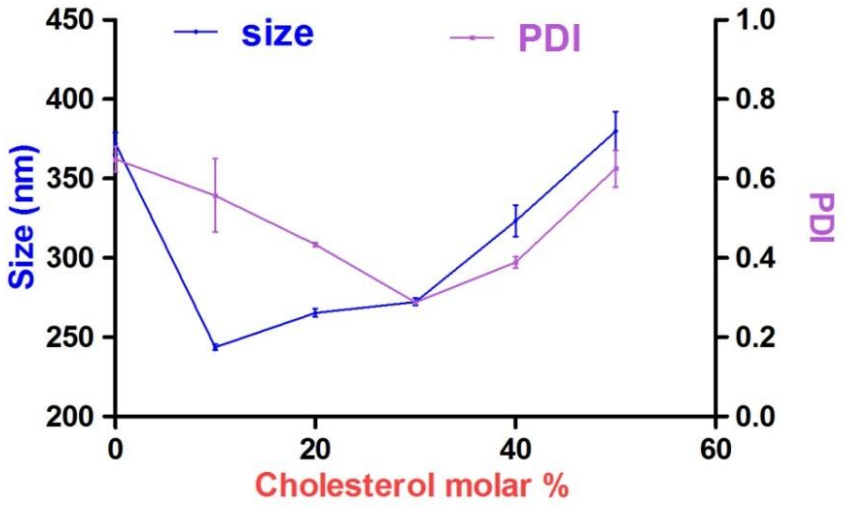

Figure (2): Effect of CH molar percent on PS and PDI of MXF loaded NLs

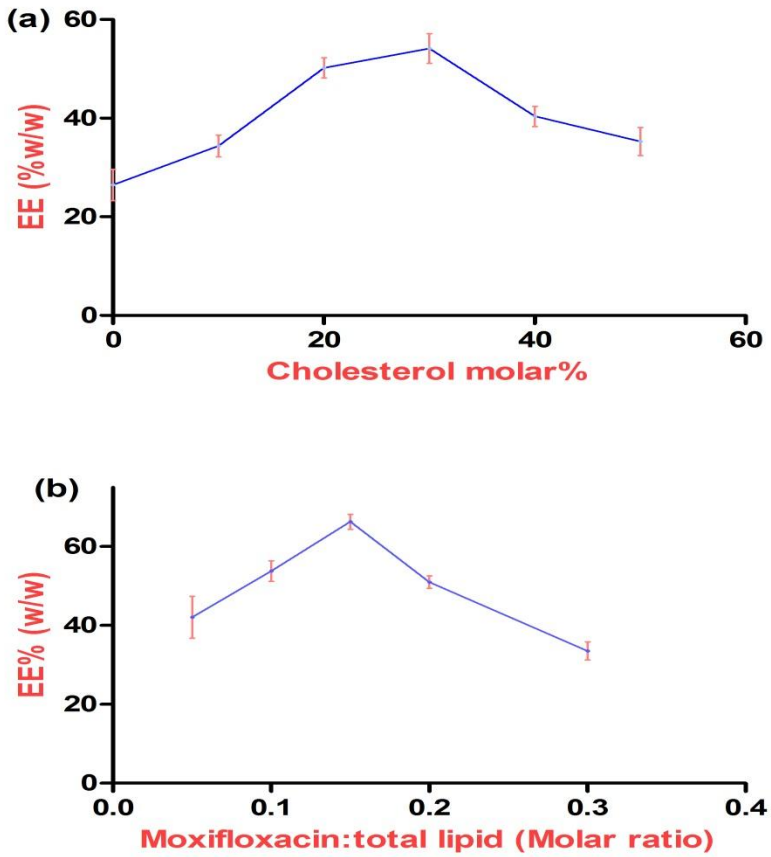

Figure (3): Effect of (a) cholesterol molar percent and (b) MXF: Total lipid (molar ratio) on the entrapment efficiency of MXF in NLs.

4.1.3. Effect of MXF concentration

Using a PC/CH molar ratio of 7:3, formulae F9, F10, F11 and F12 containing MXF to total lipid molar ratio of 0.05:1, 0.15:1, 0.2:1 and 0.3:1 respectively were prepared by REV method. Formula F6 containing 0.1:1 (MXF to total lipid molar ratio) showing optimum NLs characteristics was also included in this study.

Decreasing the drug loading from 0.1 to 0.05 significantly decreased ($P<0.05$) the EE from 54.12 to 42.07 as can be seen by comparing F6 to F9 in Table (2). On the other hand, the EE increased reaching 66.22± 1.46 as MXF to total lipid molar ratio increased from 0.05:1 to 0.15:1. Further increase in MXF content was accompanied with significant decrease in EE as demonstrated in Figure (3b).

The presence of an optimum amount of MXF that can produce maximum EE can be explained based on two factors. The first relates the EE of a hydrophilic compound with the inner volume of vesicle **[13]**. While the second explanation is the increase of EE with the increase in MXF concentration keeping total lipid at constant level. Fixed amount of lipid produces constant number of vesicles in the system that can entrap certain amount of the drug inside **[19]**. Further increase in MXF ratio beyond certain level in this study MXF to total lipid 0.15:1 molar ratio, was not associated with EE improvement indicating vesicles saturation.

It is worthy to mention that the increase in MXF content in NLs did not have a significant effect on PS and PDI as shown in Table (2). Also, all formulae with different MXF contents had non-significantly different negative zeta potential **[8].**

To conclude, MXF to total lipid molar ratio (0.15:1) was the optimum ratio that gave homogenously distributed NLs with the highest EE. Hence, MXF loaded NLs were prepared at PC: CH: MXF molar ratio 7:3:1.5 using REV method.

4.1.4. Effect of buffer pH

To study the effect of varying the pH of the aqueous buffer on the drug EE, formula F13 was prepared at the same composition of F10 using PBS buffer of pH 5.5 instead of 7.4. As showed

in Table (2), a significantly lower drug EE of 59.42 ± 1.31% was obtained with F13 compared to 66.25 ± 1.89% with F10. MXF possesses two pka values: 6.3 and 9.3 **[23, 24].** At pH 5.5, MXF amino groups are protonated and hence the drug carries positive. The slight negative charge of the NLs was not enough to promote electrostatic attraction between the drug and the NLs. While at pH 7.4 the drug is probably in its unionized form which could be better loaded in the conventional NLs. So, a pH of 7.4 was found to be the optimum pH for the preparation of conventional nearly neutral liposomes.

4.2. Characterization of selected MXF loaded NLs

For this study, formula F10 that was prepared at pH 7.4 with a PC/CH/MXF molar ratio of 7:3:1.5 was selected. Formula F10 representing the optimum formulae with the highest EE%, suitable PS and PDI.

4.2.1. Morphology of MXF loaded NLs

TEM examination revealed spherical NLs with homogenous distribution and smooth surfaces. The lipid bilayers of NLs appeared as dark ring surrounding the internal brighter aqueous phase **[25]**. The non-agglomerated particles existed dispersed in the system. The sizes obtained from TEM measurements were quite smaller than the results obtained from the PS measurements by DLS. This smaller size might be attributed to hydration and swelling of NLs in the aqueous buffer while the solvent was allowed to evaporate during TEM sample preparation **[26]**.

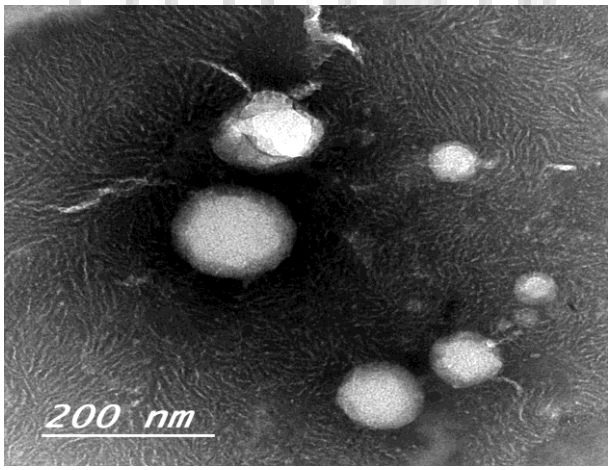

Figure (4): Transmission electron micrograph of MXF loaded neutral NLs (F10).

4.2.2. *In vitro* release of MXF from NLs

In vitro release of MXF from optimized formulations of NLs was shown as cumulative percent release over a 48h study period (Figure 5). Diffusion of free MXF in PBS through the dialysis membrane was used as a control.

Pure MXF showed approximately 100% dissolution and diffusion in 2h. Expectedly, entrapment of MXF in NLs reduced both the drug release rate and the cumulative amount released. In general, the release profiles of liposomal dispersions were biphasic, showing a relatively fast initial release phase (burst release) over the first 2h followed by a slower release phase. The initial fast release could be attributed to the surface located and loosely bound drug.

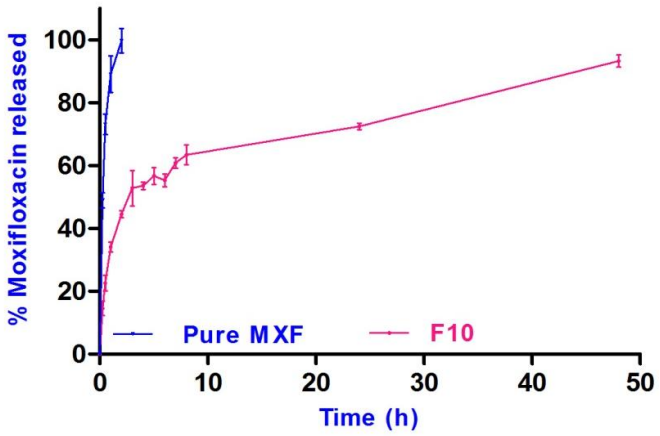

Figure (5): MXF cumulative percent released from F10 and Pure MXF solution.

4.2.3. Physical stability of MXF loaded NLs

4.2.3.1. Stability of MXF loaded NLs colloidal dispersion

Physical stability studies on the selected formula stored at refrigeration temperature (4^0C) for a period of three months were conducted by monitoring:

a) The leakage of encapsulated MXF from NLs.

b) The change in PS distribution of the vesicles after storage.

A- Effect of storage on MXF leakage from NLs

MXF leakage from NLs was evaluated after 1, 3, 7, 14, 30, 45, 60 and 90 days after storage at 4^0C and the results are graphically illustrated in Figure (6a) in terms of EE% of MXF in NLs.

From Figure (7a), we can note that MXF EE in NLs, F10 was not significantly (P>0.05) decreased for the first two weeks after which the decrease value became significant reaching 41.47 ±1.3% in 90 days. MXF EE tended thus to decrease with increasing the storage period. The decrease in MXF EE was 24.76%. Based on the previous data, we can conclude that formulae F10 was stable only for 2 weeks when stored at 4˚C. The leakage of the drug from liposomes may be attributed to many reasons such as the fluidity and mobility of phospholipid bilayer of the liposomes, the size of the entrapped molecule related to its penetrability to the bilayer membrane and hydrolysis and degradation of phospholipid bilayer **[13, 27].**

B- Effect of 3 months storage at 4˚C on NLs PS distribution

Figure (6b) revealed the effect of storage at 4˚C for 3 months on PS of NLs. It was noticed that there was non-significant (P>0.05) increase in PS of NLs within the first 30 days after that a significant increase in PS was noticed. Storage of liposomal colloidal dispersion resulted in a) Increase in vesicle diameter of the stored liposomes. b) Changes in PS distribution (PDI). This may be due to fusion and aggregation of liposomes after storage [28]. Finally, we can conclude that the vesicular system was not stable as colloidal dispersion for long storage period. Freeze drying was tried to enhance the stability.

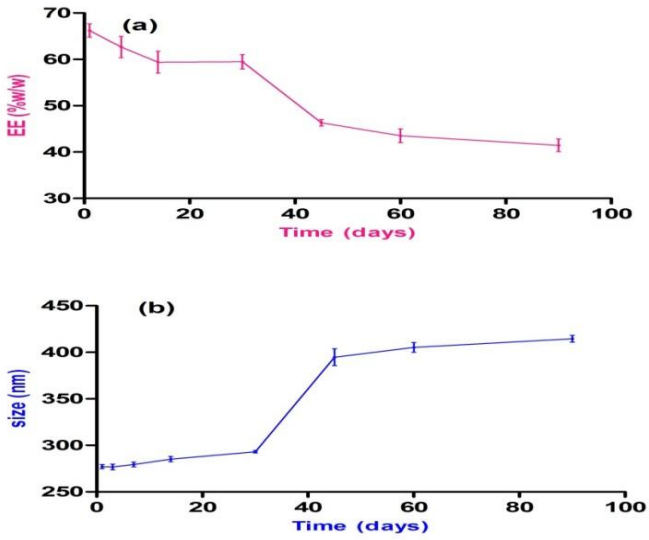

Figure (6): Effect of 3 months storage at 4˚C on (a) MXF entrapment efficiency in NLs and (b) Particle size of MXF loaded NLs.

4.2.3.2. Freeze drying of NLs

4.2.3.2.1. Optimum cryoprotectant mass ratio

Conventional NLs, formula F10 was prepared and mixed with trehalose as a cryoprotectant in different lipid to cryoprotectant mass ratio. Table (3) shows the characterization of FDNLs using different trehalose mass ratio.

FD of NLs in absence of cryoprotectant resulted in sticky mass that gave large particle size (737.85 ± 9.43) and a significant (P>0.05) reduction in EE (29.68%) after re-dispersion. Similar results were reported by Ozer and Talsma [29] during the preparation of FD liposome of 5-flurouracil. While the presence of trehalose during FD produce powder varied from sticky mass to dry cake. Increasing trehalose mass ratio improved the quality of FDNLs in terms of lower PS increase (565.14 ± 5.11 to 298.53 ±3.88 in formula T1and T5 respectively) and smaller decrease in the EE (33.02 ±2.69% to 63.68 ± 1.94% in formula T1 and T5 respectively) compared to NLs suspension before FD, formula F10. The stability of the liposomal membrane is the main issue should be considered in drying the liposomal formulation. Since these can be easily disrupted during the drying process either via ice crystals or sublimation of water from the liposomal surfaces **[30, 31].** Therefore protecting the liposomal membrane during drying process is essential. Sugars like sucrose and trehalose are well known with their cryoprotective effect and often used to protect delicate structures like protein, DNA and liposomes during drying process **[1]**. This effect could be ascribed to their hydroxyl groups form H-bonding with polar head group of phospholipid after sublimation of water during FD (water replacement theory) **[32].** So trehalose was important during FD process to keep NLs integrity. Various mass ratios of trehalose were tried to determine the optimum for our formulae as inadequate concentration of the cryoprotectant cannot provide sufficient hydroxyl groups needed for stabilization of NLs membrane resulting in drug leakage **[33]**. Figure (7) reveal that formulae T4 and T5 were the best formulae nearly similar to F10 (NLs before FD). Yet there was non-significant difference between PS and EE of T5 and T4. Since T4 had lower trehalose content so the optimum trehalose ratio was 1:6, in formula T4. Freeze drying process had non-significant effect on zeta potential as revealed in Table (3).

Table (3): Effect of trehalose ratio on the characteristics of FDNLs

Formula	Lipid/trehalose mass ratio	PS (nm)	PDI	EE (%w/w)	ζ (mV)
F10	Before FD	277.07 (2.18)	0.303 (0.024)	66.25 (1.89)	-12.31 (0.76)
T0	0	737.85 (9.43)	0.851 (0.009)	29.68 (4.03)	-12.2 (0.86)
T1	1:1	565.14 (5.11)	0.777 (0.03)	33.02 (2.69)	-11.7 (0.67)
T2	1:2	495.08 (4.61)	0.621 (0.03)	41.02 (4.09)	-12.13 (0.69)
T3	1:4	435.57 (5.97)	0.614 (0.02)	47.68 (2.33)	-12.25 (0.64)
T4	1:6	301.21 (6.32)	0.416 (0.02)	62.35 (2.77)	-12.47 (0.56)
T5	1:8	298.53 (3.88)	0.421 (0.02)	63.68 (1.94)	-12.37 (0.52)

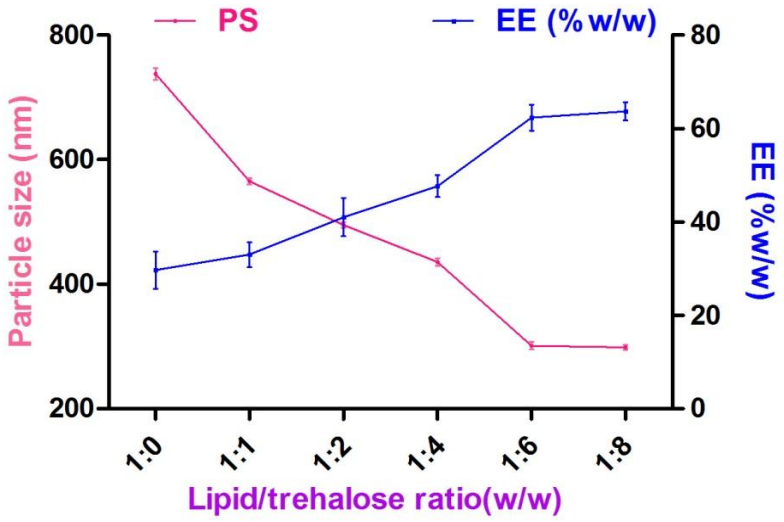

Figure (7): Effect of trehalose mass ratio on the particle size and entrapment efficiency of FDNLs.

4.2.3.2.2. Differential scanning calorimetry

DSC measurements were performed to study closely the physicochemical properties of liposomal membranes and their interactions with encapsulated MXF. DSC can also give an insight onto the physical state of MXF loaded in NLs and explaining EE results. DSC thermograms of pure MXF, PC, CH as liposomes constituents and unloaded and MXF-loaded NLs (formulae F10) and FDNLs (T4) are shown in Figure (8).

MXF shows a sharp endothermic peak, corresponding probably to the melting of its crystalline structure at 235.9^0C. CH exhibited a sharp melting peak at 146.92^0C**[34]**. DSC of PC showed no characteristic melting peak and a degradation endotherm of PC above 191.48^0C. Phospholipids do not undergo a simple melting process on heating, passing from solid to liquid state; instead, depending on the amount of water present, they exist in one or more intermediate liquid-crystalline or mesomorphic forms **[35]**.

There was no obvious difference between thermogram of unloaded and MXF loaded NLs (Figure 8). The DSC of plain and drug loaded NLs exhibited less pronounced lipid peak or shifted to lower temperature with the absence of CH melting peak. According to these results, we can conclude that the lipid components interact with each other to a great extent while forming the lipid bilayers. The absence of MXF melting peak in the thermogram of MXF loaded (medicated) liposomes allowed to suggest that it was entrapped in liposomal vesicles. Also, DSC of FDNLs showed that they were amorphous in nature with no characteristic melting peak appeared in either plain or medicated one. Also, the drug melting peak isn't obvious in the thermogram of FDNLs. This confirmed the complete amorphization of the drug and its entrapment within the FDNLs.

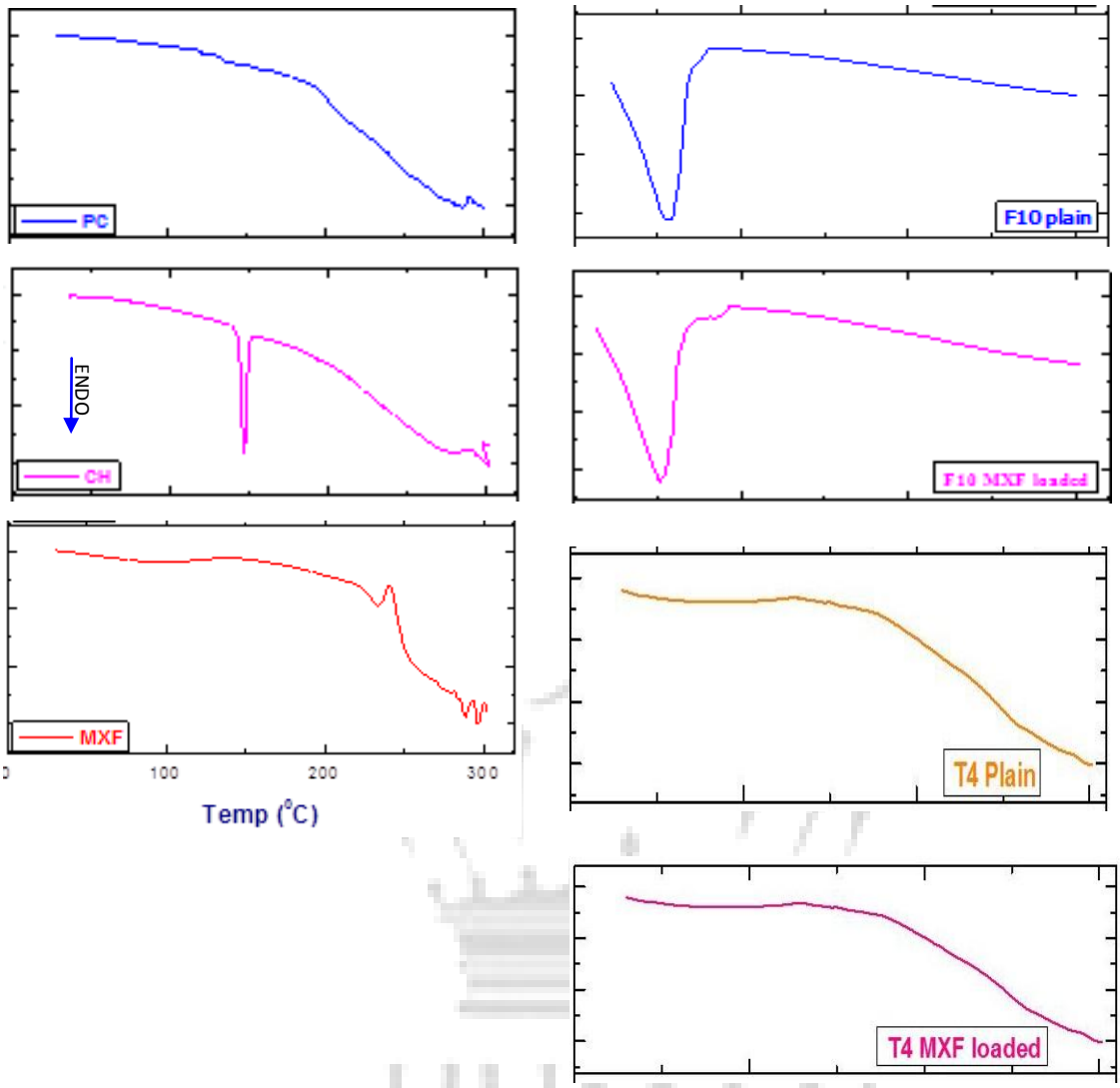

Figure (8): DSC thermogram of pure MXF, PC,CH, F10 (before and after FD) plain and MXF loaded

CONCLUSION

This study focused on the preparation of controlled release vesicular delivery system of moxifloxacin HCl. The particle size and entrapment efficiency were greatly affected by method of preparation, phospholipid to cholesterol molar ratio and drug to lipid ratio and pH of aqueous buffer used for hydration. PC/CH/MXF molar ratio 7:3:1.5 and pH 7.4 using REV method produced NLs with high entrapment efficiency and good morphological characteristics but stable for only two weeks. Freeze drying with trehalose at lipid/trehalose ratio 1:6 enhanced the stability of MXF-NLs without significant changes in their particle size or entrapment efficiency.

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