Development of novel methotrexate-loaded nanocochleates for breast cancer targeting.

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Abstract

The objective of this research was to develop stable methotrexate(MTX) loaded nanocochleates (MTX-NC) drug delivery system to achieve controlled drug release,targeted delivery and enhanced anticancer potency with reduced dose and its effect on Breast cancer cell line. The development involved the loading of MTX to nanocochleates (MTX-NC) through conversion of phosphatidyl choline and cholesterol bearing liposome on addition of calcium ions and compared with MTX and MTX-NC. Optimized NC under optimized conditions showed particle size and zeta potential of 298.7nm and -12.9mv respectively, high entrapment efficiency 75%, drug loading of 37.5% and drug content 99%. Investigation on the drug lipid interaction using FTIR confirmed the compatibility of drug with the lipid carrier. SEM and TEM images of NC indicated that the particles were in a rod cylindrical shape with a smooth surface.Formulated MTX-NC demonstrated higher in-vitro anticancer activity in human breast cancer MCF-7 cells. The concentration of the drug needed for growth inhibition of was 47% for free MTX(80µg/ml) while it was -35.6% for the MTX-NC (80µg/ml) . Furthermore, the LC50 value of free MTX and MTX-NC was NE and 75.3µg/ml respectively.GI50 value of free MTX and MTX-NC was <10µg/ml. The results indicate that the MTX- NC has the potential to be applied for targeting anticancer drug delivery.

1. Introduction

Breast cancer is the most frequent cancer occurs in women worldwide [1]. Current treatment involves chemotherapy, hormone therapy and surgery. A drawback with these therapies is lack of specificity, more side effects and poor absorption. Nanocarrier based delivery systems provides many benefit over current therapies such as active or passive drug targeting, increased encapsulation of drug, reduce side effects and degradation of drug in blood circulation [2, 3].

Methotrexate (2,4-diamino-N10-methyl propyl glutamic acid, MTX) an anticancer agent belong to Biopharmaceutical Classification System (BCS) class II used for the treatment of different tumors such as lung, osteosarcoma and breast cancer. Moreover, it is also used to treat inflammatory and autoimmune diseases such as Crohnsdiseases, *rheuma* → *rthritis* and *ps* or *iasis*[4 - 6]. However, ∴ s clinical use is limited [6]. Therefore, there was need to develop a formulation which could improve therapeutic efficacy at low dose, thus reducing systemic toxicity too.

Vesicles have been the preferred route for medication delivery in recent years [6]. Encapsulation of the drug in these vesicular structures, prolong the existence of drug in the systemic circulation. The vesicular systems are highly ordered assemblies of one or several concentric lipid bilayers [7]. The drug delivery system for cochleates and nanocochleates is based on encapsulating the medicine in a multilayered, lipid matrix in order to administer the drug safely and effectively [8] .Cochleates were first discovered by Dr. Dimitrious Papahadjoupoulos and his co-workers in 1975 as precipitates formed by the interaction of negatively charged phosphatidylserine and calcium. This was cylindrical in shape and he named these cylindrical structures as "cochleate" means Shell" i.e. rolled-up form [9]

Nanocochleates are cigar-like structures made up of a sequence of lipid bilayers (Figures 1.1 and 1.2), which arise when small unilamellar negatively charged liposomes condense. The small phosphatidylserine (PS) or phosphatidyl choline (PC) liposomes merge and produce huge sheets in the presence of calcium. These sheets have hydrophobic surfaces and tend to coil up into a cigar-like cochleate (Figure 1.3) to reduce their interactions with water [9].

Cochleates differ from liposomes in having water-free interior, rod-shaped, and rigid stable structure as shown in Fig no.1.3

Nanocochleates formulation technology is particularly applicable to macromolecules as well as to drugs that are hydrophobic and hydrophilic. It is also for drugs that undergo first pass metabolism and drug that degrades at physiological pH like proteins and peptides. These novel carriers have been used to deliver drugs belong to class of antifungal, antibiotics, protein, anti-leprosy, anticancer, and DNA 70 subunit to increase their therapeutic efficacy [10,11].

To further advance the therapeutic utility of MTX, the present study investigates the potential of nanocochleates as a vehicle for oral delivery of MTX [12]. Methotrexate-loaded nanocochleates (MTXNC) were developed through conversion of phosphatidylcholine (PC) and cholesterol bearing nanoliposomes into the nanocochleates structures on addition of calcium ions by trapping method. Cholesterol was included to stabilize the phospholipid membranes as well as to form complex with the drug [10]. The MTX-NC were characterized by their particle size, zeta potential, entrapment efficiency, *in-vitro* drug release, compatibility studies (FTIR), SEM, TEM, stability studies and *in vitro* cell lines studies.

2. Materials & Methods

2.1 Materials

Methotrexate was obtained as a gift sample from Neon Laboratories, India. Lipoid S75 and Phospholipon 90H was obtained as generous gift from Lipoids, Germany and used as vesicle forming phospholipid, cholesterol (Sigma-Aldrich) provides flexibility to the formed liposomal vesicles thus maintaining its shape. Calcium chloride was used as the cochelation agent. All other Chemicals used were analytical grade.

2.2 Preparation of liposomes by solvent injection method

MTX-loaded nanoliposomes (MTXNL) were prepared by ethanol injection method as shown in fig.no. 2.1. Briefly, different amount of phospholipon 90H, Lipoid S-75 and, cholesterol were dissolved in 10 mL of ethanol as shown in table no. 2.1. Drug was dissolved in phosphate buffer (pH – 7.4) and kept on a magnetic stirrer under constant rpm. Ethanolic phase was rapidly injected into the aqueous phase under constant magnetic stirrer for a specific time using Teflon-coated beads and stirring was continued until complete evaporation of under vacuum. Further, phosphate buffer was added to adjust the volume of final nanoliposomes suspension to 20 mL. Finally, the nanoliposomes were purified by passing through a 0.45

mm membrane filter to obtain a MTXNL suspension [15–17]. Batch which shows higher encapsulation efficiency was selected for the preparation of MTX-Nanocochleates.

Table No. 2.1 Trial batches with combination of Phospholipids with different ratio

Sr. No	Lipid Content	Phospholipid 90H	Lipoid S-75	Phospholipids : Lipoid S-75	Lipid: Cholesterol	Apperrance	%EE
1	0.1%	Yes	-	-	1:0.3	Less Turbid	25
2	0.1%	-	Yes	-	1:0.3	Less Turbid	52
3	0.1%	Yes	Yes	1:1 (50:50)	1:0.3	Less Turbid	75
4	0.1%	Yes	Yes	0.6:0.4 (60:40)	1:0.3	Less Turbid	45
5	0.1%	Yes	Yes	0.4:0.6 (50:50)	1:0.3	Less Turbid	60
6	0.5%	Yes	Yes	1:1 (50:50)	1:0.3	Turbid	35
7	1%	Yes	Yes	1:1 (50:50)	1:0.3	Turbid	38
8	2%	Yes	Yes	1:1 (50:50)	1:0.3	Turbid	40

2.2 Formulation of MTX-loaded nanocochleates

MTX-loaded nanocochleates (MTXNC) were prepared by trapping method [14-15] as shown in fig.no. 1.2 . Fifty microliters of calcium chloride solution (0.1 M) was added dropwise into the prepared MTXNC under stirring. The MTXNL phase immediately turned turbid because nanocochleates formation. The MTXNC dispersion was evaluated in terms of particle size, zeta potential, drug loading, *in-vitro drug release*, encapsulation efficiency, SEM, and *in-vitro* cell line (MCF-7 breast cancer) study.

Table No. 2.2.1 Trial Batch by Trapping Method

Sr. No.	%Lipid	L:CH	Speed	Time	%Transmittance		Divalent	%Transmittance	
					30mins	60mins	CaCl ₂	30mins	60mins
1	0.1	1:0.3	1500	2	84.20	91.08	0.1M	83.02	82.02

2.3 Experimental Design

Design-Expert software was utilized for experimental design and statistical analysis. General full factorial design with three independent variables was applied to determine the optimal levels. Each numeric factor was varied over 2 levels thus by selecting general full factorial design 8 experiments were generated by the software. Encapsulation efficiency percentage of MTX-Liposomes and encapsulation MTX-NC values were chosen as quality attributes for optimizing the formulation.

The response was assessed by the nonlinear quadratic equation:

$$Y: \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_{12}AB + \beta_{13}AC + \beta_{23}BC + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2$$

where Y is the predicted response, A, B and C are independent variables, A_2 , B_2 and C_2 symbolize the interaction and the formulations were prepared by same procedure as mentioned in the preliminary studies.

All the 8 experiments in these experiments were prepared and evaluated for Nanocochleates prepared by trapping method.

Table No. 2.3.1 Design Summary for Nanocochleates

File Version	11.0.5.0		
Study Type	Factorial	Subtype	Randomized
Design Type	Full Factorial	Runs	8
Design Model	2FI	Blocks	No Blocks
Center Points	0	Build Time (ms)	1

Table no. 2.3.2 Preoptimized batches for nanocochleates

STD	RUN	L:CH	SPEED	TIME
1	1	1:03	1500	1
3	2	1:03	2000	1
2	3	1:0.5	1500	1
5	4	1:03	1500	2
6	5	1:0.5	1500	2
8	6	1:0.5	2000	2
7	7	1:03	2000	2
4	8	1:0.5	2000	1

Encapsulation efficiency and transmittance percentage of MTX-NC values was chosen as quality attributes for optimizing the formulation.

The impact of independent variables on responses was assessed using analysis of variance (ANOVA) and $p < 0.05$ was accepted to be statistically meaningful. To evaluate the convenience of the model, the calculated predicted and adjusted correlation coefficient (R^2) were analysed. The influences of selected independent variables on the quality of the nanocochleates formulation were assessed by fit statistics and coefficient factors for the dependent variable. The optimum MTX-NC formulation was decided by considering responses having maximum encapsulation efficiency and % transmittance

The optimized nanocochleate formulation was prepared three times and the achieved outcomes were matched with the predicted values.

2.4 Evaluation of optimized nanocochleates

2.4.1 Microscopic evaluation.

A drop of nanocochleates dispersion was observed under optical microscope (45X magnification) for any cluster formation.

2.4.2 In- vitro drug release.

The in-vitro release of MTX from MTX-NC was carried out in phosphate buffer (PBS, pH 7.4 and using dialysis bag diffusion technique. Formulation equivalent to 2mg of MTX was introduced into a dialysis bag (cellulose membrane, molecular weight cut off 12, 0000 Da), hermetically sealed and immersed into 200 ml of release medium. The entire system was kept at 37 ± 0.5 °C with continuous magnetic stirring at 100 rpm. At selected time intervals, 2 mL sample was removed and 2 mL phosphate buffer was added to maintain sink condition. Absorbances of the solutions were recorded at λ_{max} of 302 nm using the Shimadzu Jasco UV-visible spectrophotometer (JASCO V-630, Japan) [6,18]

2.4.3 Size measurement and Zeta potential of Cochleate dispersion.

The vesicle size and size distribution of nanocochleates were measured by Malvern Zetasizer 2000 (Malvern, UK). Vesicular suspensions were mixed with the appropriate medium (PBS, pH 7.4) and the measurements were taken in a multimodal mode. All measurements were performed at 25°C after 5 min of thermal equilibration. The Malvern Zetasizer 2000 (Malvern, UK) detects backscattering at an angle of 173° for an improved sizing of especially larger particles at higher concentrations. Viscosity of the medium of 0.89 mPas and refractory index of 1.33 were assumed [19-26].

2.4.4 Entrapment efficiency

2 ml of formulation was subjected to centrifugation for 45 minutes at 10000 rpm at 4°C, the supernatant and pellets were separated. The 0.5 ml of supernatant was withdrawn and volume was made up to 10 ml with phosphate buffer and analyzed [19-26].

2.4.5 Transmission Electron Microscopy (TEM):

TEM was employed to study the morphology of the nanocochleates. TEM images were formed using Philips Electron Optics transmission electron microscope, model no. CM200. The operating voltage was 200 kV, with a resolution of 2.4 Å and magnification of 45,000X (66). The optimized formulation was reconstituted in distilled water and a drop was introduced on a carbon coated mesh like grid and dried. The images were resolved on a fluorescent screen and the analysis of the x-rays produced by interaction between accelerated electrons with the sample allows determining elemental composition of sample with high spatial resolution [19-26].

2.4.6 Scanning Electron Microscopy (SEM):

Liposome and nanocochleates sample for SEM was prepared by dispersing sample in 5 mL DDW. A drop of nanoparticle dispersion was deposited on carbon tape, which was then air-dried after being glued to metal stubs with a double-sided adhesive tape. SEM was used to determine the morphology of nanoparticles (Zeiss DSM 940A SEM; Oberkochen, Germany). After covering the particles with a thin layer of gold under vacuum using a sputtering apparatus, imaging was done on SEM [19-26].

2.4.7 In-vitro cell line studies:

The cytotoxicity tests were carried out on the MCF7 human breast cancer cell line. RPMI 1640 medium with 10% foetal bovine serum and 2 mM L-glutamine was used to culture the cell lines. Cells were injected into 96 well microtiter plates in 90 L for the screening experiment. Following cell inoculation, the microtiter plates were incubated for 24 hours at 37°C, 5% CO₂, 95% air, and 100% relative humidity before adding experimental samples. The cytotoxic activity was assessed using the Sulforhodamine B (SRB) assay. One plate of each cell line was fixed in situ with trichloroacetic acid (TCA) after 24 hours to reflect a measurement of the cell population at the moment of drug addition. The needed final drug concentrations were achieved by adding aliquots of 10 L of various drug dilutions to appropriate micro-titer wells already holding 90 L of medium. Plates were incubated at standard conditions for 48 hours after drug addition, and

the test was finished with the addition of cold TCA. The cells were fixed in place by gently adding 50 l of cool 30% w/v) TCA (final concentration, 10% TCA) and incubating for 60 minutes at 4°C. The supernatant was removed, and the plates were rinsed five times with tap water and air (w/v) TCA (final concentration, 10% TCA) before incubation at 4°C for 60 minutes. The supernatant was discarded, and the plates were rinsed and air dried five times with tap water. Sulforhodamine B (SRB) solution (50 µl) at 0.4 % (w/v) in 1 % acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1 % acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base and the absorbance was read on an Elisa plate reader at a wavelength of 540 nm with 690 nm reference wavelength. The optical density of drug-treated cells was compared to that of control cells, and the percentage of growth inhibition was computed. At four distinct concentrations of 10-7M, 10-6M, 0-5M, and 10-4M, each drug was evaluated in triplicate. For each experiment, an Adriamycin positive control was employed to check that the experimental setup was correct [6,27-29].

Formulations that are given for the in-vitro cell line studies are

- 1) Drug
- 2) Liposomes
- 3) Nanocochleates prepared by trapping method containing Ca+2
- 4) Nanocochleates prepared by trapping method containing Ba+2
- 5) Nanocochleates prepared by hydrogel method containing Ca+2
- 6) ADR (Adrinomycin)

Table No. 2.3.7.1 Concentration taken for the *In-vitro* Cell line studies

Sample Code	Experiment µg/ml			
	1	2	3	4
All samples	10	20	40	80
ADR*	10	20	40	80

2.3.8 Stability studies

Stability testing is used to assess expiration dating and storage conditions for pharmaceutical products. Nanocochleates dispersion was packed glass vials and they were subjected to stability testing at various storage conditions as per ICH QA1 R2.

Stability conditions:

Storage in refrigeration: 2 - 8°C ± 2°C for 2 months; sample withdrawn at 7, 14, 28, and 56 days.

3. Results And Discussion

3.1 Experimental Design.

Table No. 3.1 Preoptimized batches for NC (Ca²⁺)

STD	RUN	L:CH	SPEED	Time	EE (%)	TT (%)
1	1	1:0.3	1500	1	61	93.87
3	2	1:0.3	2000	1	65	91.63
2	3	1:0.5	1500	1	50.68	81.63
5	4	1:0.3	1500	2	75	101.76
6	5	1:0.5	1500	2	51.36	90.63
8	6	1:0.5	2000	2	51.68	78.67
7	7	1:0.3	2000	2	50.83	80.78
4	8	1:0.5	2000	1	56.63	92.63

From the table 3.1 we can found out that batches with Run4 showed maximum entrapment efficiency of 75% and transmittance 101.76%.

3.1.1 Model analysis for Entrapment efficiency

Anova for Entrapment efficiency

Table No. 3.1.1.1 Anova for Entrapment Efficiency

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	352.85	1	352.85	11.43	0.0148	Significant
A-L:CH	352.85	1	352.85	11.43	0.0148	
Residual	185.25	6	30.87			
Cor Total	538.10	7				

From the table no. 3.1.1.1 we can see that

Factor coding is **Coded**.

Sum of squares is **Type III – Partial** paraphrase it

The **Model F-value** of 11.43 implies the model is significant. There is only a 1.48% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A is a significant model term. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Fit Statistics

Table No. 3.1.2.1 Fit Statistics for Entrapment Efficiency

Std. Dev.	1.78	R²	0.8883
Mean	78.67	Adjusted R²	0.8045
C.V. %	2.50	Predicted R²	0.8254
		Adeq Precision	19.248

The **Adjusted R²** of 0.8045 is reasonably close to the **Predicted R²** of 0.8254; that is, the difference is less than 0.2.

The signal-to-noise ratio is measured by Adeq Precision. It is preferable to have a ratio of more than four. Your signal strength is adequate, as indicated by your ratio of 19.248. The design space can be navigated using this concept.

Coefficient in Terms of Coded Factors

Table No. 3.1.2.2 Coefficient for Entrapment Efficiency

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	57.77	1	1.37	53.96	61.57	
A-L:CH	-6.64	1	1.37	-10.45	-2.84	1.0000
C-Stirring Time	2.85	1	1.37	-0.9562	6.65	1.0000
AC	-2.74	1	1.37	-6.55	1.06	1.0000

The coefficient estimate provides the estimated change in response per unit change in factor value while all other factors are kept constant. The intercept in an orthogonal design is the overall average response of all

the runs. The coefficients are adjustments depending on the factor settings in the vicinity of the average. When the factors are orthogonal, the VIFs are 1; VIFs greater than 1 indicate multi-colinearity, and the higher the VIF, the more severe the factor correlation. VIFs of less than ten are regarded as acceptable.

3.2. Evaluation of optimized formulation

3.2.1. Microscopic evaluation.

From the figure no. 3.2.1 Homogenous dispersion, with very few clusters was observed under microscope

3.2.2 *In-vitro* drug release

Table No. 3.2.2.1 *In-vitro* drug release of marketed and optimized formulation

Time(hrs)	%CUMULATIVE RELEASE	
	Marketed Formulation	Nanocochleates
0	0%	0.12%
0.15	8.96%	11.52%
0.30	13.56%	14.25%
0.45	21.84%	22.36%
1	28.36%	35.36%
2	30.79%	45.36%
3	43.36%	51.85%
4	-	65.36%
24	-	86.23%

From the release studies it was observed that nanocochleate formulation led to prolong release of the drug from the system. The release from the system at the end of 24 hours was found to be 86.01%. for the marketed formulation the cumulative release at the end of 3 hrs was 43.36%. In nanocochleate formulation although a sustained release is achieved, at the start of studies the diffusion is higher than the latter half. This could be because of the drug at the surface of the nanocochleates which is first diffused due to wetting.

From Fig No. 3.2.2 it can be seen that release of the Model Drug was sustained as compared to marketed formulation

1) Paired t test

T test were applied to analyse a significant change between the Formulation and the marketed. The *t* test is one type of inferential statistics. (*p* < .05 is a common value that is used). T test for all the mediums were found to be 0.027 which is less than 0.05. Indicating significant change in the drug release rate of formulation and marketed.

2) Mathematical Order Model

The model which drives the release of the drug from the formulation was evaluated. The best fit profile was understood by plotting the graphs for zero order (% CR vs time), higuchi release model (%CR vs time^{1/2}), hixon crowell release model, korsmeyer peppas release model and first order model. The results of the following is shown in Table No 3.2.1

Table No. 3.2.2.2 R² value for model in the *in vitro* diffusion studies

Formulation	R ² value for model in the <i>in vitro</i> diffusion studies				
	Zero Order	Higuchi	Hixson	Kors-peppars	First Order
DRUG LOADED NC	0.8047	0.9484	0.8342	0.9361	0.8481

From table no. 3.2.1, it is understood that the release of the drug from the nanocochleates follows higuchi model

3.2.3 Particle size measurement and polydispersity index (PDI):

Particle size of nanocochleate was found to be 298.7nm and 199.6nm respectivelywhile PDI of nanocochleate was 0.295.

3.2.4 Zeta Potential

From the above figure it was found that zeta potential of nanocochleate and was found to be -12.9mv

3.2.5 Transmission Electron Microscopy (TEM):

Surface morphology revealed nanocochleates showed rolled-up layers and rod structure

3.2.6 Scanning Electron Microscopy (SEM):

SEM analysis showed that the nanocochleate formulated had a cylindrical rod like shape.

3.2.7 Cytotoxicity Studies:

Table No. 3.2.7.1 Control Growth Inhibition Values

Formulation	% Control Growth on MCF-7 (Average Values)			
	Drug concentrations (µg/ml)			
	1 (10)	2 (20)	3 (30)	4 (40)
Drug	43.0	50.1	47.1	47.9
Liposomes	38.1	20.7	4.7	-20.8
NC (Trapping) Ca ⁺²	29.3	14.8	-3.3	-35.6
NC (Hydrgel) Ca ⁺²	24.9	15.1	-3.1	-46.5
NC (Trapping) Ba ⁺²	44.3	46.3	44.2	43.2
ADR	-5.0	-11.3	-21.3	-39.9

Table No. 3.2.7.2 LC-50, TGI and GI50 Dose response parameter

	LC-50	TGI	GI-50
Drug	NE	NE	<10
Liposomes	75.3	38.0	<10
NC (Trapping) Ca ⁺²	64.7	30.9	<10
NC (Hydrgel) Ca ⁺²	59.6	28.5	<10
NC (Trapping) Ba ⁺²	NE	NE	<10
ADR	NE	NE	<10

The minimum detectable anticancer activity on MCF7 cell line was observed in the Model Drug loaded nanocochleate at a concentration of **40µg/mL**, with an inhibition of **-3.3 %** cell death, and reaching a maximum inhibition of **-35.6 %** cell death at a concentration of **80µg/mL**. Simultaneously Model Drug loaded liposome showed at a concentration of **80µg/mL**, with an inhibition of **-20.8%** cell death. LC-50 of Model Drug loaded nanocochleate and in Model Drug loaded liposome were found to be **75.3µg/mL** and **64.7µg/ mL** respectively. TGI of Model Drug loaded nanocochleate and Model Drug loaded liposome were found to be **38µg/mL** and **30.9µg/ mL** respectively. From the results, it clearly indicates Model Drug loaded nanocochleate was showing better cancer cell death as compared to Model Drug loaded liposome (p<0.05).

The above significant activity could be due to nanosize and presence of lipid facilitating better penetration into the cancer cell and improved intracellular drug accumulation into MCF-7 cells.

This is also indicated by shrinking of cell seen in fig.no 3.2.7.3.

3.2.8 Stability studies of optimized nanocochleates

Table No. 3.2.8.1: Stability Study Result for Nanocochleates at 2-8°C

Parameters	Results for Nanocochleates					
Time (days)	0	7	14	28	56	84
Appearance	Clear, translucent	Clear, translucent	Clear, translucent	Clear, translucent	Clear, translucent	Clear, translucent
%transmittance	90.63	89.75	91.23	90.74	90.53	90.52
% Entrapment Efficiency	75	74.87	72.86	72.76	71.84	73.85
Particle Size (nm)	298	-	-	-	-	268.7
Zeta Potential (mv)	-12.9	-	-	-	-	-9.1
Drug content (% w/w)	99	98.20	98.54	97.74	97.50	98.50
% Drug release	86.63	86.76	86.50	85.71	85.40	85.64

Appearance: The stability batch of optimized nanocochleates was found to be clear and translucent in appearance. No change in the appearance was observed over a period of three months at long term stability conditions of 5°C±3°C but at room temperature precipitation was observed after 1 month

Drug Content: Drug content was found to be in the range of 99%-98.77%. Initial drug content for batch nanocochleate was found to be 82.5% and 86.5% respectively. Further after 1, 2 and 3 months the drug content of nanocochleate was found to be 97.74%, 97.50 % and 98.50 % respectively at 5°C±3°C. Slight decrease in the drug content was observed during the storage period. Drug content at Room temperature was not determine because precipitation was observed.

Drug Release: Drug release of nanocochleate was found to be in the range of 85.0% to 87% over a period of 24 hours. Initial drug release of nanocochleate was found to be 86.63 and after 1, 2, 3 months drug release was 85.74%, 85.50%, 85.60% respectively. Slight decrease in the drug release was observed during the storage period. Drug release at Room temperature after 1 month was not determine because precipitation was observed.

Entrapment Efficiency: Entrapment Efficiency of nanocochleate was found to be in the range of 71% to 75%. Initial entrapment efficiency of nanocochleate was found to be 75 and after 1, 2, 3 months drug release was 72.76%, 71.84%, 73.85% respectively. Slight decrease in the entrapment efficiency was observed during the storage period. Entrapment efficiency at Room temperature after 1 month was not determined because precipitation was observed.

From the above results, it can be seen that the optimized batches of nanocochleate at refrigeration condition of 2°C-8°C showed minimum changes as compared to the results obtained for batches stored at

room temperature. This may be due to destabilization of the nanocochleate surface at higher temperatures. Thus, refrigerated conditions were found to be optimum for storage of nanocochleate.

Conclusion

We have developed and investigated nanocochleates composed of phosphatidylcholine (PC), cholesterol and calcium ions as an effective oral nanocarrier for the delivery of a anticancer drug MTX. The developed nanocochleates exhibited higher encapsulation efficiency and sustained release of MTX. Moreover, the MTXNC demonstrated higher in vitro anticancer activity in human breast cancer MCF-7 cells. As a result, these nanocochleates could be used to augment PTX's anticancer potential and provide an alternative to the current oral delivery. This approach could be applied to other anticancer medications with oral delivery issues due to their low water solubility and bioavailability.

Declarations

Conflicts of interest

The authors declare no competing interests.

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Figures

Figure 1

1.1 & 1.2 Cigar like structure of Nanocochleates

Figure 2

1.1 & 1.2 Cigar like structure of Nanocochleates

Figure 3

1.3: Structural difference between liposomes and cochleates

FORMULATION OF LIPOSOMES BY SOLVENT INJECTION METHOD

Figure 4

2.1 Schematic representation for preparation of Liposome by solvent injection method

Figure 5

3.2.1 Microscopic Image of Optimized Liposome

Figure 6

3.2.2 Cumulative Release Graph

Figure 7

3.2.3.1 P.S of nanocochleate

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-12.9 mV	-0.000100 cm ² /Vs
2	— mV	— cm ² /Vs
3	— mV	— cm ² /Vs

Zeta Potential (Mean) : -12.9 mV
Electrophoretic Mobility Mean : -0.000100 cm²/Vs

Figure 8

3.2.4.1 Zeta of Nanocochleate

Figure 9

3.2.5.1 TEM of Nanocochleate

Figure 10

3.2.6.1 SEM of Nanocochleate

Figure 11

3.2.7.1 Growth inhibition graph at various concentrations

Figure 12

3.2.7.2 MCF CONTROL

Figure 13

3.2.7.3 MCF NCT Ca

Figure 14

3.2.7.4 MCF Liposomes

Figure 15

3.2.7.5 MCF Drug

Figure 16

3.2.8.1 Nanocochleate at 2-8°C on left and RT on right after 3 months