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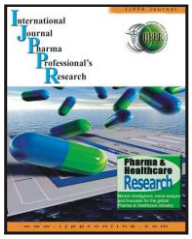

Short Communication

DEVELOPMENT AND CHARACTERIZATION OF CITICHOLINE LIPOSOMES

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Abstract
We developed pH-responsive liposomes containing Citicholine and evaluated their properties both in vitro and in vivo with the aim of constructing an efficient liposome-based systemic drug delivery system. Loading of the drugs into liposomes has proved to be a measure of their utility. If there is poor loading, there is a great loss of the active drug and the use of liposomes as the pharmaceutical vehicle becomes uneconomical. Several methods exist for improved loading of the drugs, including remote (active) loading methods which load drug molecules into preformed liposomes using pH gradients and potential differences across liposomal membranes.

Introduction
Liposomes or lipid vesicles are spherical structures composed of curved closed lipid bilayers that encapsulate liquid or gas. Their size ranges from nanometers to microns and the thickness of the membrane is in the order of nanometers. The most obvious features of the liposome that make it attractive to medicine (pharmacology in particular) include its ability to dissolve, protect and carry hydrophilic or hydrophobic molecules, its biocompatibility with cell membranes, its low antigenicity and its nanometer size, allowing it entering organs such as the brain, lung, spleen and liver through circulation and the relative ease of adding special ligands to their surface. Current research on the possible uses of liposomes in medicine include therapeutic [1, 2, 3] as well as diagnostic [4,5, 6] applications.

Citicholine is a neuroprotective agent used clinically to treat for instance Parkinson disease, Stroke, Alzheimer's disease and Brain Ischemia. Chemically Citicholine is 2-[[[5-(4-amino-2-oxo-pyrimidin-1-yl)-3, 4-dihydroxy- oxolan -2-yl] methoxy –hydroxy -phosphoryl] oxy- hydroxy-phosphoryl] oxyethyl-trimethyl-azanium. It is an intermediate in the biosynthesis of Phosphatidylcholine. Citicholine bears +1 charge containing an 'amino' group which can be used for its improved loading into preformed liposomes against transmembrane pH gradient created across the lipid bilayer [7].

Materials and Method
Citicholine is obtained as free gift sample from , Lyka Laboratories Pvt. Ltd. (Ankaleshwar, Gujarat, India), Hydrogenated egg Phosphatidylcholine (HEPC), Cholesterylhemisuccinate(CHEMS), Phosphatidyl – ethanolamine - polyethylene glycol (PE-PEG), Cholesterol (CHOL) were obtained from, Lipoid, (GmbH, Germany), all other reagents and excipients were purchased from S.D. Fines Chemicals (Mumbai, Maharashtra, India).

Multilamellar vesicles comprising HEPC, CHEMS and CHOL with entrapped ammonium sulfate were prepared by Thin Film hydration (TFH) technique [8]. Briefly, the lipids were dissolved in a mixture of chloroform and methanol (ratio 3:1 v/v) in a 250ml round bottom flask in different molar ratios . The solvent was evaporated in the rotary flask evaporator under vacuum. The thin dry lipid film thus formed was hydrated using aqueous ammonium sulfate of different molarity (Such as 80mM, 100mM and 120mM) as hydrating medium at 60 ± 3 ℃ i.e. above phase transition temperature of lipid(Tg) [9]. The formed liposomal dispersion was sonicated (5 cycles, 60 % Amp, 0.6 sec, 2 min., Temp. 55± 3℃) in probe sonicator. The sonicated liposomes were then allowed to stand undisturbed for about 60 min, for annealing. Resultant Liposomes were subjected to centrifugation at 3,000 rpm, 4^{o}C for 10 minutes using Remi centrifuge to remove unhydrated lipid, if any. To establish a transmembrane ammonium sulfate gradient, dialysis exchange against aqueous 10% sucrose was carried out [10]. Briefly, A 4 cm long portion of the dialysis tubing was made into a dialysis sac by folding and tying up one end of the tubing with thread, taking care to ensure that there would be no leakage of the contents from the sac. The sac was then soaked overnight in 10% sucrose solution in distilled water.

The wet sac was gently opened and washed copiously with 10% sucrose solution. Then the sac was filled with ammonium sulfate liposomal preparation (2-4ml). The sac was once again examined for any leaks and then was suspended in a glass beaker containing 100 ml of 10% sucrose solution, which acted as a receptor compartment. The

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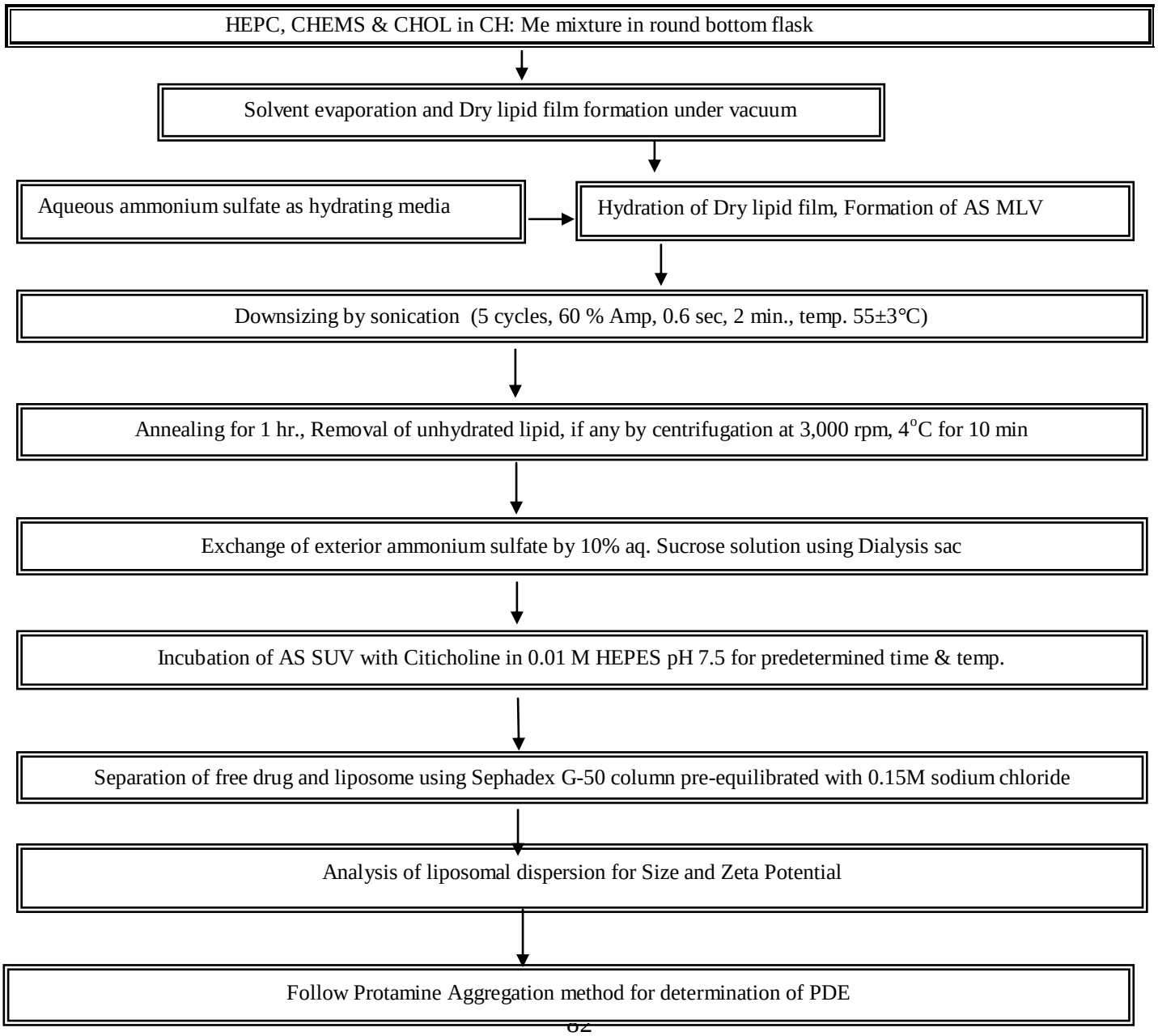
contents of the beaker were stirred using Teflon coated bar magnet (length = 2.5 cm, d = 0.5 cm) and the beaker was closed with the aluminium foil to prevent any evaporative losses during the experiment run. The dialysis was carried out for 18+ hours for sufficient exchange of exterior (unentrapped) ammonium sulfate against 10% sucrose solution to establish a transmembrane pH gradient [11].

After creation of gradient, liposomes were incubated with the drug solution (15 mg/ml) in 0.01M HEPES (pH-7.5) buffer for definite period of time at 55±3°C i.e. above Tg. For separation of free drug and liposome 'gel exclusion' chromatography was followed as reported in literatures [8]. Briefly, Sephadex G-50 column was prepared by soaking Sephadex G-50 into 0.15 M sodium chloride overnight. 0.15M sodium chloride was optimized to exchange with free citicholine. Then column of 2cm was prepared by pouring sephadexG-50 slurry into a 2 ml syringe. The syringe was put into 10ml centrifuge tube and centrifuged at 1000 rpm for 10 min. to remove excess solvent in Remi cooling centrifuge. The column was pre-equilibrated with 0.15M sodium chloride by three consecutive passes and each time centrifuged to remove excess 0.15M sodium chloride. Then 1ml liposomal suspension was applied at top of column and centrifuged at 1000 rpm for 10min.The elute was collected which contains citicholine liposome while the free drug was got entrapped into the column.

A flowchart depicting the process of preparation of liposomes with pH gradient loading of drug is shown in FIG.1

FIG. 1 Flow chart for preparation of AS liposomes prepared by TFH method. Drug loaded into liposome by pH Gradient Approach.



Results and Discussion

1. Characterization of liposomes

Citicholine entrapped liposomes were evaluated for morphology, particle size and zeta potential, internal pH of liposome and percent drug entrapment [12]. The photomicrograph obtained by Optical microscope and transmission electron microscopy (TEM) confirms the multilamellar nature of unsonicated liposome with spherical shape of liposomes respectively [13]. The TEM photograph shows size of a particle 138.4nm. The average particle size of optimized batch before and after citicholine loading was found to be 115.5nm and 124nm respectively. The zeta potential of optimized batch was recorded as -25.8mV. The internal pH of liposome prior to and following drug loading was found to be 5.52 and 7.33 respectively. The percent drug entrapment in optimized batch was found to be 39.88 ± 2.707 %.

Table 1. Characterization of Optimized Citicholine Liposomal dispersion

Characterization Parameters	Results
*Particle Size (PDI) ± SEM	124nm (0.081)± 4.1
Zeta Potential	-25.8 mV
*PDE ± SEM	39.88±2.707

*n=03

2. *In- vitro* drug diffusion study

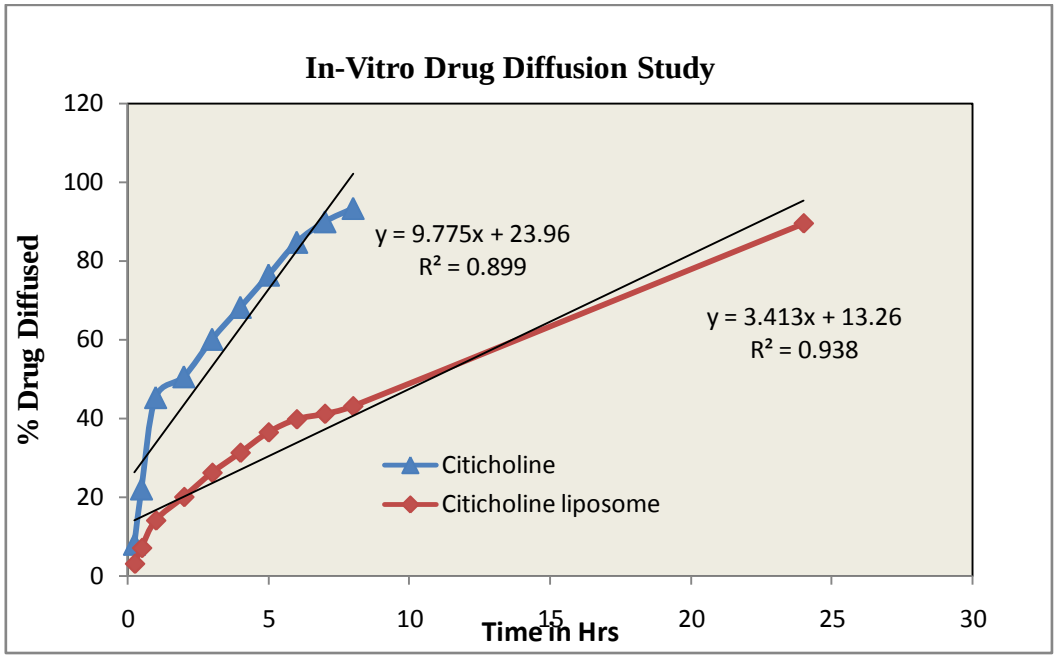
In-vitro drug diffusion study was performed using dialysis sac, in which drug release was observed in phosphate buffer saline (PBS) pH 7.4 [14]. The plain drug took 8 hours for about 93.25% of drug release whereas only 89.54% drug released from the liposomal formulation after 24 hours. The release kinetics was studied by fitting different models of drug release kinetics [15]. The data obtained indicate that citicholine plain drug follows 'First Order Release (R^2 0.978) whereas citicholine liposomal formulation obeys 'Higuchi's Diffusion Controlled' model for drug release (R^2 0.993).

Table -2. Comparative *in-vitro* drug diffusion of Citicholine plain drug and Citicholine liposome in PBS 7.4

Time	Mean Cumulative % Drug Diffused across the membrane (±SEM)*	
	Citicholine plain Drug	Citicholine Liposome
0.25 hr.	8.21±1.41	3.3±2.41
0.5 hr.	22.22±1.05	7.29±1.06
1 hr.	45.35±2.31	14.26±2.23
2 hr.	50.62±2.20	20.21±3.05
3 hr.	60.11±3.04	26.35±1.56
4 hr.	68.2±2.51	31.42±2.43
5 hr.	76.36±1.87	36.58±2.20
6 hr.	84.67±2.08	39.89±1.62
7 hr.	89.87±1.52	41.28±3.10
8 hr.	93.25±1.26	43.21±2.65
24 hr.	--	89.54±2.83

* n=03

FIG. 2 Comparative *in vitro* drug diffusion of Optimized Citicholine Liposome formulations and Plain Citicholine.



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