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Formulation and Evaluation of Liposomes Containing Erlotinib Hydrochloride

Zingade Sarika G [1], Nagoba Shivappa N.[1*], Swami Avinash B.[1], Vijayendra Swamy S. M.[1]
[1]Department of Pharmaceutics, Channabasweshwar Pharmacy College, Latur, Maharashtra, India.
Email: nagobashivraj@gmail.com, nshivraj11@rediffmail.com
*Corresponding Author

ABSTRACT
The aim of the present study is to formulate and evaluate liposomes containing Erlotinib hydrochloride. Erlotinib is a tyrosine kinase inhibitor which specifically inhibits epidermal growth factor receptors involved in angiogenesis of human non small cell lung carcinoma and inhibit growth of lung tumor. The present study conducted with the aim of preparing a site targeted nano-sized liposome to enhance the efficacy of Erlotinib which belongs to BCS class II. Total nine formulations were prepared by modified thin film hydration technique in which rotating flask contains glass beads for vortexing; lecithin (encapsulator), cholesterol (rigidator), and organic solvent. These formulations of liposome were evaluated and characterized for physical appearance, pH, drug content, % drug entrapment efficiency, microscopic determination and in vitro drug release. The results inferred F8 batch is most promising among all with highest drug entrapment i.e. 84.81% with drug release (~90%). Stability studies were conducted on optimized batch at 4°C, 40°C and room temperature for up to three months and formulation was found as stable at refrigerated temperature 4ºC.
Keywords: *Liposome; Erlotinib; BCS class; Stability.*

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INTRODUCTION

In 1960, Bangham introduced the liposome drug delivery; that phospholipids combined with water immediately formed a sphere and is capable of carry drug to the site of action [1]. Liposomes are concentric spherical vesicles of phospholipid bilayers having size 20- 1000 nm that are formed spontaneously in aqueous solution. The word liposome made of two Greek words, lipos (fat) and soma (body or structure). Lipid bilayered membrane encloses a central aqueous core of hydrophilic drugs while lipophilic drugs are entrapped within the bilayered membrane [2, 3].

Liposome bi-membrane is composed of natural and synthetic lipids, which are relatively biocompatible, biodegradable and non-immunogenic material. Because of amphipathic bilayer structure properties, liposomes are used as carriers for both lipophilic and water-soluble molecules. Liposomes have good biological properties of biocompatibility and biodegradability. They show promise as active vectors due to their capacity to enhance the entrapment performance by increasing drug solubility, and stability; delivering encapsulated drugs to specific target sites, and providing sustained drug release [4-6].

The liposomes help the drug to penetrate the cancer cells more selectively and decrease the possible side effects (nausea, hair loss and vomiting). Erlotinib is an EGFR-specific tyrosine kinase inhibitor which blocks the catalytic activity of the kinase responsible for non small cell lung cancer, thereby stopping complex network of downstream signaling pathway i.e. responsible for angiogenesis [7]. However, poor aqueous solubility and undesirable side effects limit the clinical application and local treatment of erlotinib. These side effects might be overcome by use of liposomes for tumor delivery and controlled release of erlotinib.

MATERIAL AND METHODS

Materials:

Erlotinib tosylate were obtained as a gift sample from Naprod life sciences Pvt. Ltd., Mumbai, India. Soyalecithin and HSPC gifted by Lipoid GMBH, Germany. Cholesterol were purchased from Research Lab Fine Chem Industries, Mumbai. The other chemicals, reagents and solvents used like potassium chloride,

sodium chloride, potassium dihydrogen phosphate, disodium hydrogen phosphate, chloroform, and methanol are of analytical reagent grade.

Methods:

Preformulation study:

Standard calibration curve:

Concentration of 1mg/ml standard working solution of drug was prepared in methanol. 1ml of this solution is then added to PBS 7.4 to make 10µg/ml of stock solution. By taking 2, 4, 6, 8, 10, and 12 ml of stock solution, dilutions were made with a PBS 7.4 to make concentrations of 0.2, 0.4, 0.6, 0.8, 1, and 1.2 µg/ml. The absorbance of these solutions was measured at 262nm, using UV- Visible spectrophotometer. Absorbance Vs concentration graph were plotted to obtain standard calibration curve [8].

Drug-excipient compatibility studies

FTIR spectroscopy is used to investigate and predict any physicochemical interactions between drug and lipids chosen in a formulation. The FTIR study performed using Perkin Elmer FTIR. The drug and excipients were taken in equal proportion (1:1) and analysed in near IR range (4000 cm^{-1} to 400 cm^{-1}). Compatibility was concluded by comparing interpretations of peaks obtained [9, 10].

Formulation method of Erlotinib liposome:

Nine batches of liposomes were prepared using Erlotinib HCl (API), soyalecithin, HSPC, PEG 2000 (cryoprotectant), tween 80 (surfactant), cholesterol and organic solvent as shown in table no. 2; by modified thin film hydration technique in which rotating flask contains glass beads for vortexing. In this method, the lipid phase (Phospholipid: Cholesterol, 7:3) 50 mg was dissolved in a 5 ml organic solvent (Chloroform: Methanol, 2:1) for 1 min in a 250 ml RBF containing adequate amount of 4 mm glass beads. Erlotinib hydrochloride is dissolved within this lipid phase. The organic solvent was evaporated under vacuum using rotary flash evaporator, which allows lipid to form a thin dry film on the walls of RBF. This system was maintained at vacuum and 45°C for an additional 10 min, for complete removal of organic solvent as indicated by visual observations. Sticking of beads to the wall indicate complete evaporation. 10 ml of hydrating media (PBS 7.4 pH) maintained above the Tm of the lipid, was then added immediately to form crude liposome vesicles. Flask is vortexed for 1 hr and to this crude liposome addition of polyethylene glycol (2 mg), tween 80 (0.25 ml) and remaining lipid takes place. Further vortexing for 1 hr yields drug loaded liposome [11-16].

Liposomes formed were ultrasonicated for 10 min to reduce the size of the vesicles and kept for overnight to mature the liposomes. The formed liposomal dispersions were centrifuged to separate supernatant liquid and to form dry liposomes for better stability and for further evaluation test.

Table 1: In-process checks during formulation of erlotinib hydrochloride liposomes

	Film formation	Hydration
RPM	65-70	50-55
Temperature	40-45°C	65-70°C

Table 2: Formulations of liposomes

Formulation batches	Ingredients			
	Drug (mg)	Soyalecithin (mg)	HSPC (mg)	Cholesterol (mg)
F1	5	60	-	18
F2	5	70	-	21
F3	5	80	-	24
F4	5	-	60	18
F5	5	-	70	21
F6	5	-	80	24
F7	5	50	10	18
F8	5	50	20	21
F9	5	50	30	24

Evaluation of liposomes:

Physical appearance and pH:

Liposomal dispersion was observed with naked eyes to determine its appearance and pH of the preparation were noted using digital pH meter [16].

Drug Entrapment Efficiency:

To determine % of drug entrapped; liposomal dispersion have subjected to high speed centrifugation on a laboratory refrigerated centrifuge at 12,000 rpm for a period of 30 min at 4°C and further 5 min is to

ensure complete sedimentation of erlotinib liposomes. The clear supernatant has removed carefully to separate non-entrapped erlotinib hydrochloride. The sediment in the centrifugation tube was washed with acetonitrile and both supernatant, sediment has diluted to 100 ml with PBS 7.4 and the absorbance of this solution was recorded at 262 nm. Amount of erlotinib in supernatant and sediment gives a total amount of erlotinib in whole formulation.[17, 18] The entrapment efficiency was calculated using the formula:

$$\%\ Entrapment\ efficiency\ = \frac{Amount\ of\ drug\ in\ sediment}{Total\ drug\ added} \times\ 100$$

Drug content:

Drug content in liposome is determined on UV spectroscopy. Liposomes (5 mg) were dissolved in the mixture of chloroform and methanol (2:1 v/v ratio) by shaking manually for 2 min. One ml of the resultant solution was taken and diluted with PBS 7.4 upto 10 ml and then absorbance was recorded at 262 nm using spectrophotometer and the concentration was obtained by using equation of standard calibration curve [18].

$$Drug\ content = \frac{Sample\ absorbance}{Standard\ absorbance} \times\ 100$$

Optical microscopy:

To ensure the formation of liposome vesicles, small amount of liposome dispersion were subjected to simple optical microscopic detection. Sample placed were seen from light background. [19]

***In Vitro* Drug Release Study:**

The in vitro release study of drug has carried out using dialysis tube method. In this method, 4 ml of liposomal formulation containing known amount of drug was placed in a test tube attached with dialysis membrane. Sample containing dialysis membrane was placed in beaker of 100 ml PBS 7.4, maintained at 37°C and stirred with the help of a magnetic stirrer. Samples from receptor compartment were withdrawn at different time intervals and replaced with fresh PBS 7.4 to maintain sink condition. Sample withdrawn further treated with 1 ml of acetonitrile to precipitate the lipids. After filtration samples were analyzed at 262 nm on UV [20-23].

Stability studies:

As per ICH guidelines, the stability study was conducted to monitor physical and chemical stability of erlotinib hydrochloride liposomal formulations at 4°C, 40°C and room temperature for up to three months. The stability parameter, such as physical appearance, pH, drug content, entrapment efficiency &*in vitro* drug release was determined as function of the storage time [24, 25].

RESULTS AND DISCUSSION

Standard calibration of Erlotinib:

The UV absorbance's of erlotinib standard solutions in the range of 0.2-1.2 µg/ml of drug in buffer pH 7.4 showed linearity at λ max 262 nm. The linearity was plotted for absorbance (A) against concentration (C) with R^2 value 0.995 and with the slope equation y=0.111x + 0.021. The absorbance values and standard curve were in below table 3 and figure 2.

Table 3: Absorbance values of erlotinib at 262 nm

Sr. No.	Concentration (µg/ml)	Absorbance at 262 nm
1.	0.2	0.048
2.	0.4	0.062
3.	0.6	0.087
4.	0.8	0.109
5.	1.0	0.133
6.	1.2	0.157

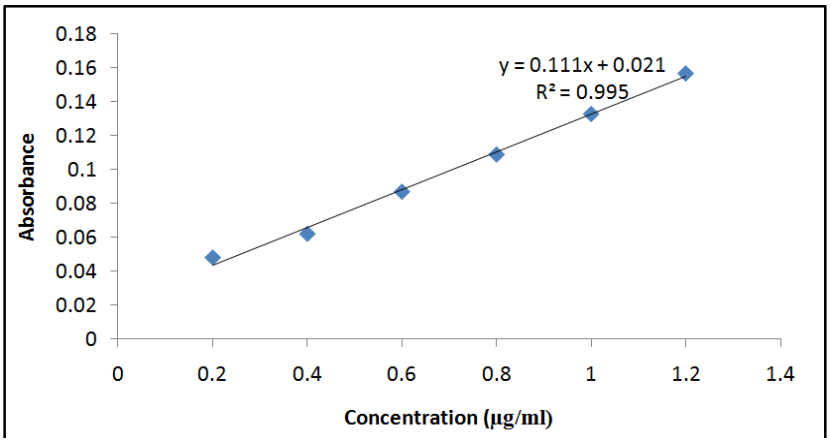

Figure 2: Calibration curve of erlotinib hydrochloride

Drug-Excipient Compatibility Studies:

By analyzing the FTIR study of pure drug and lipids used in formulation it can be considered that there is no significant peak enlargement or shortening were occurred by interaction between drug and excipients.

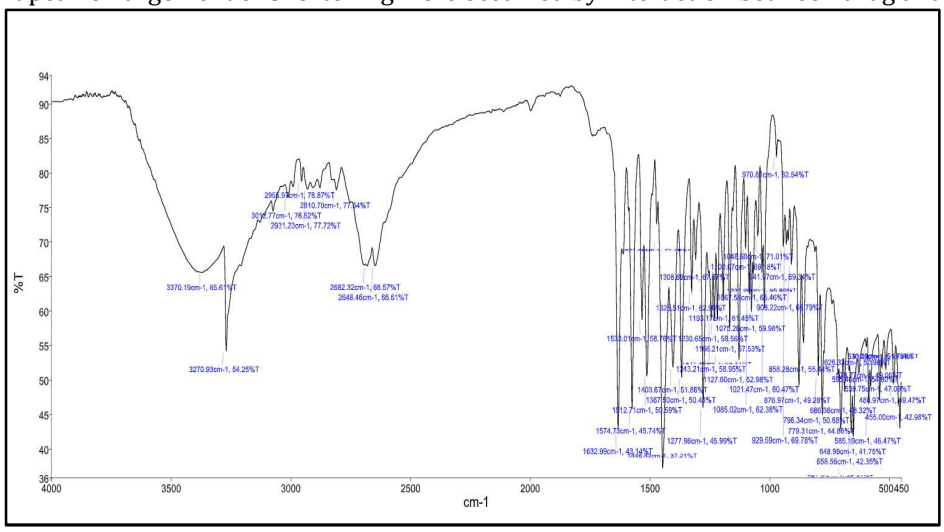

Figure 3: FTIR of erlotinib hydrochloride

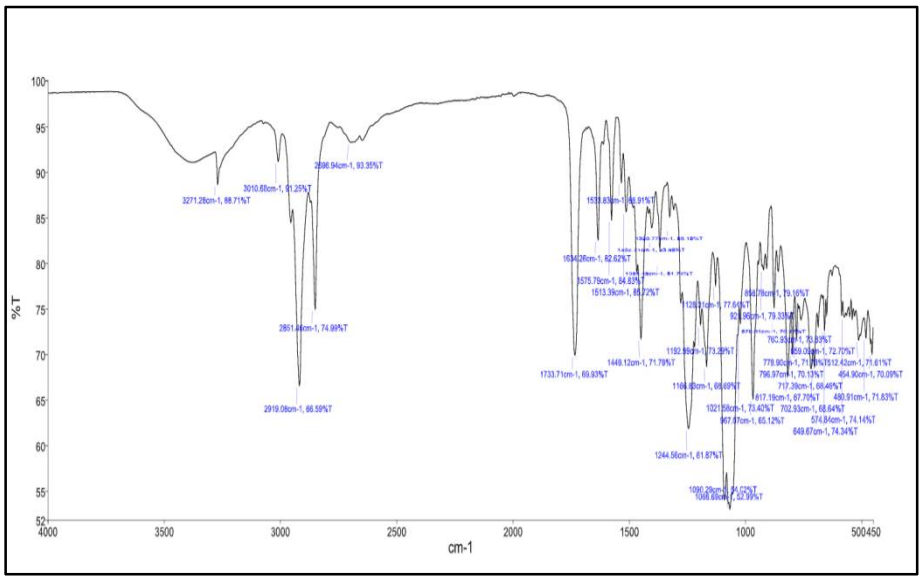

Figure 4: FTIR graph of Drug + Soyalecithin + HSPC

Table 4: Interpretations of IR spectra

Sr. No.	Functional groups	Standard frequency	Observed peak for pure drug	Observed peak for drug + excipients
1.	C-H	3040-3010	3012.77	3010.61
2.	C=C	1680-1710	1632.99	1684.25
3.	C=O	1750-1735	1651.88	1735.85
4.	N-H	1620-1590	1632.99 1574.73 1533.01 1512.71	1634.25 1575.48 1533.77 1514.11
5.	C-O	1410-1300	1403.67 1325.51	1367.50

Physical appearance, Drug content and pH:

All liposomes are found as milky white dispersion. The drug content study of all six batches have been calculated by dispersing the liposomes in mixture of buffer & methanol solution and assay was made by UV spectrophotometer. From all these batches F8 batch showed highest drug content (92.86 %) of drug.

Table 5: Physical appearance, Drug content and pH

Formulations	Physical Appearance	pH	Drug Content (%)
F1	Milky white dispersion	7.28	76.23
F2		7.37	75.86
F3		7.33	69.23
F4		7.27	74.71
F5		7.31	85.16
F6		7.35	85.81
F7		7.29	81.53
F8		7.38	92.86
F9		7.43	90.26

Drug Entrapment Efficiency:

The entrapment efficiency of all batches has been determined by centrifugation method. From all these batches F8 batch showed highest entrapment (84.81 %) of drug. Hence, F8 batch was considered as an optimized batch as given in below table 6.

Table 6: % Drug Entrapment Efficiency

Sr. No.	Formulation Code	% Drug Entrapment Efficiency
1	F1	69.75 %
2	F2	65.48 %
3	F3	61.62 %
4	F4	43.41 %
5	F5	51.86 %
6	F6	62.37 %
7	F7	80.45 %
8	F8	84.81 %
9	F9	76.23 %

Microscopic Determination:

Liposomal preparation when saw under light background; aqueous interior of vesicles spread the colors like rainbow which confirms the vesicle formulation. Below figure 5 shows the microscopic photos of liposome bed seen under light and dark background.

Figure 5: Microscopic determination of liposome

***In Vitro* Drug Release Study:**

The in-vitro release of erlotinib hydrochloride liposomal formulations checked using the dialysis membrane and measured the release at 1 hr, 2 hr and 3 hr upto 24 hrs. After, examining percentage cumulative drug release graph of all formulation, the F8 batch showed a highest drug release (83.36 %) at 24 hrs. Hence, the F8 batch was concluded as an optimized batch.

Table 7: % Drug release

Time (hr)	% Drug release								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
0	0	0	0	0	0	0	0	0	0
1	3.16	3.55	4.25	6.32	6.89	7.05	8.26	8.48	7.28
2	6.01	6.43	6.19	7.25	9.22	10.03	10.04	10.38	10.93
3	11.28	12.61	13.91	14.47	14.44	15.48	21.43	23.41	17.56
4	16.03	15.26	17.91	17.59	18.22	19.42	25.95	28.22	21.25
5	19.58	19.79	20.45	22.72	21.88	23.52	28.04	31.52	25.22
6	23.15	24.69	25.29	25.59	25.15	27.81	32.22	34.91	29.26
7	27.15	28.56	29.56	29.37	30.86	31.59	37.68	41.65	33.11
8	31.93	32.65	33.37	32.25	35.36	35.95	42.04	43.81	38.84
9	36.94	36.15	38.43	36.13	38.16	39.83	46.98	47.55	42.09
10	42.09	42.51	43.41	39.95	41.93	42.52	50.25	52.54	46.91
11	46.55	45.97	45.09	42.62	43.73	45.81	52.68	55.92	52.12
12	50.73	51.98	52.85	47.15	47.19	51.62	58.34	62.75	57.13
16	58.67	59.29	60.94	56.57	58.52	60.43	62.95	68.47	59.72
20	65.29	65.93	67.12	66.47	67.25	68.88	69.05	74.37	71.83
24	72.54	73.76	75.78	75.69	75.56	77.34	80.43	83.36	81.09

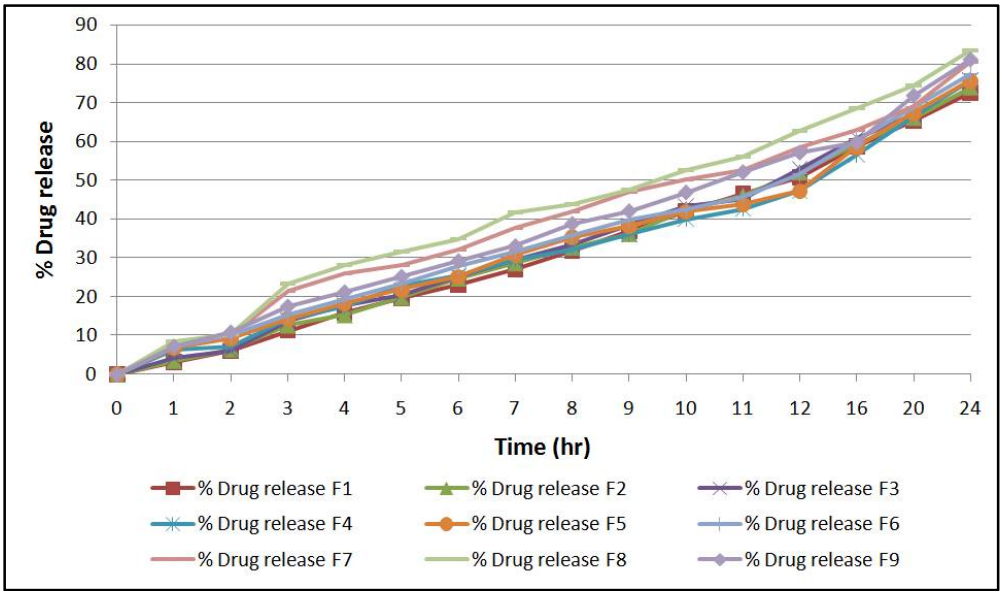

Figure 6: Graph of % cumulative drug release

Stability Studies:

Stability studies on optimized F8 batch of Erlotinib hydrochloride liposome preparation have been conducted for 90 days in different temperature conditions and have evaluated for physical appearance, pH, drug entrapment efficiency, and in-vitro drug release as a function of the storage condition. The liposomes stored at 4ºC were found to be stable for the duration of 90 days as compared to room temperature and accelerated stability temperature. The results were showed in table 7 and figure 7.

Table 7: Stability study on optimized formulation

Sr. No.	Number of days	Physical Appearance	pH	% Drug Entrapment efficiency		
				At 4 °C	Room Temp.	At 40 °C
1	Initial	Milky white dispersion	7.48	84.41	84.41	84.41
2	15	Milky white dispersion	7.45	84.36	84.11	83.15
3	30	Milky white dispersion	7.35	84.33	83.86	82.96
4	45	Milky white dispersion	7.35	84.28	82.16	80.62
5	60	Milky white dispersion	7.32	84.21	80.23	77.89
6	75	Milky white dispersion	7.30	84.15	78.12	74.48
7	90	Milky white dispersion	7.27	83.98	76.89	72.68

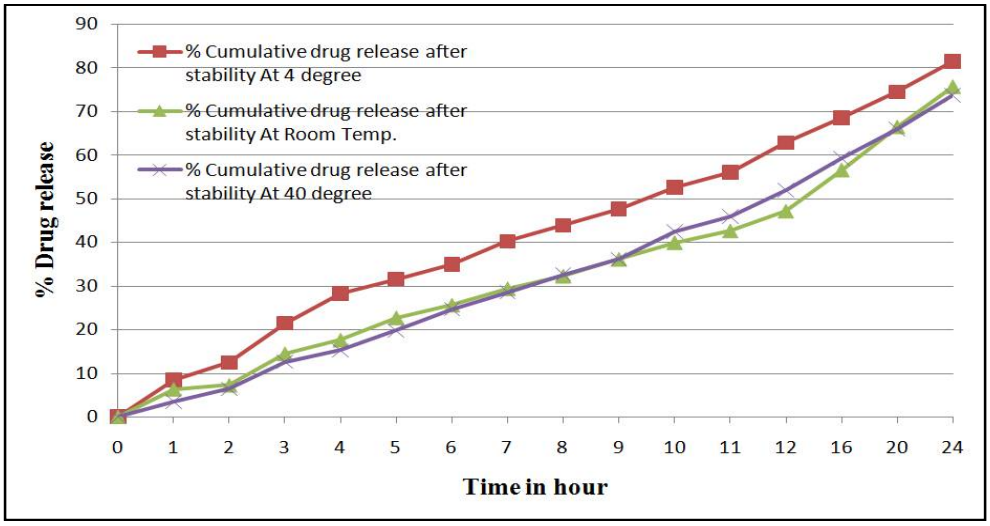

Figure 7: Graph of % cumulative drug release after stability

CONCLUSION

The research conducted has successfully encapsulated poorly bioavailable drug erlotinib hydrochloride into a vesicular liposome dosage form. Result has showed maximum drug release within 24 hrs (83.36 %) with good entrapment efficiency (84.81%) which can be achievable with liposome encapsulation. Additionally liposome requires only small amount of drug to get therapeutic action, hence it does not give any side effect, by directing drug to specific site. Although if liposome technology has better results; no one can predict its efficacy hence clinical studies are needed to understand targeting capacity of the liposomes in the cancer treatment for human.

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