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Research Article

## Formulation and evaluation of doxorubicin hydrochloride liposomes by dried thin lipid film technique

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### ABSTRACT

The main objective of the work was encapsulating of Doxorubicin hydrochloride in liposomal formulation for treatment of cancer chemotherapy. Conventional compositions of Doxorubicin hydrochloride were available as freeze-dried product or as a solution of doxorubicin hydrochloride in water. Both these products have been associated with a number of toxicities when administered intravenously. To overcome these problems, in the present study, inclusion of Doxorubicin hydrochloride in liposomal formulation has proved to be good approach to eliminate the toxicities and improve drug antitumor activity. In this study, Doxorubicin hydrochloride liposomes containing Hydrogenated Soy Phosphatidyl Choline, Cholesterol, various stabilizers and ammonium sulphate prepared by dried thin film hydration method. The characterization of liposomes was carried out by vesicle size, zeta potential, %free drug and in-vitro dissolution. The release kinetics of formulations containing neural, negative and positive stabilizers followed zero-order release kinetics. Hence it could be concluded that stabilizers like Stearylamine and Phosphotidyl glycerol along with Hydrogenated Soy Phosphatidyl Choline (HSPC) and Cholesterol were suitable carriers for the preparation of Doxorubicin HCl liposomes.

**Keywords:** Doxorubicin hydrochloride; Liposomes; HSPC; Stearylamine; Phosphotidyl glycerol; Ammonium sulphate; cholesterol

### INTRODUCTION

The goal of any drug delivery system is to provide a therapeutic amount of drug to the proper site in the body, to achieve promptly and then maintain the desired drug concentration. Liposomes are Microscopic, Fluid-filled pouch, whose walls are made up of layers of Phospholipids identical to the Phospholipids that make up cell membrane (Chein YM, 1992).

Doxorubicin hydrochloride ( $C_{27}H_{29}NO_4$ ) with Molecular weight 579.98 gm/mol, an anthracycline derivative isolated from *Streptomyces Peucetius* Var. *Caesius*. The mechanism of action of Doxorubicin hydrochloride was it interact with DNA by intercalation and inhibition of macro-molecular biosynthesis, this inhibits the progression of the enzyme topoisomerase-II, which relaxes super coils in DNA transcription. In clinical trials Doxorubicin hydrochloride is successfully used, mostly against ovarian cancer, Multiple Myeloma and Kaposi Sarcoma, the main side effects of this drug are cardiac toxicity, Infusion reactions and Myelosuppression (Ajay

Patidar *et al.*, 2010).

To overcome these problems, an alternative approach is needed. In the present study doxorubicin Hcl liposomes are formulated using various Phospholipids and Stabilizers (Positive and Negative) to check effect of drug loading and particle size. Several approaches has taken in an effort to increase the circulation time of liposome by coating the liposomal surface with a hydrophilic polymer such as polyethylene glycol (PEG) to prevent adsorption of various blood plasma proteins to the liposome surface. These liposomes appeared to reduce some of the toxic effects caused by the release of their contents, but have new toxic effects appeared like skin toxicity generally known as "Hand-Foot Syndrome" and the presence of large molecules (PEG) on the liposomal surface may reduce the interaction of liposomal with cells & hinder entry of liposomes in to tumor tissue (Gautam Vinod Daftary *et al.*, 2008; Vyas SP and Sihorkar V, 2001).

Thus, these remains a need for stable, long circulating liposomes that do not cause such deleterious effects such as the "Hand-Foot Syndrome" as well as methods of manufacturing such liposomes & composition based on them. The present formulation meets this need, and testing the effect of stabilizers on particle size analysis, percent free drug, Assay, *In-vitro* drug release studies, release kinetics & stability studies (Martin C. Woodle *et al.*, 1994).

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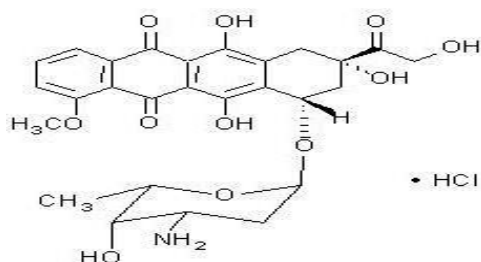
The main objective of the study was designed to prepare and evaluate the neutral and charged Doxorubicin hydrochloride liposomes and study the effect of various stabilizers based on the Physicochemical and *in vitro* release studies.

## MATERIALS AND METHODS

### MATERIALS

Doxorubicin is an anthracycline topoisomerase inhibitor isolated from *Streptomyces peucetius var. caesius*.

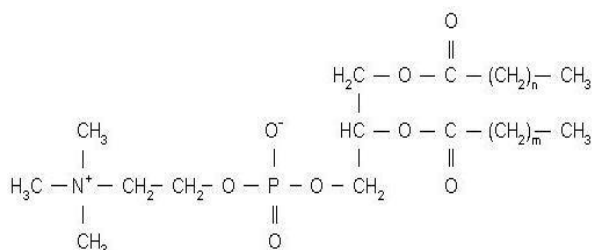
Doxorubicin HCl, which is the established name for (8S,10S)-10-[(3-amino-2,3,6-trideoxy- $\alpha$ -L-lyxohexopyranosyl)oxy]-8-glycolyl-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacenedione hydrochloride, has the following structure:



The molecular formula of the drug is  $C_{27}H_{29}NO_{11} \cdot HCl$ ; its molecular weight is 579.99.

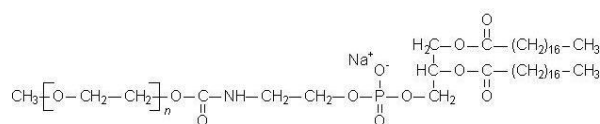
### HYDROGENATED SOY PHOSPHATIDYL CHOLINE

Phosphatidylcholine is a major constituent of cell membranes and pulmonary surfactant, and is more commonly found in the exoplasmic or outer leaflet of a cell membrane. It is thought to be transported between membranes within the cell by phosphatidylcholine transfer protein (PCTP). Phosphatidylcholine also plays a role in membrane-mediated cell signaling and PCTP activation of other enzymes. Hydrogenated soy phosphatidyl choline has the following structure



### CHOLESTEROL [methoxy-poly (ethylene glycol)-distearoylphosphatidylethanolamine]

N-(carbonyl-methoxypolyethylene glycol 2000)-1, 2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt (MPEG-DSPE). The structure of [MPEG-DSPE] is as follows,



Doxorubicin hydrochloride, Hydrogenated Soy Phosphatidyl Choline and Cholesterol were obtained as a sample from Celon labs Hyderabad. Stearylamine and Phosphotidyl glycerol were obtained from Sigma Aldrich. The ingredients were analytical grade. The laboratory grade chemicals used for the work are Ammonium sulphate, Sucrose, Histidine, Chloroform, Sodium hydroxide, Triton X-100, Acetonitrile are purchased from Merck Chemicals Pvt., Ltd. Mumbai.

### Preformulation study

#### General Procedure for the Preparation of calibration curve by UV

A stock solution of (1mg/ml) of standard drug was prepared, later required dilutions were made with a phosphate buffer pH 7.4. To a series of 10ml volumetric flasks aliquots standard solutions were taken and the volume was made up using a phosphate buffer pH 7.4. The absorbance of these solutions was measured at respective wave length of maximum absorbance, using 1cm quartz cuvette in UV- Visible spectrophotometer. Absorbance values were plotted against respective concentration to obtain standard calibration curve.

#### Compatibility studies

IR spectroscopy can be used to investigate and predict any physicochemical interactions between different components in a formulation and therefore it can be applied to the selection of suitable chemically compatible excipients. The aim of the present study was to test, whether there is any interactions between the carriers and drug; The following IR spectroscopy were recorded (Ganesh GNK et al., 2011).

#### Preparation of doxorubicin liposome

The preparation of liposomes with Hydrogenated Soy Phosphatidyl Choline was prepared by dried thin film hydration technique using rotary evaporator.

Accurately weighed quantities of Hydrogenated Soy Phosphatidyl Choline, cholesterol, Stearylamine and Phosphotidyl glycerol are dissolved in chloroform and rotated in a rotavapor by applying vacuum of about 25mmHg at 250c, until it forms a thin film. Required quantities of ammonium sulphate and sucrose (5%) are dissolved in W.F.I and it is added to the above thin film in R.B flask and rotated until it forms a milky white suspension. The above solution is homogenized for 15 cycles to reduce particle size of liposomes. The above solution is undergone for 15 cycles of dialysis, by using sucrose solution (5%) to remove free ammonia and sulphate from the lipid solution. Drug solution is prepared by adding the required quantities of Drug and Histidine in a W.F.I and pH is adjusted to 6.4 to 6.7 and this drug solution is added to the solution in a R.B flask (lipid solution) and rotated for 1hr (Sanjeevani Desai et al., 2011; Xue Ming Li et al., 2009).

**In-process Checks RPM:** 50rpm (Film formation), 70rpm (Hydration), 60rpm (Drug Loading).

**Table 1: Diffusion exponent and solute release mechanism for cylindrical shape**

S.No	Diffusion	Exponent (n) Overall solute diffusion mechanism
1.	0.45	Fickian diffusion
2.	0.45<n<0.89	Anomalous (non-Fickian) Diffusion
3.	0.89	Case-II transport
4.	n>0.89	Super case-II transport

**Table 2: Formulation variables of Doxorubicin liposome injection**

Ratio of ingredients	Types of liposomes		
	Neutral	Positive	Negative
Lecithin: cholesterol: Stearylamine : Di-acetyl phosphate : Ammonium Sulphate	5:5:0:0:30	5:5:1:0:30	5:5:0:1:30
	5.5:4.5:0:0:30	5.5:4.5:1:0:30	5.5:4.5:0:1:30
	6:4:0:0:30	6:4:1:0:30	6:4:0:1:30
	6.5:3.5:0:0:30	6.5:3.5:1:0:30	6.5:3.5:0:1:30
	7:3:0:0:30	7:3:1:0:30	7:3:0:1:30
	7.5:2.5:0:0:30	7.5:2.5:1:0:30	7.5:2.5:0:1:30
	8:2:0:0:30	8:2:1:0:30	8:2:0:1:30
	4.5:5.5:0:0:30	4.5:5.5:1:0:30	4.5:5.5:0:1:30
	4:6:0:0:30	4:6:1:0:30	4:6:0:1:30
	3:7:0:0:30	3:7:1:0:30	3:7:0:1:30

**Temperature:** 40°C (Film formation), 65°C (Hydration), 60°C (Drug Loading).

**Physical characterization of liposomes:** Determination of particle size, Zeta potential and SEM analysis was carried out by using the Malvern Zeta Sizer and Scanning Electron Microscopy in Star tech labs and Indian Institute of Chemical technology, for the optimized formulations (Luigi Cattel MN et al., 2004; Jorge JCS et al., 1994; Timothy D Heath et al., 1985).

**Percent free drug** Absorbance of solution was measured at 590nm using sucrose - Histidine solution as blank. 0.1ml of sample was transferred in to 20ml stoppered test tube, add 8ml of Sucrose- Histidine solution to it, mix well, Absorbance was measured at 590nm using calibrated UV spectrophotometer. Transferred the solution from the cell to test tube (A1).To the above test tube containing solution, added 1ml sodium hydroxide solution, mix well and absorbance was measured at 590 nm using UV transfer the solution from the cell to test tube (A2).To the above test tube containing solution, added 1ml of Triton X-100 solution, mixed well and measured the absorbance at 590 nm using calibrated UV (A3). Percent Free Doxorubicin HCl =  $[(A2 \times 1.125) - A1/A3 \times 1.25] \times 100$ .

**Doxorubicin HCl Assay** A standard and sample solution were prepared, Inject separately 20 microlitre of the standard and sample solution in chromatographic condition and record the chromatogram. Calculate the content of drug per ml in liposomal injection as follows. Assay=  $A/B \times W/200 \times 5/50 \times C/100 \times 100 - D/100 \times 50/5 \times 100/5$  Where, A = Area corresponding to Doxorubicin HCl in sample. B = Area corresponding to Doxorubicin HCl in working standard. C = % purity of Doxorubicin HCl in working standard. D = % water content of

working standard. W = Weight of working standard in mg (Howard G et al., 1977).

**In vitro dissolution studies of Doxorubicin hydrochloride liposome** The *in vitro* release of drug from the liposomal formulation was carried out by using dialysis membrane employing in two sides open ended cylinder. 4 ml of liposomal suspension containing known amount of drug was placed in a dialysis membrane previously soaked overnight. The two sides open cylinder was placed in 200ml of PBS (pH 7.4), maintained at 37°C and stirred with the help of a magnetic stirrer. Aliquots (4ml) of release medium were withdrawn at different time intervals and the sample was replaced with fresh PBS (pH 7.4) to maintain constant volume. 1 ml of Acetonitrile was added to each aliquot to precipitate the lipids and dissolve the entrapped Doxorubicin hydrochloride and then the samples were analyzed by UV spectrophotometry at a  $\lambda$  max of 254nm (Ahmad et al., 1993).

#### Release kinetics

To analyze the *in vitro* release data various kinetic models were use to describe the release kinetics. The zero order rate Eq. (2) describes the systems where the drug release rate is independent of its concentration. The first order Eq. (3) describes the release from system where release rate is concentration dependent. Higuchi (1963) described the release of drugs from insoluble matrix as a square root of time dependent process based on Fickian diffusion (Korsmeyer RW et al., 1983).

**Zero order kinetics** Zero order release would be predicted by the following equation:  $A_t = A_0 - K_0t$  When the data is plotted as cumulative percent drug release versus time, if the plot is linear then the data obeys

**Table 3: Optimized formulation of Doxorubicin liposomes injection**

Formulation code	Drug (mg/ml)	Soy lecithin (mg/ml)	Cholesterol (mg/ml)	Stearyl amine (mg/ml)	Dicetyl phosphate (mg/ml)	Ammonim Sulphate (mg/ml)
F1	2	7	3	-	-	30
F2	2	7.5	2.5	-	-	30
F3	2	7	3	1	-	30
F4	2	7.5	2.5	1	-	30
F5	2	7	3	-	1	30
F6	2	7.5	2.5	-	1	30

**Table 4: Physicochemical characteristics of Doxorubicin hydrochloride Liposomes**

S.No.	Formulation code	Average vesicular size (nm)	Zeta Potential (mV)	Poly dispersive index (Pdi)
1.	F2	356nm	5.21	0.635
2.	F4	564nm	24.66	0.762
3.	F6	317nm	-23.4	0.645

**Table 5: percentage free drug & Assay of F1, F2, F3, F4, F5, & F6 formulations**

S. No.	Formulation code	Percentage of free Drug	Doxorubicin Hcl Assay
1.	F1	13.36±1.1%	98.8±0.32%
2.	F2	12.36±1.1%	99.7±0.65%
3.	F3	12.56±4.6%	95.6±0.65%
4.	F4	11.36±1.3%	97.3±0.96%
5.	F5	8.45±5.9%	97.2±0.58%
6.	F6	6.83±0.8%	98±1.32%

**Table 6: Release rate profile of Formulations F3, F6 & F9**

Type of Formulation	Zero-order (R <sup>2</sup> )	First-order (R <sup>2</sup> )	Higuchi (R <sup>2</sup> )	Korsmeyer – Peppas (n)
F3	0.995	0.992	0.988	0.9617
F6	0.992	0.990	0.985	0.9331
F9	0.989	0.955	0.985	0.898

**Table 7: Effect of temperature on Assay of doxorubicin HCl at 4°C**

Formulation Code	Effect of stability on Assay at 4oc				
	0day	15 days	30 days	40 days	60 days
F2	99.7±0.65%	99.2±0.47%	98.8±0.72%	98±1.0%	97.6±0.65%
F4	97.3±0.96%	97.1±0.73%	96.6±1.12%	95.8±0.43%	95.2±0.96%
F6	98±1.3%	97.5±1.0%	96.7±0.9%	96.2±0.63%	95.7±1.3%

Zero – order kinetics and its slope is equal to Zero order release constant K<sub>0</sub>.

**First order kinetics** First - order release could be predicted by the following equation:  $\log C = \log C_0 - Kt / 2.303$  When the data plotted as log cumulative percent drug remaining versus time, yields a straight line, indicating that the release follow first order kinetics. The constant 'K1' can be obtained by multiplying 2.303 with the slope value.

**Higuchi's model** Drug release from the matrix devices by diffusion has been described by following Higuchi's classical diffusion equation.  $Q = \sqrt{DE} / \tau(2A - \epsilon C_s)$  Cst]1/2 When the data is splitted according to equation i.e. cumulative drug release versus square root of time yields a straight line, indicating that the drug was re-

leased by diffusion mechanism. The slope is equal to 'K' (Higuchi's, 1963).

**Korsmeyer equation / Peppas's model:** To study the mechanism of drug release from the liposomal solution, the release data was also fitted to the well-known exponential equation (Korsmeyer equation/ Peppas's law equation) mentioned in table 1, which is often used to describe the drug release behavior from polymeric systems.

$$M_t / M_\infty = Kt^n$$

Where,

$M_t / M_\infty$  = The fraction of drug released at time 't'.

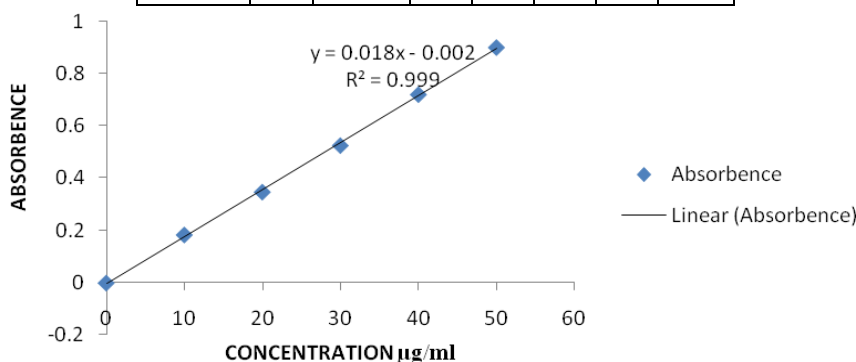
K = Constant incorporating the structural and geometrical characteristics of the drug / polymer system.

**Table 8: Effect of temperature on Assay of doxorubicin HCl at room temperature**

Formulation Code	Effect of stability on Assay at room temperature.				
	Oday	15 days	30 days	40 days	60 days
F2	99.7±0.65%	97.8±0.48	96.4±0.54	94.92±0.32	93.56±0.85
F4	97.3±0.96%	96.2±0.92	94.8±0.62	92.58±0.58	89.35±0.53
F6	98±1.3%	97.8±0.65	95.9±0.69	92.52±0.86	90.24±0.74

**Table 9: In vitro cumulative % drug release profile of doxorubicin hydrochloride liposomal formulations**

Time (hrs)	Cumulative % drug release						
	F1	F2	F3	F4	F5	F6	M
1	1.29	1.38	1.65	1.74	2.12	2.15	2.05
2	2.36	2.92	3.21	3.37	4.02	4.07	3.99
3	4.32	4.41	5.01	5.11	6.12	6.19	6.06
4	5.65	5.93	6.56	6.82	8.32	8.43	8.13
6	8.53	8.77	9.85	10.5	12.92	13.01	12.95
8	11.21	11.65	13.56	13.9	17.53	17.62	17.41
10	15.25	15.63	17.04	17.38	21.45	21.62	21.31
12	18.65	18.91	20.53	20.86	25.35	25.56	25.14
16	25.98	26.73	29.62	29.82	35.95	36.47	35.023
20	33.85	34.25	36.56	36.78	44.86	45.07	44.97
24	45.85	49.31	49.59	52.84	55.25	58.67	58.44
36	75.45	79.12	80.22	86.15	88.05	92.93	90.01

**Figure 1: Standard graph of Doxorubicin hydrochloride in phosphate buffer of pH 7.4**

N = Diffusion exponent related to the mechanism of the release.

Above equation can be simplified as follows by applying log on both sides,

$$\text{Log } M_t / M_\infty = \text{Log } K + n \text{ Log } t$$

### Stability Studies

The stability of a pharmaceutical delivery system may be defined as the capability of a particular formulation, in a specific container. The short-term stability was conducted to monitor physical and chemical stabilities of the liquid form of doxorubicin hydrochloride liposomal formulations at 4°C and room temperature for up to three months. The stability parameter, such as Assay was determined as function of the storage time.

## RESULTS AND DISCUSSIONS

### Standard calibration curve of Doxorubicin hydrochloride in UV spectrophotometer

The UV absorbance's of Doxorubicin standard solution in the range of 10-50 µg/ml of drug in buffer, pH 7.4 showed linearity at  $\lambda_{\text{max}}$  254nm. The linearity was

plotted for absorbance against concentration with R<sup>2</sup> value 0.9995 and with the slope equation  $y = 0.0179x - 0.003$ . The absorbance values and standard curve shown in Fig 1.

### Compatibility studies

The compatibility between the drug and the selected lipid and other excipients was evaluated using FTIR 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.

### Doxorubicin liposomal formulation

The Liposomes were prepared by dried thin film hydration technique using rotary evaporator with drug and carrier (Hydrogenated soy phosphatidyl choline). The formulation containing Doxorubicin were prepared with different stabilizers like Phosphatidyl glycerol and Stearylamine and all other parameters like temperature, vacuum and RPM were kept constant. The composition and ratios of compounds showed in Table 2. Among those compositions 9 Formulations are se-

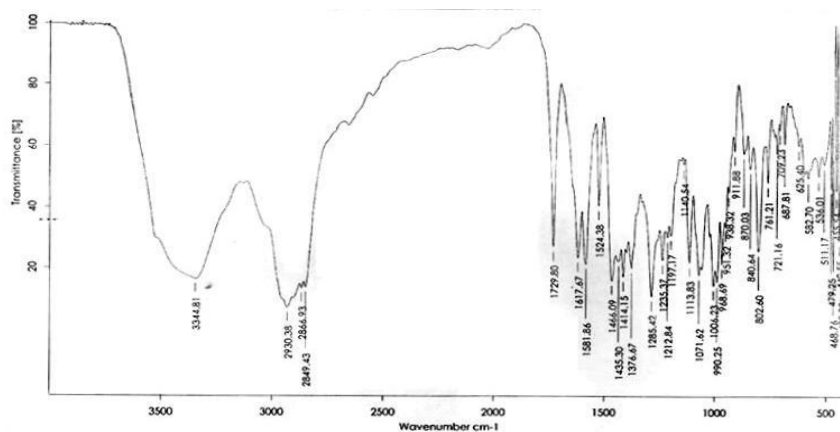


Figure 2: FTIR spectrum of Doxorubicin HCl

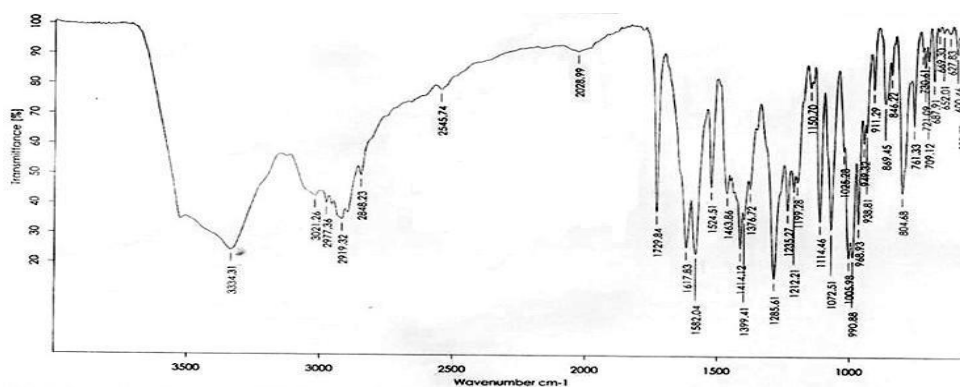


Figure 3: FTIR of Doxorubicin HCl, Soy lecithin, Cholesterol, Stearyl amine

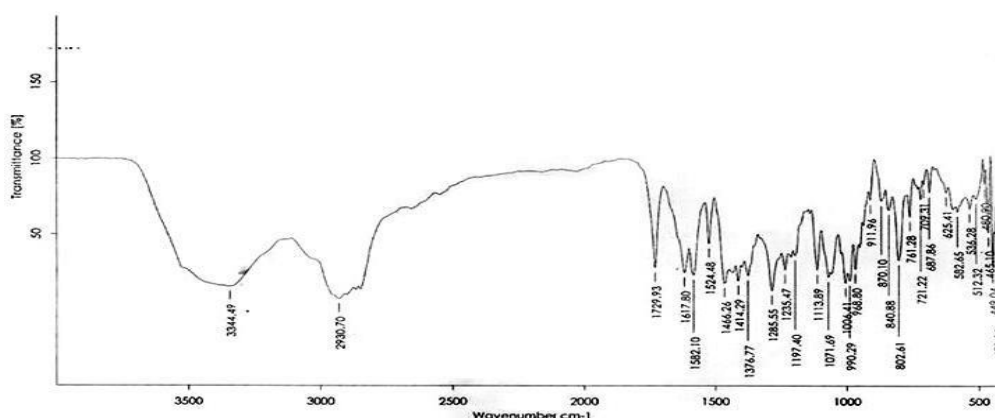


Figure 4: FTIR of Doxorubicin HCl, Soy lecithin, Cholesterol, Dicyl phosphate

lected as optimized batches for further evaluation, 3 from each of neutral, positive and negative as showed in Table 3.

**Physicochemical characterization Particle size distribution**

The particle size distribution was analyzed for F3, F6, F9 formulations of doxorubicin Liposomes by wet method. The particle size was optimum in F9 Formulation, when compared to F3 and F6, The results were shown in Table No: 4.

**Scanning Electron Microscopy**

The Morphology and surface appearance of Liposomes were examined by using SEM. The SEM photographs of F6 and F9 formulation showed that the particles have

smooth surface. The SEM images were shown in Figure 5 and 6.

**Zeta Potential analysis**

The zeta potential report of liposomal solution for F3, F6, F9 formulations are 4.31mV, 23.68mV, -23.4 which lies near to the arbitrary value. The report shows good stability value for formulated liposomal solution, the results were shown in Table 4.

**In vitro characterization Percent free drug**

The percent free drug is determined for all the formulations F1to F9. The percent free drug was optimum in F9 formulation, which is within the limit (10%), the percent free drug was as shown in the Table 5.

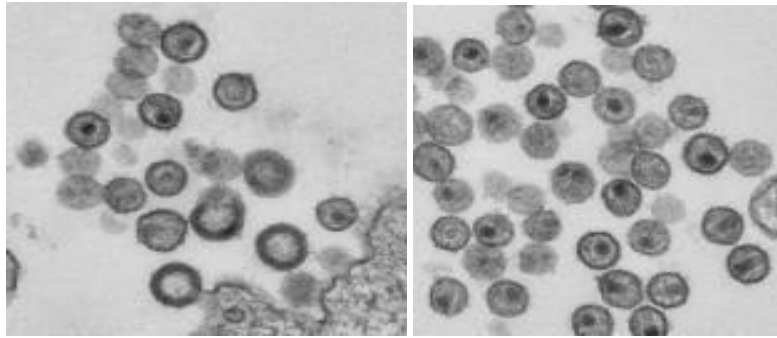


Figure 5: SEM of F6 and F9 formulation

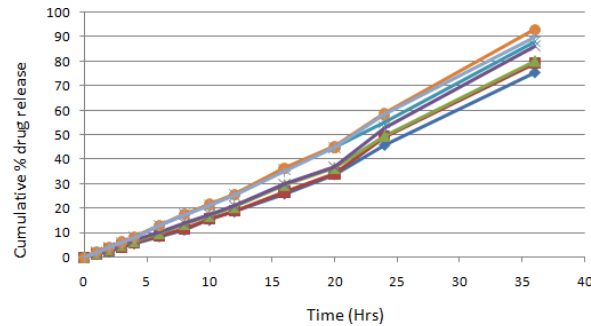


Figure 6: Comparison of in vitro release studies for optimized formulations F1to F6 & Market Samples

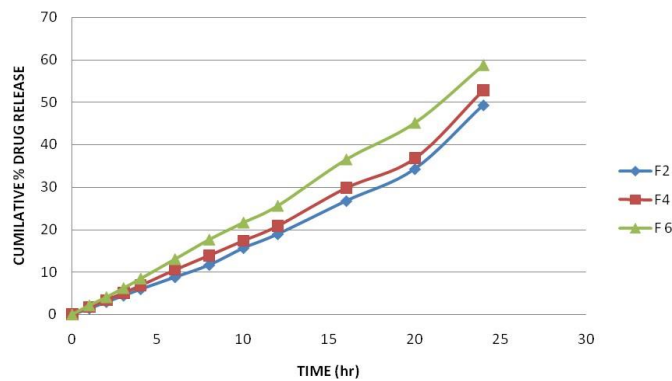


Figure 7: Comparison of in vitro release studies for optimized formulations F3, F6, F9

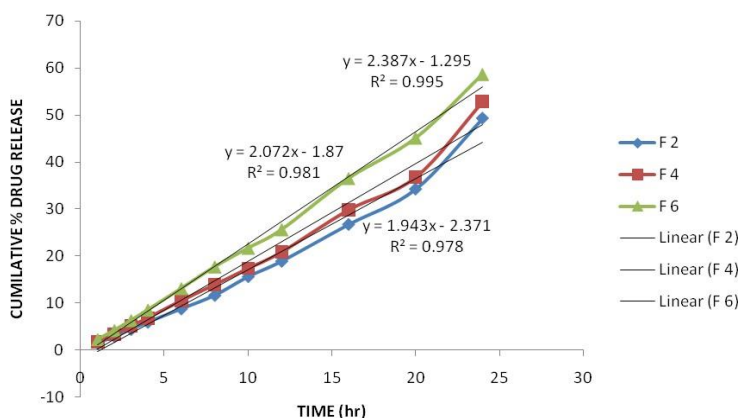


Figure 8: Zero order release studies for optimized formulations F3, F6 & F9

**Assay**

The assay value is determined for all the formulations from F1to F9. The assay value is within the limit (90%) for all the formulations, the results were shown in the Table 5.

**In vitro Dissolution data**

The *in vitro* dissolution profile of prepared formulations was determined by membrane diffusion method. The dissolution was carried out for a period of 24 hrs in 7.4 pH phosphate buffer. The cumulative percent re-

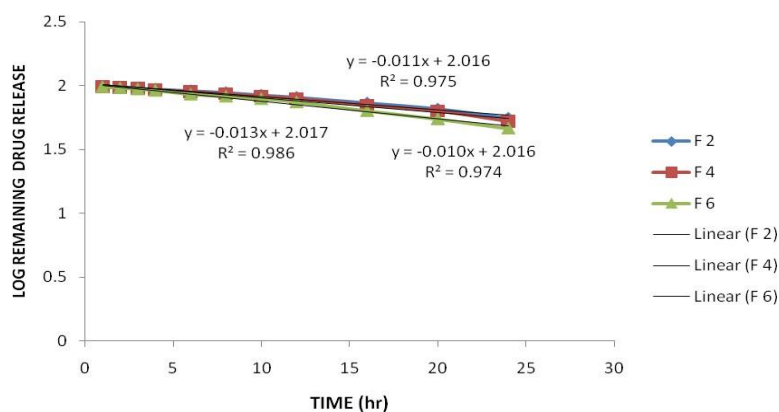


Figure 9: First order release studies for optimized formulations F3, F6 & F9

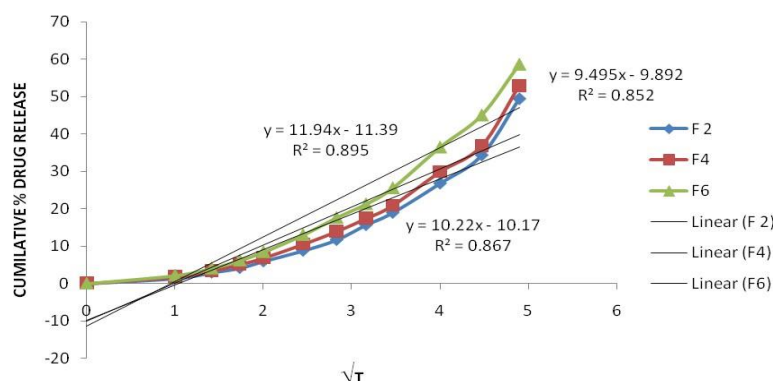


Figure 10: Higuchi's order plot for optimized formulations F3, F6 & F9

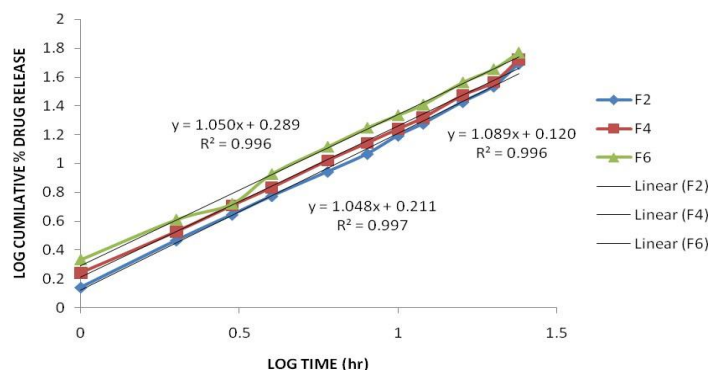


Figure 11: Korsmeyer–Peppas's model for optimized formulations F3, F6 & F9

lease of F1 to F6 formulations at various time intervals was calculated. The F7, F8 formulations was not determined dissolution studies, it has utilized for only estimation of order of reactions. The cumulative percent drug release in F3, F6, F9 formulations was plotted against time in Figure 7. The Maximum percent of drug release was found in F9 formulation which contains maximum drug entrapment.

### Release Kinetics

The release kinetics of F3, F6, F9 formulations was studied. All formulations follow Zero order release kinetics and follow case II transport when it applied to the Korsmeyer-Peppas's Model for mechanism of drug release. F9 formulation has better kinetic results when compared to F3 and F6 formulations. The results are shown in Figure 8, 9, 10 & 11 and in Table 6.

### Stability data

The stability of the Doxorubicin Liposomes was evaluated after storage at 2-8°C and 25°C for 90 days. The assays of the samples were determined as a function of the storage time. The Liposomes stored at 2-8°C were found to be stable for duration of 90 days. The results were showed in Table 6 & 7.

### CONCLUSIONS

From the executed experimental results, it could be concluded that the stabilizers like Stearylamine and Phosphotidyl glycerol along with Hydrogenated Soy Phosphatidyl Choline and cholesterol were suitable carrier for the preparation of Doxorubicin Liposomes. Though the preliminary data based on in-vitro dissolution profile, release kinetics and stability studies proved that the suitability of such formulations, Still a



thorough experiment will be required based on the animal studies. There after we can find the actual mode of action of this kind of dosage form.

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